CHEMICAL COMPOSITION AND ANTI-MICROBIAL ACTIVITY OF ESSENTIAL OILS OF THE PLANTS: *TARCHONANTHUS CAMPHORATUS,* *LEONOTIS NEPETIFOLIA* AND *SATUREJA BIFLORA*

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A thesis submitted to the Graduate School in partial fulfilment for the requirements of the Degree of Master of Science in Chemistry of Egerton University.

EGERTON UNIVERSITY

May 2007

DECLARATION AND RECOMMENDATION

I declare that this thesis is my original work and that it has not been previously presented in this or any other University for any degree

Signature…….. Date……

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This thesis has been presented for examination with our approval as the University supervisors

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Signature……. Date……

DR. OKANGA F.I

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DEDICATION

To Kimutai, Jebii and Kiprotich

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ABSTRACT

In sub-Saharan Africa, the high infant mortality is mainly due to diarrhoea, respiratory infections, typhoid and measles. The control of these diseases relies heavily on the use of synthetic drugs. However, due to the high cost of these drugs and increased bacterial resistance to available anti-biotics, their use is increasingly becoming unpopular. Plant–derived herbal medicines are feasible alternatives that can be used to reduce incidence of these diseases. Since they are readily available and are not known to develop resistance. This study involved extraction of essential oils from fresh leaves of medicinal aromatic plants; *Tarchonanthus camphoratus, Leonotis nepetifolia* and *Satureja biflora* by hydro distillation using a modified Clevenger–type system. The oils were analyzed by gas chromatography–mass spectroscopy (GC–MS) and evaluated for anti-microbial activity. Subsequently, minimum inhibitory concentration was determined by serial dilution of the oils. Essential oil from *Tarchonanthus camphoratus* was obtained in 0.2% w/w yield with thirty-eighty identified components constituting 95.75%. The oil was dominated by monoterpenes, (80.86%). The main constituents were fenchol (15.86%), 1, 8-cineole (14.27%) and α-terpineol (13.21%). The oil showed broad-spectrum anti-fungal and anti-bacterial activity however *Pseudomonas aeruginosa* was found to be resistant to the oil. *Leonotis nepetifolia* oil was obtained in 0.004% w/w yield. A total of 36 compounds constituting 94.80% of the oil were identified. The oil was characterized by a high percentage of sesquiterpenes (75.32%). The major compounds were: germacrene D (37.16%), germacrene–D-4-ol (6.02 %,) and *cis*-ocimene (4.79%). This oil exhibited negligible anti-fungal and anti-bacterial activities against *Staphylococcus aureus, Bacilllus spp, Salmonella typhi, Klebsiella pneumonia* and *Proteus* mirabillis. *Satureja biflora* oil was obtained in 0.2% w/w yield; twenty-two components representing 99.29% of the total oil were identified. The

oil was dominated by monoterpenes (62.02%). The major compounds were linalool (50.60%), germacrene D (10.63%) and linoleic acid (4.48%). The oil showed anti-bacterial activity against all the bacterial strains tested except *Pseudomonas aeruginosa*, and anti-fungal activity against *Candida albicans*. These findings may be used as leads in the search for new drugs from non-microbial sources.

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ATP	Adenosine Triphosphate
CDP	Cytidine Diphosphate
CDP-ME	4-Diphosphocytidyl-2-C-Methyl-D-Erythritol
CDP-ME2P	4-Diphosphocytidyl-2-C-Methyl-D-Erythritol-2-Phosphate
CMP	Cytidine monophosphate
CTP	Cytidine Triphosphate
DMAPP	Dimethylallyl Diphosphate
DOXP	1-Deoxy-D-Xylulose-5-phosphate
FPP	Farnesyl diphosphate
GAP	Glyceraldehyde-3-phosphate
GGPP	Geranyl geranyl diphosphate
GPP	Geranyl Diphosphate
HMBPP	1-Hydroxy-2-methyl-2-(E) butenyl-4-diphosphate
HMG-CoA	3-Hydroxy-3-methylglutanyl-CoA
HMGR	3-Hydroxy-3-methylglutanyl-CoA Reductase
IPP	Isopentyl diphosphate
MECP	2C-Methyl-D-erythritol 2, 4-cyclodiphosphate
MEP	2C-Methyl-D-erythritol-4-phosphate
MVA	Mevalonate
P	Phosphate
PP	Diphosphate
TPP	Thiamin diphosphate
WHO	World Health Organization

N/B. Other abbreviations are standard according to IUPAC

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Traditional medicine is extensively used by people in both developed and developing nations for treatment of a variety of diseases. For developing countries herbal medicine occupies a key role in the health care provisions with 80% of the population in developing countries depending on herbal medicine. The easy accessibility and affordability of herbal medication entice the marginalized population to use it for basic health provision (Luoga *et al*., 2000 & WHO, 2002). In Kenya it is estimated that over 90% of the population use medicinal plants (Nyamwaya, 1992). Although the prohibitive cost of modern medicine is one of the determinant factors, the socio-cultural traditions and societal norms promote traditional medicine as superior (Kamatenesi – Mugisha*, et al.,* 2000 ; Kakudidi *et al.*, 2000).

Indigenous medicines, in most cases, complement conventional medicine (Harjula, 1980). The choice between the two therapies depends on socio-economic status of the person. At the moment some governments are re-examining traditional medicine and its practice through multi-disciplinary studies for their efficacy and safety before any major policy change can be implemented (Baqual, 1992).

Nearly 50% of medicines in the market originate from natural sources for example plant extracts (R'acz, 1982). Examples of plant extracts used as medicine include: nisin which possess anti-microbial activity against a wide range of gram-positive bacteria. Grape seed powder is known to fight skin diseases and yacon leaves powder is an all natural herbal medicine that helps stabilize blood sugar Traditional Chinese medicine consists of several plant

extracts which have been used for thousands of years to treat everything from constipation to infertility using potent plant mixtures. There are many drugs of plant origin which are in use. These include the anti-malaria sesquiterpene artemisinin from *Artemisia annua* L. (Asteraceae) and its synthetic derivatives artemether, arteethter and sodium artesunate; forskolin from *Coleus forskoli* L (Labiateae), the acetylcholine esterase inhibitor physostigmine from *Physostigma venenosum* L (Fabaceae), galanthamine from *Galamthus nivalis* L (Amaryllidaceae); and Huperizine A from *Huperizia Serrata* L. (Lycopodiceae) (Hostettman & Hamburger, 1993).

Plants have unlimited ability to synthesize aromatic substances most of which are secondary metabolites. At least 12,000 compounds have been isolated from plants. In many cases these substances serve as plant defence compounds against attack by micro-organisms, insects and herbivores. Some, such as terpenoids, give plants their odours while others (quinones and tannins) are responsible for plant pigments. Many other compounds also known to be responsible for plant flavours are used as spices by humans to season food. Many plants have afforded useful medicinal compounds used as drugs or lead compounds in drug development (Geissman, 1963).

Volatile terpene hydrocarbons and corresponding oxygenated derivatives known as essential oils are also secondary metabolites. Unlike vegetable oils expressed from nuts and seeds, essential oils are not actually oily. Some essential oils are viscous while others are fairly solid and most are somewhat watery. They are the most concentrated form of any botanical. It takes at least one pound of any given plant to create one drop of essential oil. Essential oils provide a concentrated dose of the vast natural pharmacological active ingredients in a single drop of oil. Essential oils have been used for a long time in folk medicine and therapeutics, traditional

and alternative medicine. Volatile oils of many plants are known to have anti-microbial activity. This activity could act as chemical defence against plant pathogenic diseases. Pathogens can readily penetrate wounded sites caused, by herbivores or insects. Wounding of leaves, results in the rapture of oil glands causing the oil to flow over the wound. The existence of anti-microbial activity in the oil is of considerable benefit to the plant. Indeed, a good majority of aromatic and medicinal plants do not succumb to many of the common plant diseases (Bhattacharjee *et al*., 2004).

The anti-microbial activity of essential oils has been recognized for many years and their preparations have wide application as naturally occurring anti-microbial agents in pharmacology, medical and clinical microbiology (Consentino *et al*., 1999; Hammer *et al.*, 1999). Fungi and bacteria cause important human diseases, especially in the tropical regions. Despite the existence of potent anti-biotic and anti-fungal agents, resistant or multi-resistant strains are continuously appearing imposing the need for a permanent search and development of new drugs from non-microbial sources (Silver & Bostian, 1993).

Essential oils and extracts from several plant species are able to control micro-organisms related to skin, dental and food spoilage problems including Gram-negative and Gram-positive bacteria (Guillen *et al*., 1996). Higher aromatic plants have traditionally been used to extend the shelf life of foods, suggesting the inhibition of bacteria, fungi and yeast (Alves *et al*., 2000; Sartoratto *et al*., 2004). Most of the properties are due to essential oils produced through secondary metabolism (Adam *et al*., 1998).

Although they usually occur as a complex mixture, the activity of essential oils is related to the respective composition of the plant volatile oils, the structural configurations and functional

groups of the components and possible synergistic interactions between components (Knobloch *et al.,* 1989). Consequently, the discovery of essential oil preparations that possess anti-microbial activities has been the subject of many research investigations, through the screening of a variety of plant species (Hammer *et al*., 1999). In an effort to discover new lead medicinal and pesticidal compounds many research groups screen plant extracts to detect secondary metabolites with relevant biological activities. In this regard, this project involved extraction, screening and analysis of essential oil from aromatic medicinal plants that may be useful for development of new tools for the control of infectious and parasitic diseases.

1.2 Statement of the problem

Most of the deaths of children and the elderly are caused by infectious and parasitic diseases, like diarrhoea, respiratory infections, typhoid and measles. In developing countries, these diseases remain a public health problem where, for instance an estimate of 60 million cases and 500,000 deaths annually are caused by typhoid fever alone. Due to inadequate health facilities and the high cost of synthetic drugs, it is necessary to seriously look into the role of traditional herbal medicine, scientific efficacy of combating diseases which will not only provide cheap drugs to Africa but also eradicate the problem of increased bacterial resistance associated with the use of synthetic anti-biotics.

1.3 Objectives of the study

General Objective

To extract, characterize and identify the components of the essential oil of *Tarchonanthus camphoratus* (Asteracea), *Leonotis nepetifolia* (Lamiaceae) and *Satureja biflora* (Lamiaceae) and test for their anti-microbial activity.

Specific objectives

1. To extract essential oils from *Tarchonanthus camphoratus* (Asteraceae), *Leonotis nepetifolia* (Lamiaceae) and *Satureja biflora (*Lamiaceae).
2. To characterize and identify the essential oils from the three aromatic medicinal plants.
3. To assess the anti-microbial activity of the essential oils from the three aromatic medicinal plants.

1.4 Justification

The poorest countries in the world are most in need of inexpensive and effective treatments for diseases. WHO (2002) estimates that one-third of global population lacks regular access to essential drugs, and that in the poorest parts of Africa this figure rises to over 50%. One possible alternative source of medicine lies on traditional herbs/natural products used by indigenous ethnic groups in Africa. The main problem facing the use of traditional medicinal herbs is the lack of proof that active components contained in medicinal plants are useful, safe and effective. This proof is required to reassure the state governments, medical practitioners and the public of the efficacy and safety of medicinal plants as alternative to synthetic drugs. The proof of pharmacological activity has been mostly based on empirical experience. The scientific proof therefore becomes a priority, in order to eliminate concerns in using traditional herbs/natural products as alternatives to synthetic medicine (Rukangira, 2000). The plants: *Tarchonanthus camphoratus* (Asteraceae), *Leonotis nepetifolia* (Lamiaceae), and *Satureja biflora* (Lamiaceae) are traditionally used to treat bronchitis, asthma, headache and stomach troubles (Kokwaro, 1993). Extraction and identification of essential oil components, determination of the anti-microbial activity and effective mode of administration will add value to the medicinal plants under investigation.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Tarchonanthus camphoratus*

The plant belongs to the family of *Asteraceae*. Its common name is wild sage or camphor bush. It is a small tree that grows to a height of 6 m with moderate to strong odour of camphor. It is widely spread in all the provinces of South Africa, Lesotho, Swaziland, and Namibia. In Kenya, it is found in almost all the provinces. The leaves of the plant are alternate, borne on white-feted twigs, variable, 2 x 0.5- 12 x 5 cm, obovate to lanceolate, upper surface smooth and finely reticulate with minute golden glands over the veinlets, lower surface white-felted margin entire to denticulate (Figure 1). Flowers are cream, borne on discoid heads in terminal or auxiliary panicles, female 1-3 flowered, male with numerous flowers; fruits are densely white woolly achene (Hillard, 1977).

Figure 1: Photograph of *Tarchonanthus camphoratus*

The leaves of the plants have been used in a number of ways; infusions, tinctures or decoctions are orally taken and vapours from burning green material either inhaled or directed as a fumigant to treat inflamed joints. Fresh leaves may be chewed or smoked; a leaf poultice may be applied to the chest as an ointment to the affected areas. Decoctions are taken orally or applied externally to relieve bronchitis, asthma, headache, inflammations, chilblains or abdominal pains (Amabeoku *et al.,* 2000).

Phytochemical tests carried out on the South African species of the plant indicated the presence of tannins, saponins and reducing sugars but not alkaloids, cardiac or anthraquinone glycosides (Bishay *et al.,* 2002). However, various flavones: luteolin, apigenin and nepetin, the sesquiterpene lactone parthenolide as well as the quartenary alkaloid, tarchonanthine have been identified in Egyptian collection of this species (Bishay *et al.,* 2002). Recent investigations of anti-microbial activity of aqueous, ethanolic and hexane extracts of dried leaf did not demonstrate *in vitro* inhibitory effects against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* or *Klebsiella pneumoniae* (McGraw *et al*., 2000).

2.2 *Leonotis nepetifolia*

Leonotis nepetifolia belongs to the family *Lamiaceae* (mint family). Its common name is annual lion's ear. The plant was originally native to tropical and sub-tropical Africa. However, it is now naturalized all over the world in appropriate climates where it grows along the road shoulders in abandoned fields and in disturbed areas such as overgrazed pastures and river levee banks. It has potential to form large colonies and displace native plants (Csurhes & Edwards 1998). Lion's ear is an erect loosely branched annual plant that can grow to a height of 8 ft (2.4m) in a single season. The stems are strongly angled (square in cross section) and the leaves are in pairs opposite each other. The leaves are smooth with coarsely toothed

margins triangular in shape. The flowers are borne in rounded spiny clusters that encircle the stems so that it looks like the stems are growing right through the middle of the cluster. As the stems elongate, new flower clusters continue to develop above the older ones. The tubular flowers that peck out of the spiny heads are orange and furry like a lion's ear (Figure 2) (Wagner *et al.,* 1999).

Figure 2: Photograph of *Leonotis nepetifolia*

An infusion of the roots is used to treat stomach disorders while a decoction of the leaves is drunk for stomach-ache (Kokwaro, 1993). The anti-microbial activity of the plant essential oil is not known. However in the genus *Leonotis* two species *L. leonurus* and *L. ocymifolia* are known to exhibit anti-microbial activity. The two species have been used as traditional herbal medicine in Southern Africa (Oyedeji & Afolayan, 2005).

2.3 *Satureja biflora*

The genus *Satureja* belongs to the family of Lamiaceae and has 30 species distributed in tropical Africa and North America. *Satureja biflora* is a perennial shrub with white flowers and can grow to a height of 18 inches (Figure 3). Its common name is lemon African savory.

Figure 3: Photograph of *Satureja biflora*

The roots and the leaves of this plant are used to relieve headache (Kokwaro, 1993). *Satureja biflora*, like other *Satureja* species is known for its culinary uses (Jamzad & Sefidkon, 2004). Different *Satureja* species have been used in traditional medicine as anti-microbial, spasmolytic, analygesic, cicatrizing and diuretic agents. The anti-bacterial properties of several essential oils of *S. montana* (Melegari *et al.,* 1985*)* and *S. thymbra* (Capone *et al.*, 1989) have been demonstrated. The essential oils of *S. obovata,* (Navarro *et al.,* 1989; Cruz *et al.,* 1990),

S. cuncifolia (Tumen *et al.,* 1989) and *S. hortensis* (Hajhashemi *et al.,* 2000) have been evaluated as spasmolytia agents. Essential oils have also been extracted from four other species: *S. coerulea, S. icarica, S. pilosa* and *S. boissieri* and their anti-bacterial and anti-fungal properties evaluated (Dilek *et al.,* 2002). Little is known on the medicinal value of *Satureja biflora* species.

2.4 Essential oils

Essential oils are volatile plant oils (steam distillable), which are composed chiefly of terpenoids: mono-, sesqui- and di-terpenes plus various alcohols, ketones and aldehydes with commonly occurring aromatic compounds arising from the phenylpropanoid pathway (eugenol and safrole). In some species, alkanes, aliphatic alcohols and ketones may be obtained (Williams, 1996). They are also considered as a complex mixture of various aromatic chemicals. Each of these constituents contributes to the beneficial or adverse effects of the oil (Buchbauer, 1993). The volatile oils are more or less modified during the preparation process (Buchbauer, 1993).

Essential oils are formed in special cells or groups of cells (Koul *et al.*, 1990), and generally predominate in one particular part of the organ, such as leaves, flower calyces, fruit and roots (Lahlou & Berrada 2001). These natural products are still used as raw materials in many fields, including perfumes, cosmetics, aromatherapy and phytotherapy, spices and nutrition (Buchbauer, 1993). The composition of essential oils is dependent on such characteristics as the geographic location from which the plant is obtained, seasonal and climate variations, production techniques and purity. The effect of the plant maturity at the time of harvesting (oil production) and the existence of chemotypic differences can also drastically affect the composition suggesting that ecological or physiological condition could interfere with the presence of biologically active compounds in the plant (Cornu *et al.*, 2001). These variations

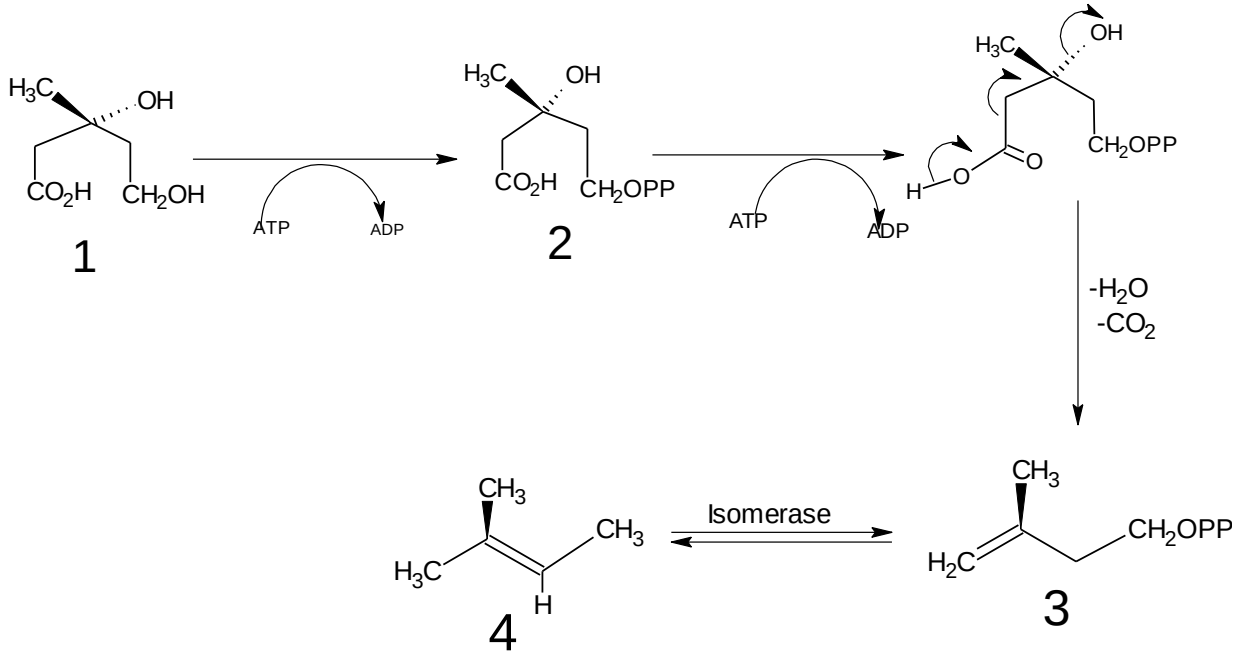
are significant in the study of biological and pharmacological activities of essential oils, because the value of the oil in aromatherapy is related to the chemical composition (Lahlou, 2003).

There are no suitable synthetic substitutes available due to the difficulty of profiling and mimicking the complex compound mixtures in the volatile oils. The original plant extracts (essential oils) will continue to be used long into the future. It is interesting to know the chemical components that nature combines to make up the oils, but it is humbling to realise that even with the best human efforts, combination of all the chemicals in the correct proportions, in a laboratory would still not give identical oil. Such reconstituted oil will not have the same therapeutic effect as the natural essential oil (Jaime &Teixera, 2004).

2.4.1 Biosynthesis of terpenoids

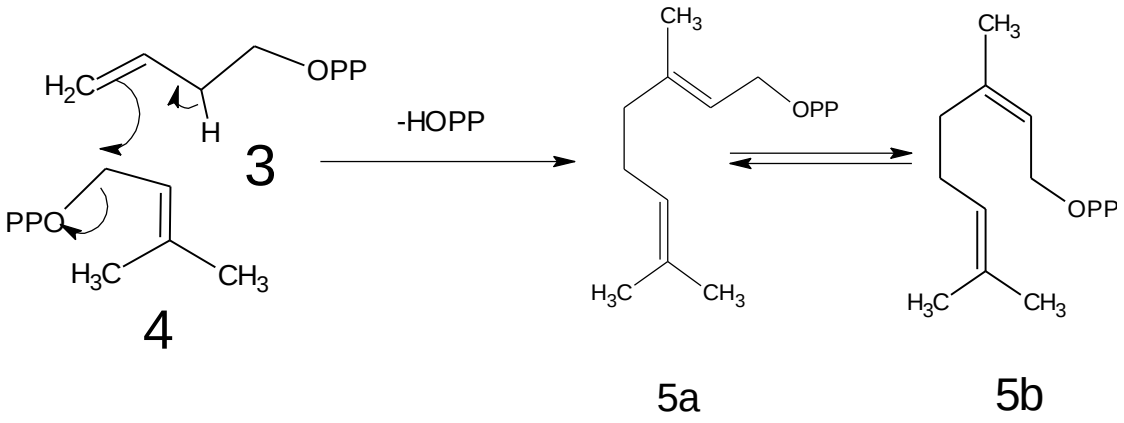
The biosynthesis of terpenoids starts from mevalonic acid (**1**) to diphosphate (**2**) which gives rise to IPP (**3**) and DMAPP (**4**) (Scheme 1) (Finar, 1975).

Scheme 1: Formation of IPP and DMAPP from mevalonic acid



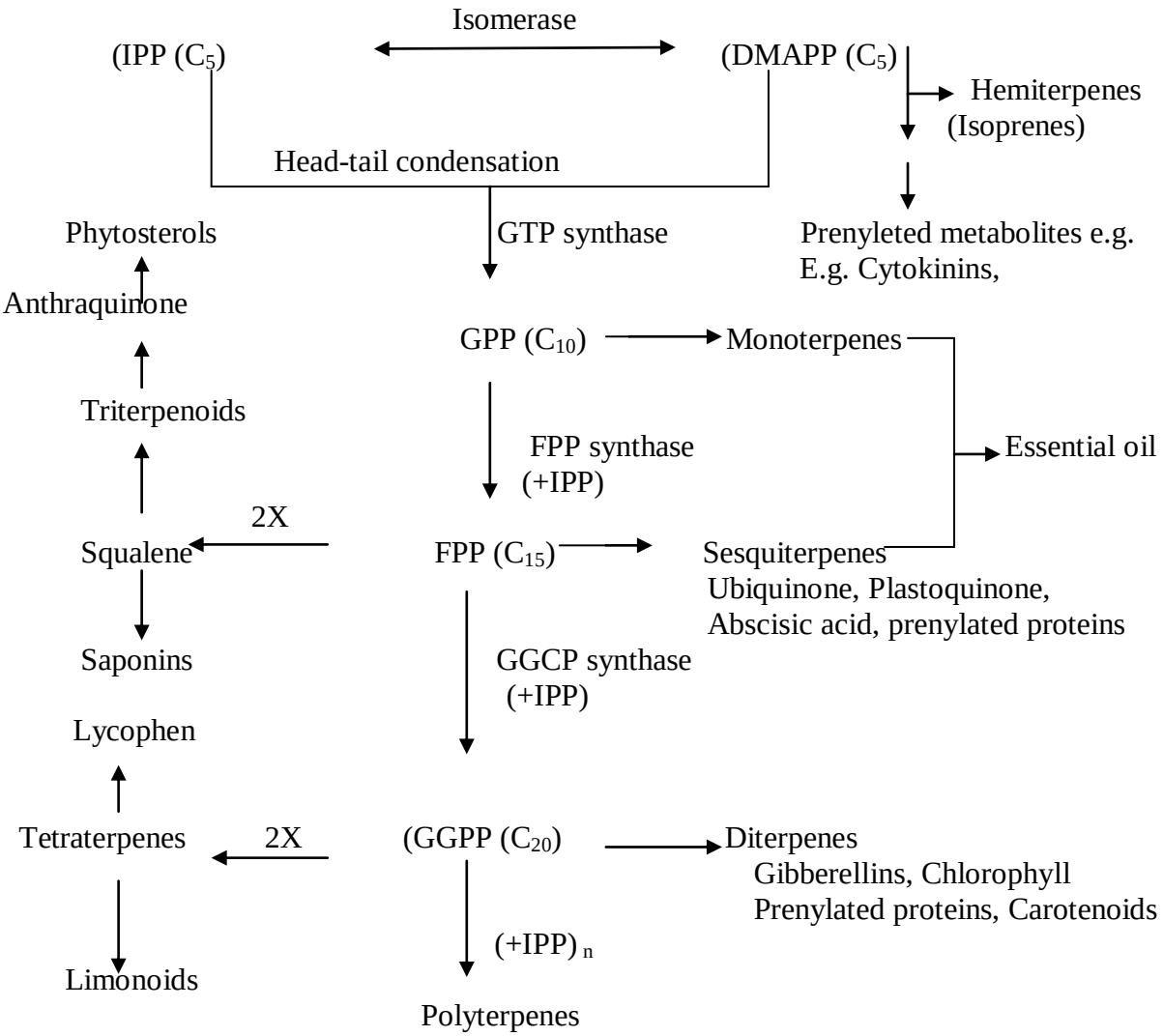
Terpernoid biosynthesis involves mostly head to tail addition of IPP, the active C_5 isoprene unit, to its isomer DMAPP to form GPP, C_{10}) (scheme 2). IPP (**3**) and DMAPP (**4**) combine head to tail to form geranyl diphosphate (GPP) (Trans and cis isomers **5a** & **5b**), IPP acting as the nucleophilic reagent and DMAPP as the electrophilic reagent as shown in the equation below (Finar, 1975).

Scheme 2: Formation of GPP from IPP and DMAPP



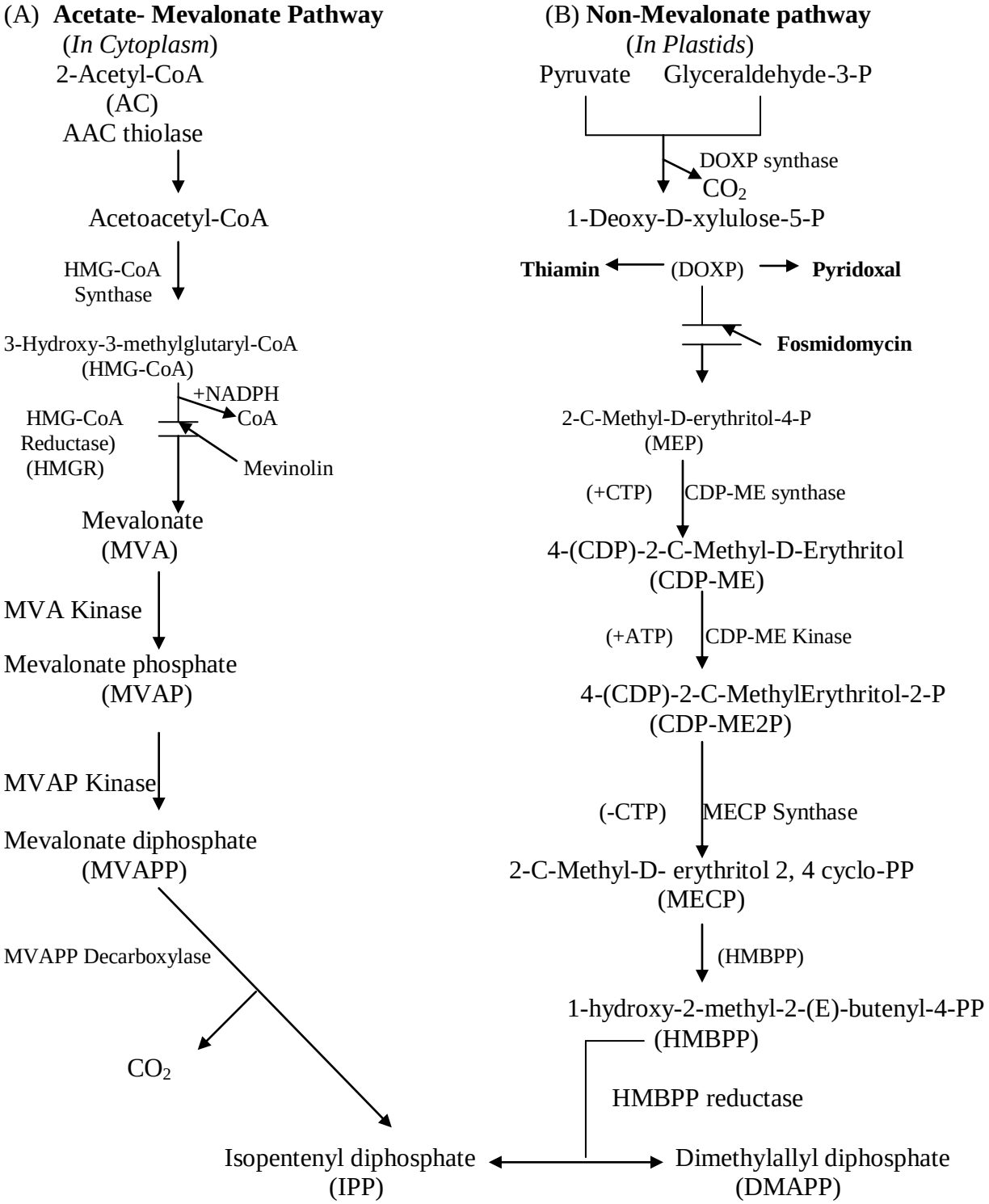
Further, condensation of enzyme-bound GPP with additional IPP units forms successively larger prenyl diphosphates like FPP $C_{15,}$ GGPP C_{20} that may undergo cyclization, coupling and/or rearrangement to produce the parent carbon skeleton of monoterpenes, sesquiterpenes and diterpenes (Figure 4) (Singh *et al.*, 1989; McGarvey &Croteau 1995; Luthra *et al.*, 1999). GPP, FPP and GGPP yield monoterpenes, sesquiterpene and diterpenes skeletons, respectively. Furthermore, FPP and GGPP dimerize to produce parental precursors to synthesize triterpenes and tetraterpenes, respectively. These precursors can undergo structural modification through oxidation; reduction, isomerization, hydration, conjugation and/or other transformations to give rise to a variety of terpenoids (McGarvey & Croteau, 1995). The biosynthetic pathway of terpenoids is summarized in scheme 3.

Scheme 3: Summary of biosynthesis of terpenoids



In plants, terpenoids are synthesized via two IPP generating pathways that is acetate-mevalonate (Ac-MVA) and non-mevalonate (non-MVA) pathways (scheme 4) (Lichtenthaler, 1999; Rohmer, 1999).

Scheme 4: Biosynthetic pathway to precursors of essential oils



In higher plants, the conventional acetate-mevalonate (Ac-MVA) pathway operates mainly in the cytoplasm and mitochondria giving rise to sterols, sesquiterpenes and ubiquinones predominantly. The plastidic non-mevalonate pathway is responsible for hemi-, mono-, sesqui-, and di-terpenes, along with carotenoids and the phytol chain of chlorophyll (Vinod *et al.*, 2003).

2.5 Functions of plant essential oils

Like all the secondary metabolites, essential oils are known to have several important functions, including protection against predators (micro-organism, fungi, insects and herbivores), attraction of pollinators, inhibition of germination and promotion of plant growth (Cowan, 1999).

Parts of many plants are odorous as a result of their essential oil content. Once produced, an essential oil either is released, as a fragrant from flower, or is stored by a plant until it eventually evaporates or deteriorates when the plant dies and is regarded as an end product of the metabolism (Williams, 1996). The fragrance of a scented flower is due to the vapours of essential oils, released from the specialized oil glands, the purpose of which is not to please our senses, but 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. This phenomenon is referred to as allelopathy.

Essential oils 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 micro-organisms and insects, so maintaining the leaves at a height sufficient to receive maximum sunlight. Those present in aromatic plant exudates may kill or inhibit the growth of pathogenic micro-organisms. Examples include gum benzoin exudates (an oleo resin), myrrh and olibanum (frankincense). They are naturally produced at a very slow rate but in much larger amounts if the bark is wounded, to seal off the living, girth-increasing and conducting tissues which perform functions vital to the continued life of the plant beneath the bark (Williams, 1996; Pichersky &Gershenzon, 2002).

The therapeutic action of essential oils is attributed to the naturally occurring chemicals within the plants. The essential oils 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, and which are harmless as recommended for use by professionally trained and fully experienced aromatherapists (Williams, 1996). In aromachology, certain essential oils are inhaled for changing the negative mood such as depression, into positive moods, such as happiness and normal physical appetite (Williams, 1996). For example linalool, a monoterpene compound prevalent in essential oil of many plant species is traditionally used as sedative. It has been demonstrated to be an anticonvulsant in several experimental models (Lahlou & Berrada 2001). 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).

Essential oils from higher and aromatic plants have been used to extend the shelf life of food suggesting the inhibition of bacteria, fungi and yeast. They are the subject of continuing

research for analytical purposes, and yield valuable information. Most of the information remains academic rather than commercial since essential oil must offer properties of importance to end-users like pleasant odour, flavour or medicinal properties, to create demand that can support large-scale cultivation and processing of the crops (Mahasneh *et al.*, 1999).

2.6 Mode of action of essential oils

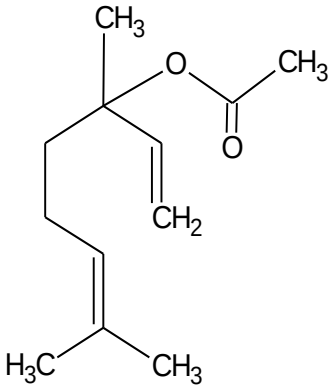
2.6.1 Specifity of action

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 localized in these areas (Buchbauer, 1993). For example, studies on skeletal and smooth muscle contraction have shown varied degree of specificity of action by depressing the contraction of the smooth muscle of the various segments of the intestines (Koul *et. al*., 2004). However, the potency of the pharmacological effect varied significantly. Thus, the essential oil from *Origanum compactum* Benth (Lamiaceae) exhibited potent molluscicidal activity on *Bullinus trucantus* snails in less than 24 h. The same oil exhibited marked niticidal activity on *Pediculus humanus capitis*, but has no significant licicidal activity in vitro up to 2h of observation. The oil was also found to have insecticidal action on *Drosophila melanogaster* adults. Larvicidal activity on *Culex pipiens*, toxicity to *Artemia salina* larvae and to mosquito fish at high concentrations were noted (Lahlou &Berrada, 2001 and Koul *et al*., 2004).

2.6.2 Effect of essential oils on micro-organisms

Anti-microbial action of the monoterpenes is through diffusion into and damage of cell membrane structures (Andrews *et al.*, 1980, Uribe *et al.*, 1985; Sikkema *et al*., 1995). Monoterpenes are lipophilic and by definition will preferentially partition from an aqueous phase into membrane structures. This causes expansion of the membrane, increased fluidity or

disordering of the membrane structure and inhibition of membrane-embedded enzymes (Sikkema *et al*., 1995). Some authors have attributed this action to the interaction of the functional groups (phenols especially) with the microbial cell envelope (Sikkema *et al*., 1995). While others have found that essential oils cause a deterioration of the cytoplasmic membrane (Helander *et al*., 1998). Bammi *et al.* (1997) demonstrated an action of essential oil of thyme on the cell lifecycle. The appearance of profound lesions in different micro-organisms (*Escherichia coli, Bacillus subtilis* and *Saccharomyces cenevisiae*) demonstrated the action of the oil (Bammi *et al*., 1997). Linalyl acetate (**6**) is known to suppress spore formation, while linalool (**7)** inhibited spore germination and fungal growth. The inhibition of sporulation appeared to arise from respiratory suppression of arial mycelia (Sikkema *et al*., 1995).



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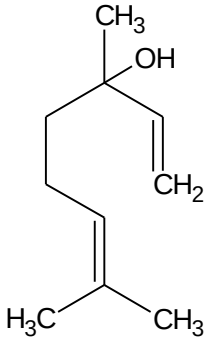

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Anti-microbial activity of volatile compounds results from the combined effect of direct vapour absorption by micro-organisms and indirect effect through the medium they absorbed the vapour (Moleyar & Narasimham, 1986). The vapour absorption by micro-organisms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

2.7 Anti-microbials

An anti-microbial is a drug from living organisms, which inhibits growth and reproduction of other micro-organisms. Anti-microbial compounds can be described as anti-biotics. Most anti-biotics 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. Bactericides on the other hand, kill bacteria outright. Chloramphenical, tetracycline and streptomycin and a number of other anti-biotics act against bacteria by interfering with their ability to synthesize the proteins essential for their body components especially the cell-wall. The end result is, of course, suppression of growth, interference with cell multiplication and ultimate death (Milton, 1990).

Penicillins on the other hand are bacteriostatics, which interfere with the ability of the bacterial cell to form a wall leading to the inevitable cellular disruption and death. Other anti-biotics, like amphotericin and nystatin, affect cell membrane of the pathogen causing it to weaken or break, and even prevent it from functioning properly (Milton, 1990). The anti-microbials under investigation in this project are plant essential oils.

2.7.1 Biological essays

Some natural product extracts contain active principles, which may not be amenable to chemical assay and are therefore analysed by bioassay for biological activities. The technique relies upon the measurement of an effect produced in a biological system, sometimes entire laboratory animals, isolated tissue preparation or cells (Brander & Pugh, 1971). Many new remedies have been introduced on the basis of a bioassay that is later replaced by chemical quantification when the active principle of the remedy has been characterized (Brander & Pugh, 1971). Bioassay is largely used in defining the strength of drugs or anti-biotics by

determining the potency of chemical or biological preparations by effects in living systems. A bioassay may be more sensitive and performed more rapidly than chemical analysis (Joshua, 1992).

Biological assays are valuable research tools in the field of medicinal chemistry. They are often the only practical means of standardizing anti-biotic substances. Many medicinal agents, particularly those derived from natural products, may consist of a mixture of biologically active substances. For such agents, we are interested in the responses before the chemical structure is ascertained (Joshua, 1992). Bioassays may be classified by laboratory techniques and culture media used or by the nature of responses, which may be qualitative or quantitative. Direct assays are used to measure the degree of response of the subject. The response can be expressed as a continuous variable or as to whether it did or did not respond (Joshua, 1992).

The bioassay is measured by its effect on a biological system. These biological systems are usually microbials, (unicellular or multi-cellular organisms). The microbials used in this project are pathogenic bacteria and fungi.

2.7.2 Fungi

Fungi are a group of organisms that lack chlorophyll, reproduce by the production of spores (sexually and/or asexually), and contain chitin or cellulose in their cell walls. They are usually considered as lower plants and in contrast to bacteria they are eukaryotic and have cell membranes that are rich in sterols such as ergosterols. They are not susceptible to the usual anti-bacterial polyene anti-microbials but are sensitive only to polyene anti-microbials that have a marked affinity for sterols (John, 1981). Anti-fungal activity in this study was tested on *Candida albicans,* which is associated with oral thrush.

2.7.3 Bacteria

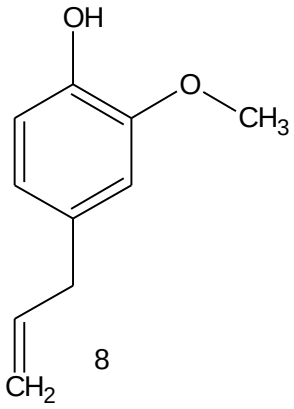
Bacteria are small organisms (1-2 µm in length or diameter). They have almost no visible internal structures and lack the nucleus that is an essential feature of the algae, protozoa, fungi and cells of higher organisms (John, 1992). Bacteria were the first disease-causing microbes to be identified, though microbiologists now recognize pathogens amongst the fungi, viruses and protozoa. There are many species and strains known and they tend to look the same. Three main shapes are known: rods (bacilli), sphere (cocci) and commas (vibrios) (John, 1992).

In this study, both gram-positive as well as gram-negative bacteria were tested against the plant essential oils. These bacteria include *Salmonella typhi,* a member of *Salmonella* genus that belongs to the Enterobacericeae family of gram-negative bacteria. Other genera in this family include *Escherichia, Shigella* and *Yersinia* all of which include species that are important causes of intestinal infections and diarrhoeal diseases in humans. *Salmonella typhi* is in Group D salmonella according to the classification of Kauffman and White (John, 1992). Other gram-negative bacteria studied are *Proteus mirabilis, Klebsiella pneumoniae,* and *Pseudomonas aeruginoseae.* Gram-positive bacteria are *Bacillus spp* and *Staphyloccocus aureus,* which is a major cause of wound infection especially as a secondary or opportunist invader (John, 1992).

2.8 Anti-microbial properties of essential oils

Essential oil components with the highest anti-bacterial coefficient are usually phenols for example eugenol (**8**) and carvacrol (**9**). These components have high anti-bacterial qualities; they also have greatly stimulating therapeutic properties. Essential oils rich in phenols should be used over short periods and proper doses at onset of treatment after which others that are easier to use should replace them, since prolonged application can lead to toxicity (Caccioni *et al.,* 1997 & Kandi, 1994). Presence of alkyls or alkenyl group(s) in the benzene ring of either phenol or guaiacol enhances the anti-microbial activity. The larger the size of the alkyl or

alkenyl groups, the stronger the anti-microbial activities (Knobloch *et al*., 1989 & Pelczar *et al*., 1993). This is because alkyl or alkenyl groups are hydrophobic; and hydrophobicity above a minimum extent is required for phenolic and alcoholic compounds to show a potent anti-microbial effect (Kurita *et al*., 1981). However, the ability of a potentially active molecule to interact with the hydrophobic cell membranes can be regarded as a result of its intrinsic hydrophobicity, which increases with the hydrocarbon chain length and/or with the presence of double bonds (Caccioni *et al*., 1997 & Knobloch *et al*., 1989).



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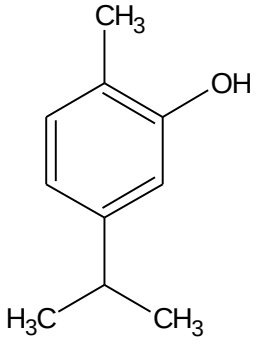

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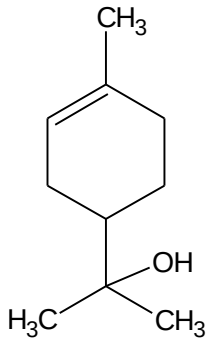
The components with phenolic structures are highly active against micro-organisms. Members of this class are known to be either bactericidal or bacteriostatic agents depending upon the concentrations used (Pelczar *et al.,* 1993). The high activity of the phenolic compounds may be further explained in terms of the alkyl substitution into phenol nucleus, which is known to enhance the anti-microbial activity of phenols (Pelczar *et al.,* 1993). The introduction of alkylation has been suggested to alter the distribution ratio between the aqueous and the non-aqueous phases (including bacterial phases) by reducing surface tension or altering the species selectivity. Alkyl substituted phenolic compounds form phenoxyl radicals, which interact with

isomeric alkyl substituents. This does not occur with esterified phenolics, possibly explaining their relative lack of activity (Knobloch *et al.,* 1989).

Monoterpenols (alcohols) such as linalool (**7**) and α-terpeniol (**10**) are reliable broad-spectrum anti-biotics which are useful in numerous cases of bacterial infection. These alcohols are practically non-toxic at physiological doses and may be applied neat on the skin or the mouth (preferably on a sugar cube) without any risk of over doses. This group of compounds is very important because of its frequent use in large number of microbial, viral and fungal pathologies and have an uplifting energizing effect (Carson & Riley, 1995; Cosentino *et al*., 1999). α-Terpineol (**10**) has been reported to inhibit growth of quite a number of bacteria and fungi, which include; *S. aureus, E. coli, S. eperdermis and C. albicans* (Carsons & Riley, 1995).



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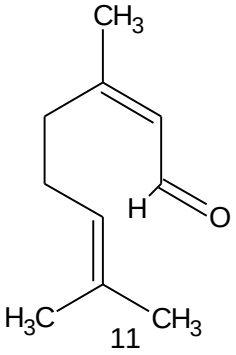
Aldehydes also have anti-bacterial properties and examples include neral **(11)** and citronellal **(12)**. Aromatic aldehydes have powerful anti-microbial, anti-viral, anti-fungal, anti-parasitic and anti-inflammatory effect. They also have sedative and disinfectant qualities. Their strong activity makes them well suited to difficult cases or those that do not respond well to other essential oil. Apart from dermocausticity and irritation of the mucous membranes, aromatic aldehydes are not toxic at therapeutic doses. However aldehydes are also unstable and easily oxidize in the presence of oxygen even at low temperatures (Harkental, 1999; Jansen *et al.*,

1987). It has been proposed that an aldehyde group conjugated to a carbon double bond is a highly electronegative arrangement, suggesting that increase in electronegativity increases the anti-bacterial activity this may explain the observed biological activity (Moleyar & Narasimham, 1986). Such electronegative compounds may interfere in biological processes involving electron transfer and react with vital nitrogen compounds such as proteins and nucleic acids, and therefore inhibit the growth of the micro-organism (Kurita *et al.*, 1979; 1981).



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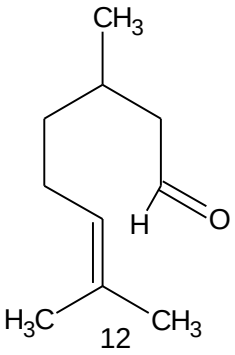

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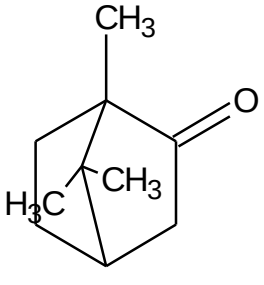
Although ketones can be toxic, as demonstrated in thujone from *Thuja* and wormwood oil, they also have some great therapeutic benefits, in the field of easing the secretion of mucus as well as skin cell regeneration, wound healing and reduction of old scar tissue in wounds, stretch marks and adhesions for example Borneone (Camphor) **(13)** and carvone **(14)** (Jansen *et al.*, 1987).



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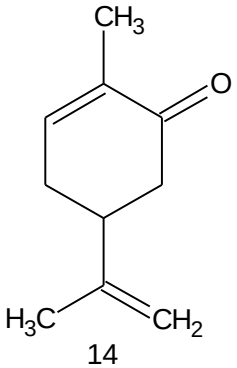

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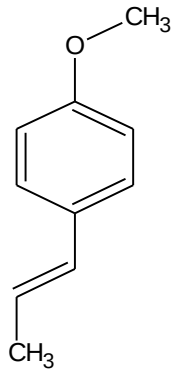
The anti-bacterial action of the ethers is certain, but irregular. Only an aromatogram can predict their use in a specific case. Anethole **(15)** is the most representative molecule in this group (Harkental, 1999; Jansen *et al.*, 1987). The main therapeutic effect of oxides is that of the expectorant with 1, 8-cineole **(16)** also commonly known as eucalyptol, being the most well known (Jansen *et al.*, 1987). 1, 8-Cineole has been known to exhibit anti-microbial activity against *E. coli, P. aeruginosae, S. aureus, Rhizobium leguminosarum* and *Bacillus subtilis* strains (Sivropoulou *et al.*, 1997). The oil from *Artemisia dracuncutus* showed the highest anti-bacterial activity and inflammatory properties due to presence of 1, 8- cineole in the oil (Deans & Svoboda, 1988).



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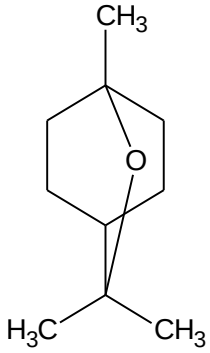

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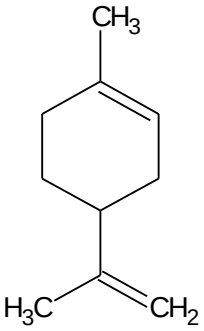
The esters found in essential oils are normally fragrant and tend to be fruity and their therapeutic effects include sedative and anti-spasmodic activity. Some esters have anti-fungal and anti-microbial properties. The most well known ester is linalyl acetate (**6**), which is found in lavender, clary sage and petit grain (Kandi, 1994). Lactones and coumarins are normally found in very low amounts and do not pose a huge problem. Though, a sesquiterpene lactone helenalin found in arnica oil seems to be responsible for the anti-inflammatory action of arnica oil (Kandi, 1994).

Finally terpenes are mostly diffused into the air (atmospheric anti-septic agents). The anti-septic power of essential oils is generally proportional to liposolubility. Other non-oxygenated mono- and sesquiterpenes with anti-bacterial properties include limonene (**17**), β-caryophellene (**18**) and α-pinene (**19**), (Kandi, 1994). α-Pinene (**19**) has been reported to be the anti-fungal principle in the oil of *Pistacia lentiscus* (Anacardiaceae). The essential oil from *P. strobus,* containing the lowest amount of hydrocarbon showed the weakest anti-fungal activity (Magiatis *et al.,* 1999). The main constituent of essential oil from *Juniperus communis* L was α-pinene **(19)** and was found to exhibit anti-fungal and anti-bacterial properties (Harrewijn *et al.,* 2001). Limonene **(17)** is active against several fungi; *Penicillin spp, Asperigillus spp* and *Trichoderma spp* (Kandi, 1994). β-Caryophellene **(18)** is a sesquiterpene known to possess anti-inflammatory and anti-carcinogenic properties. It has minimal anti-microbial activity but the oxide is highly active against bacteria and fungi (Magiatis *et al.,* 1999).



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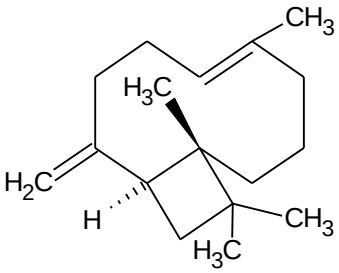

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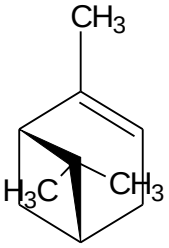

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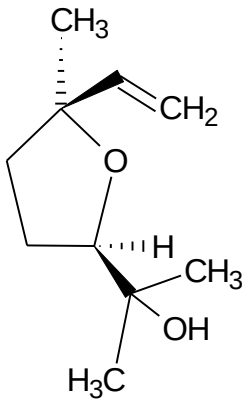
It has also been reported that essential oil from the tea tree oil was active against gram-negative bacteria *E. coli,* and gram-positive bacterium *S. aureus* and fungus *Candida albicans.* The tea tree oil inhibited respiration and increased the permeability of bacterial cytoplasmic and yeast plasma membrane. In the case of *E. coli* and *S. aureus,* tea tree oil also caused potassium ion leakage (Cox *et al.,* 2000). Differences in the susceptibility of the test organism to essential oil are attributed to variation in the rate of monoterpene penetration through cell wall and cell membrane structures. The ability of essential oil to disrupt the permeability barrier of cell

membrane structures and the accompanying loss of chemiosmotic control is the most likely source of its lethal action at minimum inhibitory levels (Cox *et al.,* 2000).

2.9 Antiviral properties of essential oils

Molecules from many chemical families have shown *in vitro* activity, among them monoterpenes and monoterpenals (Caccioni, 1998). 1, 8-cineole **(16)** is used to alleviate viral diseases of the respiratory tract (common in temperate climates). Linalool (**17**) and linalool oxide **(20)** from *Hissopus officinal* var *decumbens* have also been used for viral diseases of the lower respiratory tract. Ketones, especially pulegone have shown interesting capacity to fight naked viruses **(21)** (Consentino *et al.*, 1999). Aldehydes whether used internally or in the atmosphere are good complementary treatments for patients with viral infection. Ethers are useful in some specific clinical cases (Consentino *et al.*, 1999).



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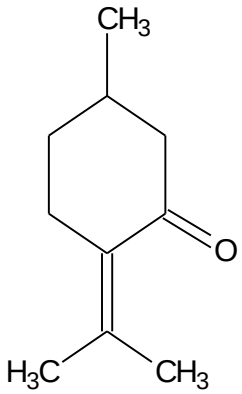

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Generally viruses are highly sensitive to the aromatic molecules and some severe viral pathologies may show a vast improvement following their use. Interestingly patients under aromatic treatment seem to acquire special resistance to viral penetration (Harkental, 1999).

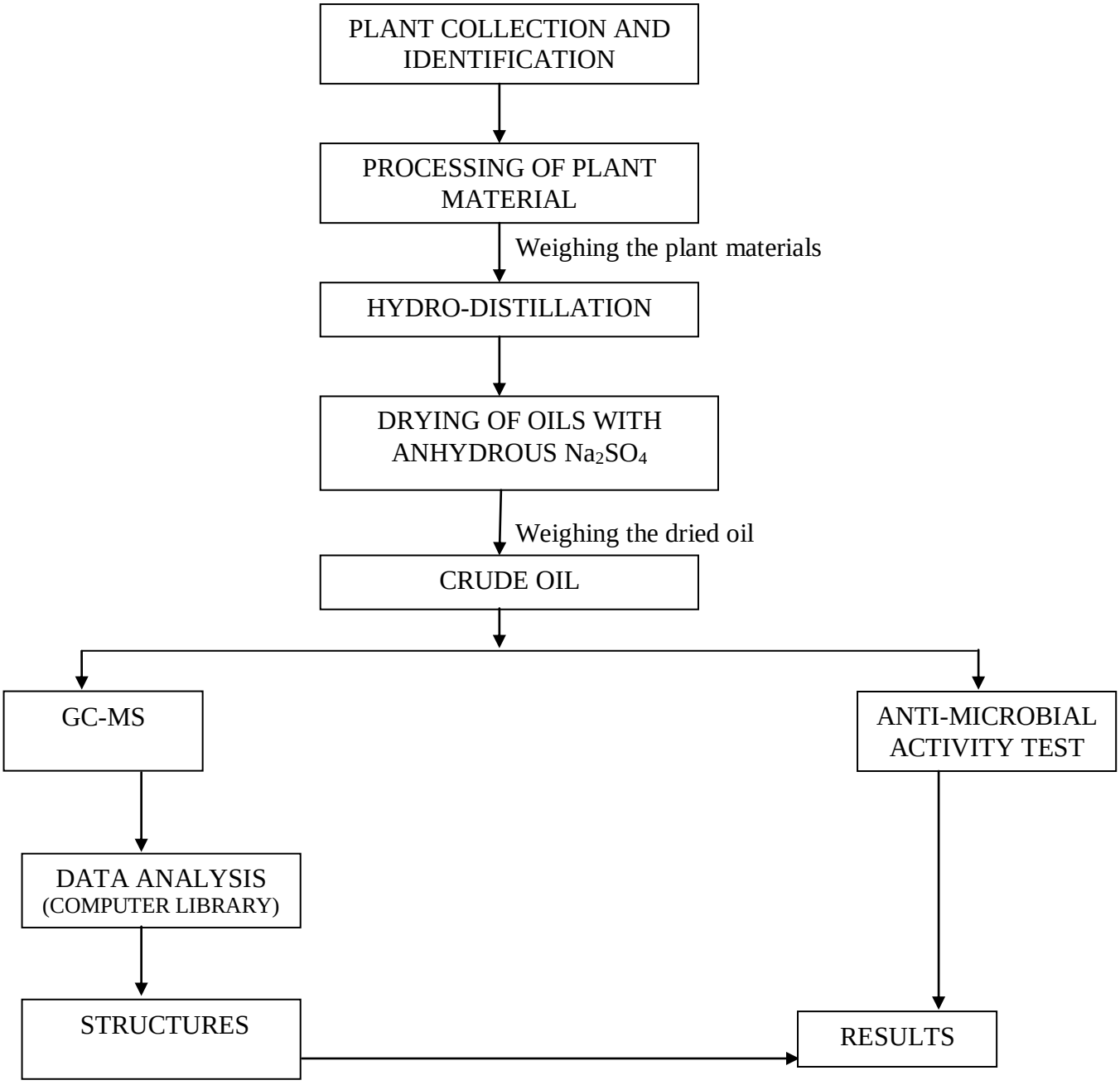
CHAPTER THREE

METHODOLOGY

3.1 Methodological procedure

The research procedures are summarized in scheme 5 and the details are in the subsequent subsections.

Scheme 5: Summary of Methodological Procedure



3.2 Collection and identification of plants

The plants were collected from Egerton University Botanical garden due to its proximity and accessibility. After identification by a taxonomist, Dr. Kariuki S.T, of the Department of Botany, Egerton University the voucher specimens were deposited. The leaves of the plants were collected in preparation for extraction.

3.3 Extraction procedures

Fresh leaves of *T. camphoratus, L. nepetifolia* and *S. biflora* were picked and then packed into 2 L flasks up to three-quarter full and weighed. About 500 mL of tap water was added and the leaves subjected to hydro-distillation, using modified Clevenger apparatus for 4 hours. The resultant mixture of steam and essential oil was passed through a Lie big condenser, cooled by a continuous flow of cold water (Figure 4). Essential oils are less dense than water and were 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 sufficient amount of oil for analysis and anti-microbial tests was obtained. The dried oil was weighed and the percentage yield calculated.

Figure 4: Photograph of hydro-distillation

3.4 Chromatographic analysis

Analysis of the crude essential oil was done by gas chromatography coupled to a mass spectrometer (GC - MS). Samples of essential oils were diluted in methyl tert-butylether (MTBE) (1:100) and analysed on an Agilent GC-MSD apparatus equipped with an Rtx-5 SIL fused-silica capillary column ('Resets') (30m x 0.25 mm internal diameter, 0.25μm film thickness). Helium (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 ^{o}C and the transfer line at 280 ^{o}C. The column was maintained at 50 ^{o}C for 2 min and then programmed to 260 ^{o}C at 5 ^{o}C /min and held for 10 min at 260 ^{o}C. The MS was operated in the EI 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 in data bases (Adams, 1995 and Wiley & QuadLib 1607 GC-MS libraries), while Kovat indices (KI) were obtained from literature (Adams, 1995). The relative proportions of the essential oil constituents were expressed as percentages obtained by peak area normalization, all relative response factors being taken as one.

3.5 Determination of Kovat Indices (KI)

Relative retention values are determined in accordance with Kovat's method. The basis of his retention index *I* is the finding that within a homologous series of *n*-alkanes (*n*-paraffins) a linear relationship exists between the logarithm of the adjusted retention time and the number of carbon atoms in the compound. The retention of a compound to be investigated is then related to that of *n*-alkanes and one defines as follows:

*The retention time of a substance is equal to 100 times the carbon number of a hypothetical n-paraffin with the same retention time as the substance of interest (*Kellner *et al., 1998).*

In accordance with the definition, the *n*-alkanes have an index of 100 times the relevant carbon number at every temperature on all separation columns, for example *n*-hexane, 600 or *n*-octane, 800. In order to determine the retention time, the substance being examined is chromatographed in a mixture, which contains at least two *n*-alkanes. In so doing, the retention times of these *n*-alkanes must encompass the retention time of the compound of interest. Normally, a graphical procedure is not required in determining retention indices. Instead, retention data are derived by interpolation from a chromatogram of a mixture of the solute of interest and two or more alkane standards. Retention index for a normal alkane is independent of temperature and column packing (Skoog & Leary, 1992) (Figure 5). Calculation of the index is undertaken on the basis of the equation:

$$I = 100y\left(\frac{\log t_{RX} - \log t_{Rz}}{\log t_{R(z+y)} - \log t_{Rz}}\right) + 100z$$

With t_{Rx}, t_{Rz}, $t_{R(z+y)}$ retention times relevant for the substance being examined, x, for the *n*-alkane with the carbon number z, and for the *n*-alkane with the carbon number (z+y), with y being the number of additional carbon atoms compared to z *(*Kellner *et al.*, 1998).

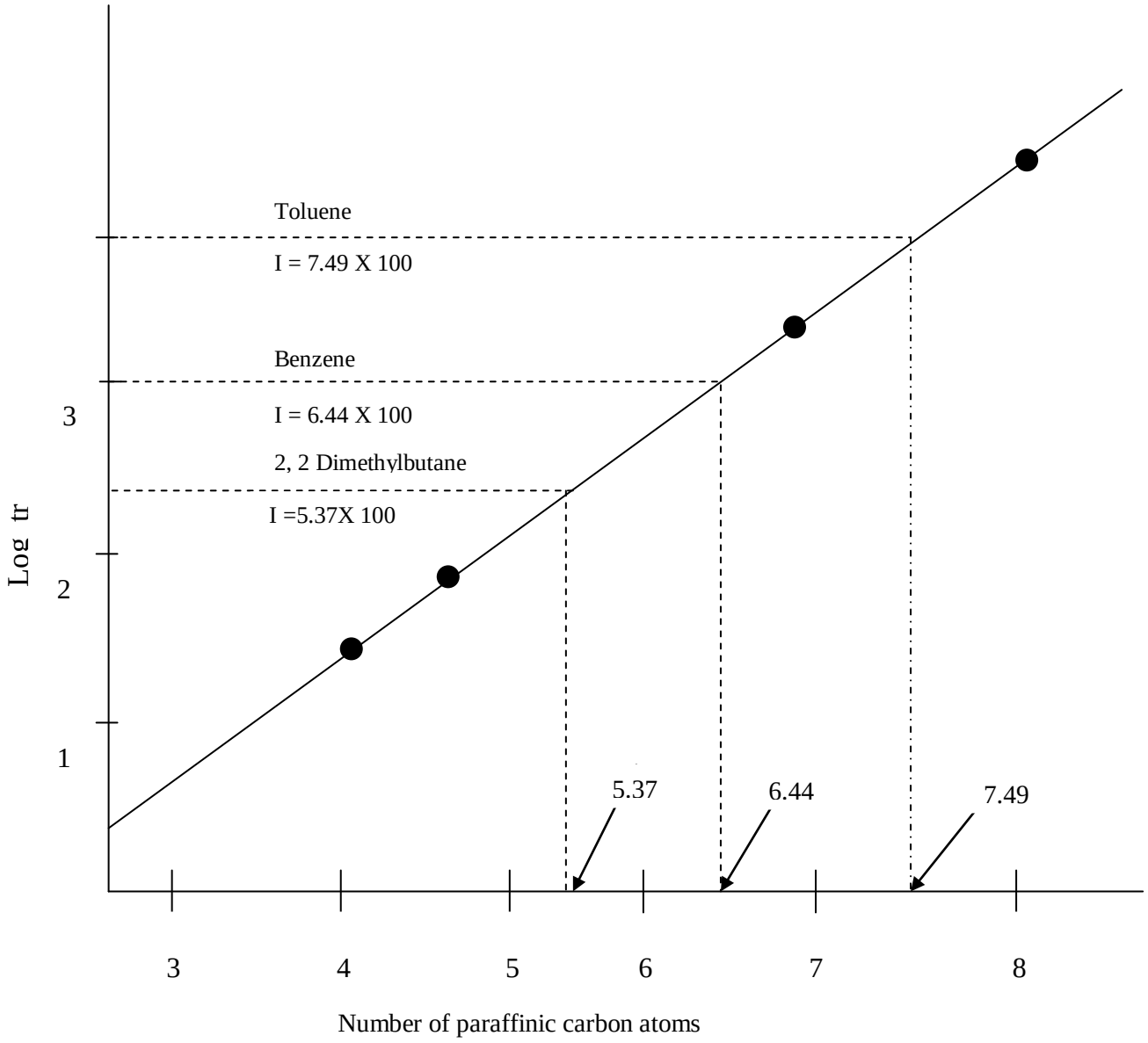


Figure 5: Graphical illustration of the method of determination of retention indices

3.6 Anti-microbial assays

Anti-microbial activity of the essential oils was tested using disc diffusion method (Baker *et al* 1983). The anti-bacterial activity was done using the American Test Culture Collection (ATCC) and clinical isolates from KEMRI. The gram-negative bacteria used were *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae, Pseudomonas aeruginosae* ATCC 27853, *Proteus mirabilis* and *Salmonella typhi* while gram–positive ones were S*taphylococcus aureus* ATCC 25923 and *Bacillus spp.* The anti-fungal activity of the essential oils was done on the pathogenic fungus *Candida albicans*.

The test organisms were inoculated in nutrient broth and incubated for 4 - 6 hours at 37 ^{o}C. To standardize the microbial inoculums for the susceptibility test, a barium sulphate standard equivalent to McFarland No. 0.5 standards or its optical equivalent was used (McFarland No. 0.5 standard gives cell density of $1.5x10^{8}$/ ml). The susceptibility of the test organism was done using disc diffusion method of Baker *et al.*, (1983). Anti-bacterial activity assay was done on Mueller Hinton agar while anti-fungal activity on Sabouraud Dextrose Agar (SDA). The media were reconstituted using distilled water and sterilized by autoclaving at 121 ^{o}C for 15 minutes then dispensed into Petri dishes aseptically and left to solidify and then stored in the refrigerator at 4 ^{o}C.

The freshly grown microbial cultures were inoculated on solid media. The blank sensitivity discs were divided into three Bijoux bottles and sterilized in the oven by air-drying at 160 ^{o}C for 1h. Test oils (1 mL) was impregnated into sterile blank disc and placed aseptically into the inoculated Petri dish. All these procedures were done in duplicate. The individual Petri dishes were covered to avoid any possible evaporation or contamination. Chloroamphenical and nyastatin were used as standard controls. The inoculated plates were incubated at 37 ^{o}C for 24

hours before the activity was determined. The activity of the test oils was established by the presence of measurable zones of inhibition (mm). The essential oil was tested for anti-bacterial or anti-fungal activity. The minimum inhibitory concentration (MIC) of the oils was determined by serial dilution.

3.6.1 Determination of the minimum inhibitory concentration (MIC)

Essential oils that exhibited anti-bacterial and anti-fungal activity in the screening assay were evaluated for efficacy by MIC determination. MIC determination was carried out by disc diffusion procedure. The freshly grown microbial suspension (approximately $1.5x10^8$ cells) was uniformly spread in the sterile Sabouraud Dextrose Agar dishes for fungi and Muller Hinton Agar dishes for bacteria using sterile cotton swabs. Sterile blank discs were impregnated with diluted essential oils. Serial dilution of essential oil was done using 10% Tween 80 in sterile distilled water. Sterile distilled water was used as control. The anti-bacterial or anti-fungal activity was established by the presence of measurable zones of inhibition (mm) after 24 h of incubation at 37 ^oC. The minimum inhibition concentration (MIC) was defined as the lowest concentration that inhibited growth of the micro-organisms detected visually. The same procedure was repeated for the standard anti-biotic (chloramphenical) and its MIC for each test organism determined.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Chemical composition and active constituents

4.1.1 Chemical composition and active constituents of *Tarchonanthus camphoratus*

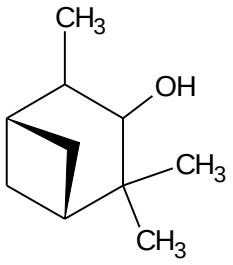
Fresh leaves of *Tarchonanthus camphoratus* (525 g), gave 1.04 g of pale yellow oil, (0.2 % w/w yield) with a moderate smell of camphor. The constituents identified by GC-MS analysis, their retention times and area percentages are summarized in Table I.

Table I: Chemical composition of *Tarchonanthus camphoratus* leaves essential oil

	Compound	KI	% Concentration	Method of identification
	Monoterpenes			
1.	Thujene	935	0.16	RI, GC-MS
2.	**α –Pinene**	**938**	**6.87**	**RI, GC-MS**
3.	**Camphene**	**952**	**3.76**	**RI, GC-MS**
4.	Sabinene	976	0.32	RI, GC-MS
5.	2- β-Pinene	978	2.03	RI, GC-MS
6.	δ-2-Carene	1006	0.93	RI, GC-MS
7.	1-Phellandrene	1011	0.48	RI, GC-MS
8.	α-Terpinene	1018	0.53	RI, GC-MS
9.	p-Cymene	1029	1.29	RI, GC-MS
10.	*DL*-Limonene	1030	1.18	RI, GC-MS
11.	**1,8-Cineole**	**1034**	**14.27**	**RI, GC-MS**
12.	γ-Terpinene	1062	1.02	RI, GC-MS
13.	*trans*-Sabinene hydrate	1068	0.64	RI, GC-MS
14.	**α** -Terpinolene	1089	1.03	RI, GC-MS
15.	Fenchone	1091	0.86	RI, GC-MS
16.	Z- β-Terpineol	1120	0.44	RI, GC-MS
17.	Nonanal	1122	0.51	RI, GC-MS
18.	**D-Fenchyl alcohol**	**1125**	**15.86**	**RI, GC-MS**
19.	***trans*-Pinene hydrate**	**1137**	**6.51**	**RI, GC-MS**
20.	*trans*-Pinocarveol	1142	0.36	RI, GC-MS
21.	1-Terpineol	1143	0.38	RI, GC-MS
22.	*trans*-Verbenol	1144	0.37	RI, GC-MS
23.	*exo*-Methyl camphenilol	1150	0.65	RI, GC-MS
24.	Borneol	1166	2.26	RI, GC-MS
25.	**Terpinen-4-ol**	**1177**	**4.74**	**RI, GC-MS**
26.	**α-Terpineol**	**1191**	**13.21**	**RI, GC-MS**
27.	Fenchyl acetate	1223	0.20	RI, GC-MS
	Sub-Total		**80.86**	
	Sesquiterpenes			
28.	α-Copaene	1376	0.32	RI, GC-MS
29.	β-Caryophllene	1418	0.63	RI, GC-MS
30.	Aromadendrene	1461	0.49	RI, GC-MS
31.	γ-Curcumene	1471	2.15	RI, GC-MS
32.	ar-Curcumene	1483	1.69	RI, GC-MS
33.	Valencene	1491	0.19	RI, GC-MS
34.	γ-Cadinene	1513	0.32	RI, GC-MS
35.	δ-Cadinene	1524	0.74	RI, GC-MS
36.	Caryophyllene oxide	1582	0.82	RI, GC-MS
37.	α-Cadinol	1653	1.75	RI, GC-MS
38.	β-**Eudesmol**	**1655**	**5.79**	**RI, GC-MS**
	Sub-Total		**14.89**	
	Grand Total		**95.75**	

KI- Kovat index **RI – Retention index**

Out of 45 peaks (99.83% of the oil), thirty eight components were identified, (95.75% of the total oil). The oil was dominated by monoterpenes, (80.86% of the oil) while the remaining portion was made up of sesquiterpenes. There was high percentage of oxygenated monoterpenes (62.29%) consisting of: fenchol (15.86%) **(22)**, 1, 8-cineole (14.27%) **(16)**, α-terpineol (13.21%) (**10**), *trans*-pinene hydrate (6.51%) **(23)** and terpinen-4-ol (4.74%) **(27**). The monoterpene hydrocarbons obtained in high yield were α-pinene (6.87%) **(19)** and Camphene (3.76%) **(24).** A similar observation was observed in sesquiterpenes with β-eudesmol (5.79%) **(25)** and α-cadinol (1.75%) as the major oxygenated sesquiterpene in the oil. Other sesquiterpene hydrocarbons, such as δ-curcumene (2.15%) and α-curcumene (1.69%) **(26)** were also present in appreciable amounts.



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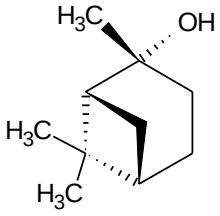

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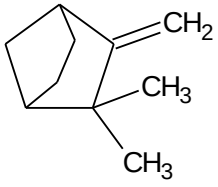

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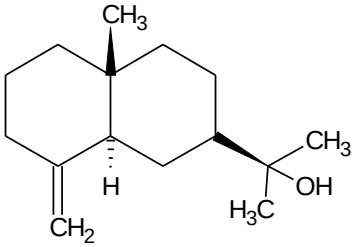

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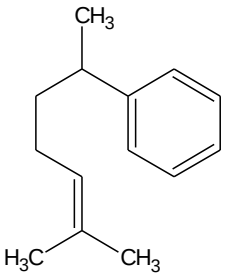

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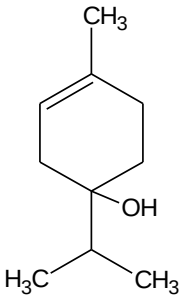

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Previous analysis of the leaf oil of *T. camphoratus* reported the three oxygenated monoterpenes as the major constituents, but with a higher concentration of fenchol (29.1%) **(22)** and 1, 8-cineole (16.5%) **(16)** while α-terpineol (8.5%) (**10**) was found in lower concentration, camphor was present in only minor quantity (0.4%) (Mwangi & Achola, 1994). The variation in the

chemical composition of *Tarchonanthus camphoratus* essential oil is thought to be due to: (i) the harvest season and plant phenological stages including vegetative, early blooming, full blooming and seeding. Plants contain a certain compound as the major constituent at the beginning of flowering while others appear after sowing. (ii) The extraction methods may also affect the composition of the oils. It is claimed that yields are highest using supercritical CO_2 followed by liquid CO_2 and then water (Lachowicz *et al.,* 1997). A hydrodistilled material contains a significantly larger proportion of the lower boiling point hydrocarbon and oxygenated terpenes. On the other hand, the CO_2 extracts contain a large number of unidentified high boiling point constituents (Lachowicz *et al.,* 1997). (iii) Different milling techniques might induce modification in the composition of the vegetable matrix and may have an adverse effect on the content of thermally labile compounds (Reverchon *et al.,* 1992). Other factors are discussed under literature review.

4.1.2. Chemical composition and active constituents of *Leonotis nepetifolia*

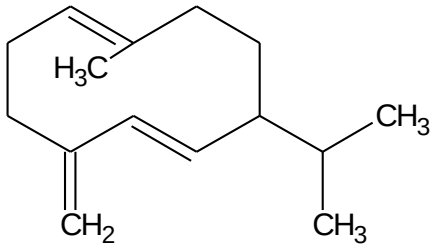
The colourless essential oil of *Leonotis nepetifolia* with a characteristic smell was obtained in 0.004% w/w yield (0.022 g) from 554 g of fresh leaves. The chemical constituents (identified by GC-MS analysis), their retention times and relative amounts are summarized in Table 2. The compounds identified were 36, constituting 94.80 % of the oil. The oil was dominated by sesquiterpenes (75.32%), and the monoterpenes (19.48%).

Table 2: **Chemical composition of *Leonotis nepetifolia* oil**

	Compound	KI	Concentration %	Method of identification
	Monoterpenes			
1.	α-**Thujene**	**935**	**2.12**	RI, GC-MS
2.	α-Pinene	938	1.71	RI, GC-MS
3.	Sabinene	976	0.89	RI, GC-MS
4.	β- Myrcene	990	0.21	RI, GC-MS
5.	1-Phellandrene	1011	0.25	RI, GC-MS
6.	p-Cymene	1029	1.29	RI, GC-MS
7.	dl-Limonene	1030	1.67	RI, GC-MS
8.	1,8-Cineole	1034	0.48	RI, GC-MS
9.	***cis*-Ocimene**	**1037**	**4.79**	RI, GC-MS
10.	***trans*- Ocimene**	**1050**	**4.32**	RI, GC-MS
11.	γ-Terpinene	1062	0.31	RI, GC-MS
12.	α-Terpinolene	1089	0.17	RI, GC-MS
13.	Linalool	1098	0.98	RI, GC-MS
14.	Terpinen-4-ol	1177	0.29	RI, GC-MS
	Sub-Total		**19.48**	
	Sesquiterpenes			
15.	α-Cubebene	1351	0.17	RI, GC-MS
16.	**α-Copaene**	**1376**	**3.43**	RI, GC-MS
17.	β-Bourbonene	1384	1.15	RI, GC-MS
18.	β- Cubebene	1390	0.87	RI, GC-MS
19.	**β -Caryophyllene**	**1418**	**4.33**	RI, GC-MS
20.	*epi*-Bicyclosesquiphellandrene	1423	0.52	RI, GC-MS
21.	α-Humulene	1454	1.33	RI, GC-MS
22.	Aromadendrene	1461	0.35	RI, GC-MS
23.	γ-Muurolene	1477	0.85	RI, GC-MS
24.	**Germacrene D**	**1487**	**37.16**	RI, GC-MS
25.	**Bicyclogermacrene**	**1494**	**3.03**	RI, GC-MS
26.	α-Muurolene	1499	0.83	RI, GC-MS
27.	β-Bisabolene	1508	0.45	RI, GC-MS
28.	γ –Cadinene	1513	0.35	RI, GC-MS
29.	**δ-Cadinene**	**1524**	**9.66**	RI, GC-MS
30.	Germacrene B	1556	0.75	RI, GC-MS
31.	**Germacrene-D-4-ol**	**1574**	**6.02**	RI, GC-MS
32.	Caryophyllene oxide	1582	0.39	RI, GC-MS
33.	τ-Cadinol	1641	0.66	RI, GC-MS
34.	τ-Muurolol	1645	0.95	RI, GC-MS
35.	α-Cadinol	1653	1.81	RI, GC-MS
36.	Phytol	1949	0.26	RI, GC-MS
	Sub-Total		**75.32**	
	Grand Total		**94.80**	

KI- Kovat index **RI – Retention index**

The major part of this oil was composed of volatile terpene hydrocarbons and a small portion by their oxygenated derivatives. The oxygenated sesqui- and monoterpenes accounted for only 10.09 and 1.75%, respectively. Considering only components with concentrations more than 2%, the major components were: germacrene D (37.16%) **(28)**, germacrene-D-4-ol **(29)**, bicyclogermacrene (3.03%) **(30)**, α-copaene (3.43%) **(31)**, β-caryophellene (4.33%) **(18)**, α-thujene (2.12%) **(32)**, *Trans*-ocimene (4.32%) **(33a)** and *cis*-ocimene (4.79%) **(33b)**. An earlier analysis of the essential oils from the leaves of *L. leonurus* and *L. ocymifolia* indicated that while the major constituent is also germacrene D **(28)**, their concentration were far much lower in both species at 18.9 and 21.5%, respectively. Other significant qualitative differences were observed in β-caryophyllene, **(18)** limonene **(17)**, *cis*-ocimene (**33b**) and α-humulene (Oyedeji & Afolayan, 2005).



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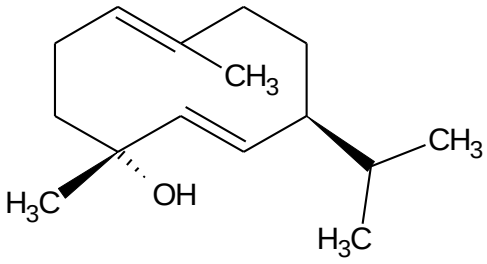

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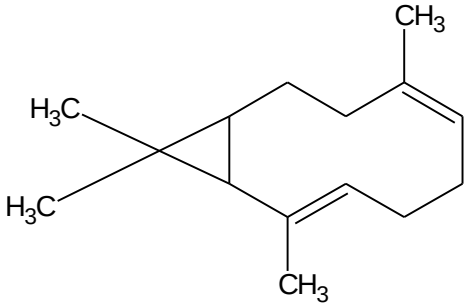

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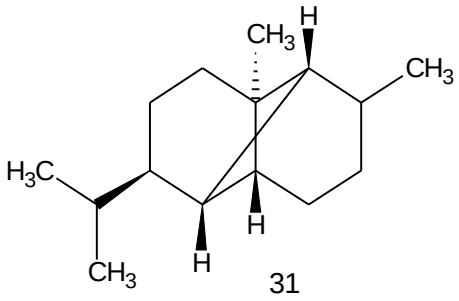

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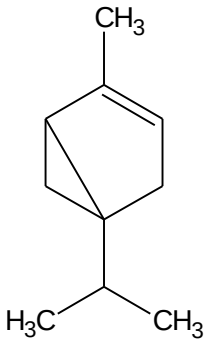

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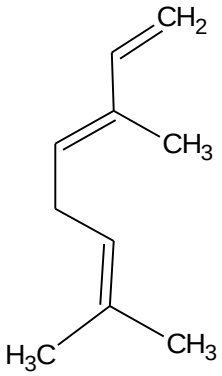

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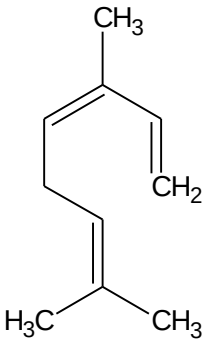

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4.1.3 Chemical composition and active constituents of *Satureja biflora*

Fresh leaves of *Satureja biflora* (534 g) gave 1.06 g of pale yellow sweet smelling essential oil, (0.2% w/w yield). The chemical constituents identified by GC-MS analysis, their retention times and relative amounts are summarized in Table 3. Twenty-two components were identified (99.29%). The oil was dominated by monoterpene hydrocarbons (62.02%). The monoterpene fraction was characterized by a high percentage of linalool (50.60%) (7). This *Satureja* species can be classified as linalool chemo-type.

Table 3: Chemical composition of *Satureja biflora* leaves essential oil

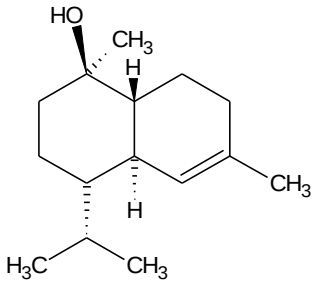
	Compound	KI	Concentration %	Method of identification
	Monoterpenes			
1.	Sabinene	976	0.49	RI, GC-MS
2.	β-Pinene	978	1.96	RI, GC-MS
3.	**β- Ocimene**	**1050**	**2.25**	**RI, GC-MS**
4.	*cis*-Linalool Oxide	1072	1.91	RI, GC-MS
5.	*trans*- Linalool oxide	1089	1.49	RI, GC-MS
6.	**Linalool**	**1098**	**50.60**	**RI, GC-MS**
7.	Terpinen-4-ol	1177	0.52	RI, GC-MS
8.	**α-Terpineol**	**1191**	**2.80**	RI, GC-MS
	Sub-Total		**62.02**	
	Sesquiterpenes			
9.	**β- Bourbonene**	**1384**	**2.33**	**RI, GC-MS**
10.	β- Elemene	1393	0.72	RI, GC-MS
11.	β-Caryophyllene	1430	1.98	RI, GC-MS
12.	β- Farnesene	1458	0.59	RI, GC-MS
13.	**Germacrene D**	**1487**	**10.63**	**RI, GC-MS**
14.	**δ-Cadinene**	**1524**	**2.19**	**RI, GC-MS**
15.	Nerolidol	1566	1.02	RI, GC-MS
16.	***endo*-1-bourbonanol**	**1570**	**2.14**	**RI, GC-MS**
17.	Caryophyllene oxide	1582	1.74	RI, GC-MS
18.	**τ-Cadinol**	**1641**	**2.17**	**RI, GC-MS**
19.	**α-Cadinol**	**1653**	**4.53**	**RI, GC-MS**
	Sub-Total		**30.04**	
	Aliphatic Hydrocarbons			
20.	1-Octen-3-ol	979	1.13	RI, GC-MS
21.	Palmitic acid	1970	1.62	RI, GC-MS
22.	**Linoleic acid**	**2030**	**4.48**	RI, GC-MS
	Sub-Total		**7.23**	
	Grand Total		**99.29**	

KI- Kovat index, RI- Retention Index

Considering components with concentration of ≥ 2%, the other major monoterpenes were: α-terpineol (2.80%) (10) and β-ocimene (2.25%) **(33a)**. The major sesquiterpene component was germacrene D (10.63%) **(28)**. Other sesquiterpenes present in appreciable amounts were: α-cadinol (4.53%) **(34)**, β-bourbonene (2.33%) **(35)**, δ-cadinene (2.19%) **(36)**, τ-cadinol (2.17%) **(37)**, *endo*-bourbonanol (2.14%) **(38)** and β-caryophyllene (1.98%) **(18)**. The aliphatic hydrocarbons represent only 7.23% of the oil, of which 4.48% is taken by linoleic acid (**39**).

Previous investigation showed variations between chemical compositions of different essential oils of *Satureja* species. The main component of the essential oils of *S. icarica*, *S. boissieri*

and *S. pilosa* is carvacrol at 59.2, 44.8 and 42.1% respectively (Azaz *et al.*, 2002). The main constituent of *S. brownie* oil from Venezuela is pulegone (64.3%), (Rojas & Usubillaga, 2002) while that of *S. paruvifolia* oil from Argentina is piperitone oxide (Viturro *et al.*, 2000). Germacrene D **(28)** has been detected to be the main compound of *S. coerulea* (Tumen *et al.*, 1998). The main components of *S. mutica*, *S. macrantha* and *S. intermedia* growing in Iran were found to be carvacrol (30.9%), *P*- cymene (25.8%) and thymol (32.3%) respectively (Sefidkon & Jamzad, 2005).



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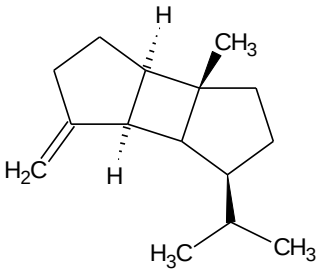

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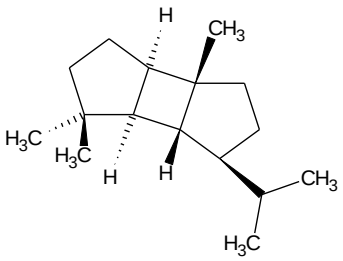

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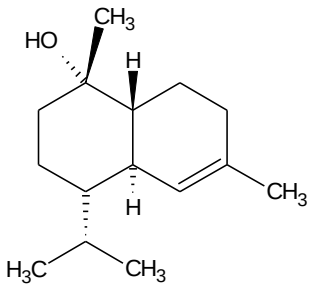

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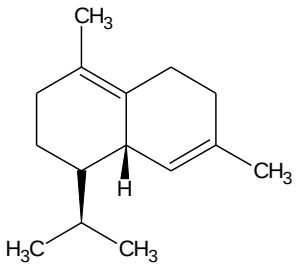

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4.2 Anti-microbial results

4.2.1 Anti-bacterial effects of *Tarchonanthus camphoratus*

In vitro studies showed the effectiveness of *T. camphoratus* oil in inhibiting several common pathogenic strains. The oil was found to be active against all the strains of bacteria except for *Pseudomonas aeruginosa*. However, the activity of the oil varies with the concentration and the micro-organism. Although the concentrations of the oil were generally in the range of 100 times more than the standard anti-biotic (chloramphenical), they showed marked anti-bacterial activity as evidenced by their zones of inhibition as shown in Table 4.

Among the gram-negative bacteria, the oil was very active against *E. coli*. The response to *E. coli* was more or less the same at 9000 μg concentration as that of chloramphenical (30 μg). *Pseudomonas aeruginosa* was considered resistant to the essential oil and the standard antibiotic chloramphenical since no inhibition zone was observed. This micro-organism is also reported to show resistance to other anti-microbial agents and diterpenes present in *Salvia* species (Darias *et al*., 1990).

Table 4: Anti-microbial activity of the essential oil of *Tarchonanthus camphoratus*

		Inhibition (mm)					STD[b]	MIC mg/ml	
Micro-organism		**Essential oil (μg x 10^2)**						**EO[c]**	**STD[b]**
Gram-ve bacteria	**Culture collection Ref. No.**	**90.00**	**67.50**	**45.00**	**22.50**	**18.00**	**chloramphenical 30 μg**		
E. coli	ATCC 25922	26. 5 ± 1.5	11.0 ± 1	10.0 ± 0	8.0 ± 0	0	30.0 ± 0	112.5	25.0
S. typhi	[a]KEMRI	11.5 ± 0.5	0	0	0	0	11.0 ± 1	900.0	25.0
K. pneumoniae	[a]KEMRI	13.0 ± 2	11.0 ± 1	0	0	0	25.0 ± 0	450.0	22.5
P. mirabilis	[a]KEMRI	13.0 ± 0	11.0 ± 0	10.0 ± 0	9.0 ± 0	0	8.0 ± 0	225.0	_
P. aeruginosae	ATCC 27853	0	0	0	0	0	0	_	_
Gram+ve bacteria									
S. aureus	ATCC 25923	26.5 ± 1.5	17.5 ± 2.5	15.5 ± 1.5	10.0 ± 1	9.0 ± 1	27.0 ± 2	100.0	31.3
Bacillus spp.	[a]KEMRI	21.0 ± 1	15.5 ± 0.5	10.0 ± 1	8.5 ± 0.5	0	30.0 ± 0	128.6	26.3
Fungus							**Nyastatin 30 μg**		
Candida albicans	[a]KEMRI	20.5 ± 0.5	15.0 ± 0	12.0 ± 0	10.5±0.5	7.5 ± 0.5	13.0 ± 0	112.5	_

a. Clinical isolate from Kenya Medical Research Institute (KEMRI)
b. Chloramphenical
c. Essential oil

N/B Control experiment showed no inhibition, chloramphenical and nyastatin used as standards.

The reference antibiotic chloroamphenical showed the highest anti-microbial activity against all the micro-organisms except *Pseudomonas aeruginosa* and *S. typhi*, which were resistant to it. Interestingly, *Tarchonanthus camphoratus* oil exhibited activity against *Salmonella typhi* at the highest concentration and the oil was superior to the standard antibiotic (chloramphenical) in this particular instance. The oil showed more or less similar activity across the

concentration range to *K. pneumoniae* and *P. mirabilis.* The essential oil also showed pronounced activity against gram-positive bacteria (*Bacillus spp* and *Staphylococcus aureus*). The MIC of the oil for gram-negative bacteria ranged from 112.5 to 900 mg/ml and 100 to 128.6 mg/ml for gram-positive bacteria.

Because of the appearance of bacterial resistance to anti-microbial agents, more effort is being made to find alternative anti-microbial components. It had been suggested that natural products are preferable to synthetic ones. The current study showed the role of essential oil of *T. camphoratus* as strong anti-bacterial agents against *S. aureus, E. coli, P. mirabilis, S. typhi, K. pneumoniae* and *Bacillus spp* under laboratory condition, and may be considered as a useful lead in the search of new drugs.

4.2.2 Anti-fungal effects of *Tarchonanthus camphoratus*

Anti-fungal activity of the oil was tested against *Candida albicans*. The oil showed remarkable activity against the fungus as compared to the standard nyastatin (Table 4). The MIC for the fungus was 112.5 mg/ml. The activity of the oil at 6750µg concentration was comparable to that of the standard drug (nyastatin) as shown by the inhibition zones in Table 4.

4.2.3 Anti-bacterial effects of *Leonotis nepetifolia*

The essential oil of *Leonotis nepetifolia* was evaluated for anti-microbial properties and found to be active against the entire gram-positive and two gram-negative bacteria *S. typhi a*nd *Proteus mirabilis as* shown by the inhibition zones in Table 5.

Table 5: Anti-microbial activity of the essential oil of *Leonotis nepetifolia*

						MIC mg/ml	
Micro-organism	**Culture collection Ref No.**	**Essential oil concentration ($\mu g \times 10^2$)**			**chloramphenical**	**EO[c]**	**chloramphenical**
Gram-ve bacteria		**70.0**	**52.5**	**35.0**	**30 μg**		
S. Typhi	[a]KEMRI	14.5 ± 1.5	10.0 ± 0	0	11.0 ± 1.0	525.0	25.0
P. Mirabilis	[a]KEMRI	8.0 ± 0	0	0	0	700.0	–
Gram+ve bacteria							
S. Aureus	ATCC 25923	13.0 ± 1.5	9.0 ± 1.0	8.0 ± 1.0	25.0 ± 0	350.0	31.3
Bacillus ssp.	[a]KEMRI	13.0 ± 1.0	10.0± 0	9.0 ± 1.0	28.0 ± 2.0	350.0	26.3
Fungus							
Candida albicans	[a]KEMRI	14.5 ± 0.5	11.0±0.5	0	0	525.0	–

a. Clinical isolates from Kenya Medical Research Institute (KEMRI)
b. Chloramphenical (standard)
c. Essential oil

NB Control experiment showed no inhibition, chloramphenical and nyastatin used as standards.

The microbes *Escherichia coli, K. pneumoniae* and *P. aeruginosae* were resistant to the oil since no inhibition zones were observed. Among the gram-negative bacteria, the oil was very active against *S. typhi*. The response of *S. typhi* was more or less the same as that of the standard antibiotic chloramphenical (30μg). *Proteus mirabilis* was resistant to the reference antibiotic chloramphenical since no inhibition zone was observed but the oil exhibited activity against it. The oil had relatively the same activity level in gram-positive bacteria (*S. aureus* and *Bacillus spp),* which was about 50% of the standard antibiotic. The MIC for the gram-positive bacteria (*S. aureus* and *Bacillus spp),* was 350mg/ml and 525 and 700mg/ml for *S. typhi* and *P. mirabilis* respectively.

4.2.4 Anti-fungal effects of *Leonotis nepetifolia*

The oil showed remarkable anti-fungal activity against *Candida albicans.* Some components of the oil such as α-pinene **(19)** and limonene **(17)** are known to possess anti-fungal properties discussed under literature review. The MIC value was 525mg/ml while no inhibition zone was observed for the standard drug (nyastatin).

4.2.5 Anti-bacterial effects of *Satureja biflora*

The essential oil of *Satureja biflora* was found to be active against all the bacteria strains except for *P. aeruginosa* (Table 6). The activity of the oil varied with concentration and the type of bacteria. Although the concentrations of the oil were generally in the range of 100 times more than the standard anti-biotic (chloramphenical), they showed pronounced anti-bacterial activity as evidenced by their zones of inhibition (Table 6). Among the gram-negative bacteria, the oil was much active against *S. typhi.* The response to *S. typhi* was three times more at 75 x 10^2 μg as that of chloramphenical (30 μ g). The inhibition zones of the neat oil against gram-positive bacteria compared very well with those of the standard anti-biotic. The oil showed more or less similar activity across the concentration range to *E. coli* and *P. mirabilis.* The MIC of the oil for gram-negative bacteria range from 83.3 to 250 mg/ml while that of gram-positive bacteria range from 93.8 to 107 mg/ml. The MIC values for chloramphenical ranged from 22.5 to 25 mg/ml for gram-negative and 26.3 to 31.3 mg/ml for gram-positive.

Table 6: Anti-microbial activity of the essential oil of *Satureja biflora*

		Inhibition zone (mm)										
		Essential oil μg x 10^2								STD[b]	MIC mg/ml	
Micro-organism	**Culture collection & Ref No.**	**75.0**	**37.5**	**25.0**	**18.8**	**15.0**	**12.5**	**10.7**	**9.4**	**8.3**	**30 μg**	**EO[c]**	**STD[b]**
Gram-ve bacteria													
E. coli	ATCC 25922	15 ± 0	14 ± 0	13 ± 0.5	12 ± 2.0	11 ±1.0	10 ±1.0	9 ± 0.5	8 ± 1.0	7 ± 0	28± 1.5	83.3	25.0
S. typhi	[a]KEMRI	31 ± .5	15 ± 2.5	14 ± 1.0	12 ± 0.5	10 ± 0	9 ± 0.5	0	0	0	10± 1.0	125.0	25.0
K. pneumoniae	[a]KEMRI	12 ±0.5	11 ± 0.5	9 ± 0.5	0	0	0	0	0	0	25 ± 0	250.0	22.5
P. mirabilis	[a]KEMRI	15 ± 0	14 ± 0.5	13 ± 0.5	12 ± 0	11 ± .5	10 ± 0	9 ± 1.0	8 ± 1.0	7±0.5	8 ± 0	83.3	_
P. aeruginosae	ATCC 27853	0	0	0	0	0	0	0	0	0	0	_	_
Gram-positive bacteria													
S. aureus	ATCC 25923	24 ± 0	20 ± 0	14 ± 0.5	12 ± 1.0	11 ±2.0	10 ±0.5	10 ±0.0	8 ± 0.5	0	24± 1.0	93.8	31.3
Bacillus spp.	[a]KEMRI	34 ± 2	32 ± 1.0	22 ± 1.0	17 ± 1.5	12 ±1.0	10 ±1.0	9 ± 1.0	0	0	26± 2.0	107.0	26.3
Fungus													
Candida albicans	[a]KEMRI	15 ± 0	12 ± 0	11 ± 0.5	10 ± 0	9 ± 0.5	7 ± 1.0	0	0	0	10 ±0.5	125.0	_

a. clinical isolate from Kenya Medical Research Institute (KEMRI)
b. Chloramphenical & Nyastatin
c. Essential oil,

NB Control experiment showed no inhibition, chloramphenical and nyastatin used as standards.

4.2.6 Anti-fungal effects of *Satureja biflora*

The anti-fungal assay showed that the essential oil possesses low activity as compared to anti-bacterial activity as shown by the inhibition zones in Table 6. The MIC was 125 mg/ml while the anti-bacterial MIC ranged from 83.3 to 250mg/ml.

4.3 Discussion

The activity of an essential oil would be expected to relate to the respective composition of the plant volatile oil, the structural configuration of the chemical constituents of the oil, their functional groups and possible synergistic interactions of the components. A correlation of the anti-microbial activity of essential oil, percentage composition, and chemical structure of the components, functional groups and configuration has been done, and a number of observations were suggested (Knobloch *et al.,* 1989). The solubility of essential oils and their terpenoid compounds in water is directly related to their anti-microbial activity on whole cells (Knobloch *et al.,* 1989). The variations in the fungicidal and bactericidal action of essential oil components seem to rely on their solubility and lipophilic properties (ability to penetrate the chitin-based cell walls of fungal hyphae) (Knobloch *et al.,* 1989).

The essential oil obtained from *Tarchonanthus camphoratus* had a high percentage of oxygenated monoterpenes (62.29%) that accounts for the good activity against both bacterial strains and fungi. Presence of an acetate moiety in the structure increases the activity of parent compound (Knobloch *et al.,* 1989). *Tarchonanthus camphoratus* oil had fenchyl acetate (0.20%) in minor quantity, which could have contributed to the pronounced activity of the oil. Borneol has been found to be less active than its acetate but active against *Bacillus subtilis, Aermonas hydrophila, Beneckea natriegens, Escherechia coli, Flavobacterium suaveolens* and *Serratia marcescens* (Pelczar *et al.,* 1988). It was also present in the oil in fairly good amount (2.26%), and shown to possess anti-microbial properties.

Tarchonanthus camphoratus had a number of alcohols, fenchol **(22)**, Z-β-terpineol, terpin-4-ol (**27**) α-terpineol (**10**) , 1-terpineol and α-cadinol (**34**). Alcohols are known to possess bactericidal rather than bacteriostatic activity against vegetative cells (Knobloch *et al.,* 1989). The alcohols exhibit activity against micro-organism by potentially acting as either protein denaturing agents or solvent dehydrating agents (Pelczar *et al.,* 1988). Fenchol **(22)** α-terpineol (**10**) and 1, 8-cineole **(16)** are known to posses anti-microbial properties. Terpinen-4-ol **(27)** that occurs in appreciable amounts in this oil is also reported to show activity against *S. aureus, E. coli, S. eperdermis and C. albicans*. It is also responsible for the broad-spectrum anti-microbial activity of the essential oil of *Melaleuca alternifolia* (tea tree oil) (Sean *et al., 2001*). *Trans*-pinene hydrate (**23**) which occurred to an extent of (6.51%) in *T. camphoratus* essential oil is known to exhibit anti-microbial properties due to its high solubility in water (Knobloch *et al.,* 1989). Caryophyllene oxide, although a minor constituent in the oil of *T. camphoratus* is known to possess efficient anti-bacterial properties. Another minor monoterpene alcohol, linalool **(17)** is also known to exhibit a wide range of anti-bacterial and anti-fungal activity (Pattnaik, *et al., 1997*).

α-Pinene (**19**), which was found in appreciable amounts in the oil of *T. camphoratus*, has been reported to exhibit anti-fungal activity (Magiatis *et al* 1999). Therefore the remarkable anti-fungal activity of this oil could be due to the presence of α-pinene (**19**). The leaves of the plant are used for the treatment of allergies and sinuses by boiling then, the patient inhales vapour. Infusions, tinctures or decoctions are orally taken and vapours from boiling green material either inhaled or directed as a fumigant to inflamed joints (Kokwaro, 1993), this means the active compounds are volatiles in steam.

Leonotis nepetifolia oil showed poor anti-bacterial effects, but good anti-fungal effects. The oil had a high percentage composition of sesquiterpenes (75.32%) and a low percentage of monoterpenes (19.48%). Sesquiterpenes possess weak anti-microbial activity than monoterpenes (Consentino *et al.,* 1999). This may be attributed to their large molecular size that may not penetrate the microbial cell wall and interact with the microbial enzymes and denaturing them (Consentino *et al.,* 1999). Previous studies revealed that *L. leonurus* and *L. ocymifolia* growing in Eastern Cape of South Africa exhibit higher anti-bacterial activity against gram-positive (*B. subtilis, B. cereus, Micrococcus kristinae, S. aureus, S. epidermis)* and gram-negative (*E. coli, P. aeruginosa, Shigella sonnei*) bacteria with MIC values ranging from 1.25-0.039 mg/ml (Oyedeji & Afolayan, 2005). These major disparities in the anti-microbial activities, of the essential oils of these two *Leonotis* species as compared to that of *L. nepetifolia* can be explained in terms of the significant differences in chemical composition.

Research has shown that gram-negative micro-organisms are less susceptible to essential oils than gram–positive ones because they posses outer membrane surrounding the cell membrane (Ratledge &Wilkinson, 1988) which restricts diffusion of hydrophobic compounds through its lipopolysacharide covering (Vaara, 1992). This explains why the MIC for gram-positive was lower than the MIC for gram-negative bacteria. The MIC for *P. mirabilis* was double that of *S. aureus* and *Bacillus spp*. *Pseudomonas aeruginosa* is less susceptible to the anti-microbial properties of essential oils than many bacteria and its tolerance is considered to be due to its outer membrane (Cox *et al.,* 2000).

Some components which appeared in low concentration in *L. nepetifolia* oil, such as α-pinene (1.71%) **(19)** are known to exhibit anti-microbial and anti-fungal properties (Lis-balchin *et al.,*

1998). Others like β-myrcene (0.89%), limonene (1.67%) **(17)**, *P*-cymene (1.29%), terpinen-4-ol (0.29%) (**27**) are also known to exhibit anti-bacterial properties. 1, 8-cineole (**16**) though a minor constituent in the oil is known to possess anti-bacterial activity. It was present in essential oil from the genus Achillea, and exhibits anti-bacterial properties (Prokopios *et al.*, 2001). Limonene **(17)** that was also present in minor quantities (1.67%) in the essential oil of *L. nepetifolia* has anti-microbial properties (Kandi, 1994). β-Caryophellene **(18)** which is one of major components (4.33%) in the oil has been reported to exhibit low anti-microbial activity, but its oxide is highly active against bacteria and fungi (Alginiannis *et al*., 2000). Bicyclogermacrene **(30)** one of the major components in *L. nepetifolia* essential oil was also found in *Eucalyptus* oil and has been known to treat a large number of diseases, cuts sores, ulcers, coughs, throat infection, cold and fever. This compound is also reported to possess anti-viral, anti-fungal and anti-bacterial properties (Caccioni, 1998).

The oil investigated by GC-MS showed that the major constituents of *Satureja biflora* essential oil were terpene alcohols (linalool). Linalool (**7**) has been found to have anti-microbial activity for example linalool from lavender essential oil had anti-mcrobial properties against 17 out of 18 bacteria and 10 out of 12 fungi. In a similar study linalool was found to have anti-microbial activity against all microbes tested except *P. aeruginosae* and *Candida albicans* (Carsons & Riley, 1995). Knobloch *et al.* (1989) evaluated the anti-microbial activity of essential oil components against gram-negative bacteria (*Enterobacter aeroginosa and P. vulgaris)* gram-positive *bacteria* (*S. aureus* and *B. subtilis*) and fungi (*Aspergillus flavus, A. niger, A. ochraceus and Penicillicum expansum).* They found that linalool, with its high water solubility, had a significant anti-microbial activity. Linalool (**7**) is also known to inhibit spore germination and fungal growth. The inhibition of sporulation appeared to arise from respiratory suppression of arial mycelia. The tertiary alcohol linalool is active against the test

micro-organisms potentially acting as either a protein-denaturing agent or as a solvent dehydrating agent (Knobloch *et al.,* 1989). Previous studies have also shown that secondary alcohols such as borneol, 2-octanol, *L*-menthol and tertiary alcohols like linalool possesses a markedly lower anti-fungal activity as compared to primary alcohols (Knobloch *et al.,* 1989). Therefore since alcohols have high water solubility they may have contributed to the significant anti-microbial activity of *Satureja biflora* oil. Specific functional group and the interference with membrane–associated enzyme protein may also affect the result (Knobloch *et al.,* 1989).

Other terpene hydrocarbons such as sabinene, ocimene, β-elemene and β-farnesene also present in the *S. biflora* oil are known to have less anti-microbial activity than phenols and alcohols when used alone. This is because they lack a hydroxyl group (OH), which is thought to play an important role in the anti-microbial activity (Ultee *et al*., 2002). Other compounds present in the oil though in minor concentration have previously been known to possess anti-microbial properties include β-caryophellene **(18)**, which also has anti-inflammatory and anti-carcinogenic activities (Tellez *et al.,* 1999; caryophellene oxide is known to possess anti-microbial properties against a wide range of bacteria and fungi. Terpinen-4-ol (0.52%) (**27**) has a broad-spectrum activity against micro-organisms (Sean *et al*., 2001).

Satureja biflora oil had 30.04% of sesquiterpenes and 62.02% monoterpenes, sesquiterpenes are known to possess weak anti-microbial activity (Consentino *et al,*. 1999). In addition, it has already been shown that the anti-microbial activity of volatile compounds results from combined effect of direct vapour absorption on micro-organisms and indirect effect through the medium that absorbed the vapour (Moleyar & Narasimham, 1986).

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

- The essential oil from the three aromatic medicinal plants can be classified into three chemo types according to the content of the major compounds. *Tarchonanthus camphoratus* oil was classified as fenchol chemo-type, *Leonotis nepetifolia* oil as Germacrene D chemo-type while that of *Satureja biflora* oil classified as linalool chemo-type.
- The fenchol and linalool-type oils had significant anti-bacterial and anti-fungal activities. This could be attributed to the presence of alcoholic compounds in these oils.
- There is a relationship between the percentage yield (w/w), anti-microbial activity and chemical composition of essential oils. The oil with high monoterpene content exhibited good anti-microbial activity while the oil from *Leonotis nepetifolia* had low monoterpene concentration and it showed weak anti-microbial activity.
- All the three oils had good anti-fungal properties; therefore essential oil from plants can be used as a useful lead in the development of a drug for treatment of fungal infection since they are used for plant defence against fungus.
- The bioactivity results 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 use of these plants as traditional herbal medicine is also scientifically rationalized as shown by the activity of their essential oil against bacteria and fungi.
- The chemical composition of the essential oils of the plants *Tarchonanthus camphoratus* and Satureja *biflora* contain compounds, which are known to relieve stress (like linalool). Therefore communities should be advised to use the essential oil to provide cheap aromatherapy for themselves. This could be done by boiling the leaves from the aromatic medicinal plants and inhaling the steam which contains the volatile essential oils.
- The research finding adds value to the three aromatic, medicinal plants under study.

5.2 Recommendations

- Other separation and spectroscopic techniques (such as UV, IR and NMR) could be done in future so as to identify the unknown compounds, which were not found in the GC-MS library.
- Further toxicological and clinical studies are required to prove the safety of the essential oils as medicine.
- Further studies could also be carried out on therapeutic application of volatile essentials oils.
- Essential oils could have greatest potential use as a food preservative since they have been known to inhibit bacteria, fungi and yeast, therefore research on this could be carried out.
- The shelf life of essential oil should also be determined, in order to assess their rate of deterioration if it is to be used as medicine.

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