THE ESSENTIAL OIL FROM *CYMBOPOGON VALIDUS*

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*** SUBMISSION APPROVED FOR EXAMINATION**

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I, **Nelisha Naidoo** (Student Number: 20428829) and **Prof. Bharti Odhav** and **Prof. H. Baijnath** do hereby declare that in respect of the following dissertation:

The essential oil from *Cymbopogon validus*

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABTS	2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)
BSL	Biosafety Level
COX	Cyclooxygenase
DEET	N, N-Diethyl-meta-Toluamide
DMSO	Dimethyl Sulfoxide
DPPH	1, 1-Diphenyl-1-Picrylhydrazyl
DXP	1-Deoxyxyulose5-Phosphate
DXPS	1-Deoxy-D-xylulose-5-Phosphate Synthase
FFA	Free Fatty Acids
GC-MS	Gas Chromatography-Mass Spectroscopy
IP	Induction Period
LOX	Lipoxygenase
MBC	Minimal Bactericidal Concentration
MFC	Minimal Fungicidal Concentration
MIC	Minimal Inhibitory Concentration
MSD	Mass Selective Detector
NDGA	Nordihydroguaiaretic Acid
TLC	Thin Layer Chromatography

LIST OF APPENDICES

ADDENDUM

It should be noted that the name *Cymbopogon validus* is considered a synonym of *Cymbopogon nardus* and may be represented as follows (Germishuizen *et al*., 2006):

Cymbopogon nardus (L.) Rendle = *Cymbopogon validus* (Stapf) Stapf ex Burtt Davy

ABSTRACT

The chemical and biological properties of the essential oil from *Cymbopogon validus* were investigated. Hydro-distillation was used to extract the oil from *C. validus*, the flower-heads, leaves, culms and rhizomes. The percentage oil yields obtained from the plant organs varied from 0.05 to 1.23%, with the greatest concentration found in the flower-heads and rhizomes, 1.23 and 1.12% respectively. A sensory evaluation of the oil revealed that the essential oil was slightly murky, pale yellow in colour, had a strong turpentine-like smell and remained liquid at room temperature. The oxidative stability of *C. validus* oil was evaluated by determining its Rancimat induction period (negative), peroxide value (60.56 meq/kg), iodine value (84.55), percentage free fatty acids (0.19%) and percentage cholesterol (3.03%). These results indicated that the oil was highly susceptible to oxidation. Chromatographic profiles of the oils from *C. validus*, as well as the plant organs were generated using gas chromatography-mass spectroscopy (GC-MS). Predominant compounds present in the oil included alpha-Cubebene, Camphene, Geraniol, Limonene, Myrcene, Palmitic acid and Sabinene.

C. validus essential oil was also investigated for its antimicrobial (disk diffusion), antioxidant (1, 1-Diphenyl-1-Picrylhydrazyl (DPPH) assay), anti-inflammatory (5-lipoxygenase assay), anti-mosquito properties (insecticidal, larvicidal and repellency assays) and toxicity profile (Brine shrimp and Ames assays). The oil showed poor antimicrobial activity and inhibited the growth of only Gram positive bacteria with a minimum inhibitory concentration (MIC) of 0.0625 (vol/vol) for *Bacillus*, *Micrococcus* and *Staphylococcus* species. The oil also exhibited excellent antioxidant activity, scavenging more than 80% of DPPH free radicals and possesses anti-inflammatory activity (IC_{50}=190 ppm). *C. validus* oil showed good adulticidal

activity (53.7% mortality) and excellent larvicidal (100% mortality) and repellent activity (100% repellency) against *Anopheles arabiensis* mosquitoes. At high concentrations, the oil was toxic to brine shrimp larvae. However, when diluted it was safe and the minimum inhibitory concentration was 0.0001(vol/vol). The absence of revertant colonies at all essential oil concentrations in the Ames test suggest that the oil is not mutagenic.

These results lead the way for exploiting *C. validus* oil as a multi-functional agent that has antibacterial, anti-oxidant, anti-inflammatory, and anti-mosquito properties.

CHAPTER ONE: INTRODUCTION

Essential oils are generally regarded as high value, low volume commodities and yet many developing countries import large quantities of oils to meet local demand for use in soaps, detergents, perfumes etc. It is widely accepted that South Africa has many indigenous plants that have potential commercial value. With agriculture providing the main source of income for rural communities, diversification to growing and even processing agricultural plants for essential oils can help protect farmers from unstable prices of cash crops (New Agriculturist, 2000). Locally produced essential oils are generally sold to two or three of the local companies that have been able to penetrate the international export market. Low volumes of smaller quantities of oils are sold into the growing local aromatherapy market. To exploit these commercially on an international scale one needs to move from wild-harvesting in order to ensure sustainability, in terms of quantity and quality. There is a common misconception that there is a ready market for new and novel products. The reality is that the market is very conservative and market acceptance of a new product must be coupled with an intensive development programme (FRIDGE, 2004).

Although there are no unique grounds upon which South Africa can claim competitive advantage in the global essential oils industry, there is a combination of factors that are in South Africa's favour. These include:

- Being in the Southern Hemisphere – many growing regions are in the Northern Hemisphere; the seasonal effect makes Southern Hemisphere suppliers attractive.
- Traditional strong trade links with Europe, a major importer of flavour and fragrance materials.
- Being established as a world class agricultural producer in a wide range of products.

- A diverse climate with a range of biomes - thus allowing for a good selection of essential oil crops to be grown.
- Good quality soils – being largely uncontaminated by centuries of exploitation as is the case in the developed world.

In spite of these competitive advantages and substantial interest from international buyers, South Africa has not yet established itself in the international essential oils market, hence there is a real opportunity to develop this industry. The other grounds of competitive advantage, in the long term, is the access to unique plant materials which could also provide a platform for the creation of new markets in which South Africa could dominate. Such domination would need to be based on good science, established trade links and reputation, all of which needs to be established in advance. Scientific knowledge is the key to creating this competitive advantage.

In this study we studied the essential oil from *Cymbopogon validus* (*C. validus*) (see Addendum). The aim was to investigate the chemical and biological properties of this oil. *C. validus* is an aromatic grass species usually found in mountainous grasslands and in the high-rainfall parts of South Africa. It is commonly used for thatching and rural communities traditionally use this plant to treat various ailments. It was chosen due to its abundant and widespread growth, its potential for developing cottage industries, its other multifarious uses, for example insect repellency and also that very little research has been done on the essential oil found in this grass species. The general characteristics and methodologies used for essential oils are reviewed (Chapter 2). Chapters (3-7) describes specific characteristics of *C. validus*.

We extracted the essential oil, conducted a sensory evaluation and analysed the chemical characteristics of the oil (Rancimat induction period, peroxide value, iodine value, percentage

free fatty acids and percentage cholesterol). To define the chemistry of the oil we evaluated its volatile composition using GC-MS (Chapter 3). The biological properties of the oil were evaluated by determining its antimicrobial properties (Chapter 4), its antioxidant properties using the DPPH assay and its anti-inflammatory activity using the 5-lipoxygenase assay (Chapter 5), and its anti-mosquito properties (Chapter 6). The safety profile of the oil was determined by evaluating its toxicity against Brine shrimps and its mutagenic potential using the Ames test (Chapter 7). The final conclusions of the potential of this oil is summarised in the Conclusions (Chapter 8).

CHAPTER TWO: REVIEW OF LITERATURE

2.1 ESSENTIAL OILS

2.1.1 Introduction

Essential oils are volatile, natural plant products generally present in low concentrations in specialized structures such as oil cells, glandular trichomes and/or resin ducts (Simon, 1990). They can be distinguished from the fatty vegetable oils, such as canola and sunflower, by the fact that they evaporate or volatilise with the air and they usually possess a strong aroma. These products are complex mixtures of organic chemicals, the nature and relative proportions of which are primarily determined by the genetics of the plant species. The purity of an essential oil can be determined by its chemical constituents. Variables that may affect these constituents include environmental factors (e.g. climate and altitude); agricultural factors (e.g. soil conditions, nutrition, time, harvest conditions and methods of post harvest handling) and process of extraction (FRIDGE, 2004). It is also necessary to extract or to isolate the essential oil as completely as possible from the mass of inert cellular matter with the minimum amount of chemical change. This may be achieved by several techniques depending on the nature of the start material. For an extract to be classified as an essential oil, only heat and water may be used in its extraction from the plant (Lucchesi *et al*., 2004).

More than 250 types of essential oils are traded in the world market. Some of these are listed in Table (2.1) (AromaWeb; Baylac and Racine, 2003; Choi *et al*., 2006; Nature's Gift; Oils 4 Life).

Table 2.1: Commercially available essential oils

Essential Oil	Plant Species
African Verbena	*Lippia javanica*
African Wormwood	*Artemisia afra*
Agarwood	*Aquilaria agallocha*
Almond	*Prunus dulcis*
Ambrette Seed	*Abelmoschus moschatus*
Amyris	*Amyris balsamifera*
Angelica Root	*Angelica archangelica*
Angelica Seed	*Angelica archangelica*
Anise Seed	*Pimpinella anisum*
Anise, Star	*Illicium verum*
Aniseed Myrtle	*Backhousia anisata*
Apricot Kernel	*Prunus armeniaca*
Arnica Flower	*Arnica montana*
Atlas Cedarwood	*Cedrus atlantica*
Avocado	*Persea gratissima*
Basil, Sweet	*Ocimum basilicum*
Basil, Holy	*Ocimum sanctum*
Bay Laurel	*Laurus nobilis*
Bergamot	*Citrus bergamia*
Black Cumin	*Nigella sativa*
Black Pepper	*Piper nigrum*
Blue Mountain Sage	*Buddleia salviifolia*
Blue Tansy	*Tanacetum annuum*
Buchu	*Agathosma betulina*
Calendula	*Calendula officinalis*
Camphor	*Cinnamomum camphora*
Cape May Bush	*Coleonema album*
Cape Snowbush	*Eriocephalus africanus*
Caraway Seed	*Carum carvi*
Cardamon	*Eletteria cardomomum*
Carrot Seed	*Daucus carota*
Cedar Atlas	*Cedrus atlantica*
Cedar Himalayan	*Cedrus deodara*
Celery Seed	*Apium graveolens*
Chamomile, Blue	*Matricaria reticulate*
Chamomile, Cape	*Eriocephalus punctulatus*
Chamomile, German	*Matricaria recutita*
Chamomile, Maroc	*Ormenis multicaulis*
Chamomile, Roman	*Anthemis nobilis*
Cinnamon	*Cinnamomium zeylanicum*
Citronella	*Cymbopogon nardus*
Clary Sage	*Salvia sclarea*
Clove	*Eugenia caryophyllata*

Table 2.1 Continued

Essential Oil	Plant Species
Clove leaf	*Sygyzium aromaticum*
Combava	*Citrus hystrix*
Copaiba balsam	*Copaifera officinalis*
Corriander	*Coriandrum sativum*
Cumin	*Cuminum cyminum*
Curcuma	*Curcuma longa*
Cypress	*Cupressus lusitanicus*
Cypress, Blue	*Callitropsis intratropica*
Dill	*Anethum graveolens*
Eucalyptus	*Eucalyptus globulus*
Fennel	*Feoniculum vulgare*
Frankincense	*Boswellia carteri*
Galangal	*Alpinia offcinarum*
Geranium	*Pelargonium graveolens*
Ginger	*Zingiber officinale*
Ginger Lily	*Hedychium coronarium*
Grapefruit	*Citrus paradici*
Helichrysum	*Helichrysum angustifolium*
Hypericum	*Hypericum perforatum*
Jasmine	*Jasminum officinale*
Jojoba	*Simmondsia chinensis*
Juniper	*Juniperus communis*
Katrafay	*Cedrelopsis grevei*
Lantana	*Lantana camara*
Lavender	*Lavendula angustifoli*
Lavender Tree	*Heteropyxis natalensis*
Lemon	*Citrus limon*
Lemongrass	*Cymbopogon citrates*
Lemon Balm	*Melissa officinalis*
Lime	*Citrus aurantifolia*
Lovage	*Levisticum officinale*
Mandarin	*Citrus noblis*
Mandarin Peel	*Citrus reticulate*
Marjoram, Sweet	*Origanum majorana*
Mastic	*Pistacia lentiscus*
Melissa	*Melissa officinalis*
Mint	*Mentha arvensis*
Monarda	*Monarda fistulosa*
Mugwort	*Artemisia vulgaris*
Muhuhu	*Brachyleana hutchinsii*
Myrrh	*Commiphora myrrha*
Myrtle	*Myrtus communis*
Naartjie	*Citrus reticulate*
Neem	*Azadirachta indica*

Table 2.1 Continued

Essential Oil	Plant Species
Neroli	*Citrus aurantium*
Niaouli	*Melaleuca viridiflora*
Nutmeg	*Myristica fragrans*
Opopanax	*Commiphora erythraea*
Orange, Bitter	*Citrus aurantium*
Orange, Sweet	*Citrus sinesis*
Palmarosa	*Cymbopogon martini*
Parsley	*Petroselinum sativum*
Patchouly	*Pogostemon cablin*
Pennyroyal	*Mentha pulegium*
Peppermint	*Mentha piperita*
Pepper Tree	*Schinus molle*
Petitigrain	*Citrus aurantium*
Pine Patula	*Pinus patula*
Psidia Altissima	*Psidia altissima*
Ravensara	*Ravensara aromatica*
Rhus Tarantana	*Rhus tarantana*
Rose	*Rosa damascene*
Rosemary	*Rosmarinus officinalis*
Rose Maroc	*Rosa centifolia*
Rosewood, African	*Pterocarpus erinaceus*
Sage	*Salvia officinalis*
Sandalwood	*Santalum spicatum*
Sassafras	*Sassafras albidum*
Schinus	*Schinus molle*
Spearmint	*Mentha spicata*
Tagetes	*Tagetes minuta*
Tangerine	*Citrus reticulata*
Tansy	*Tanacetum annuum*
Tarragon	*Artemisia dracunculus*
Tea Tree	*Melaleuca alternifolia*
Tumeric	*Curcuma longa*
Thuja	*Thuja occidentalis*
Thyme Red	*Thymus vulgaris*
Violet Flower	*Viola odorata*
Virginian cedar	*Juniperus viginiana*
Vetiver	*Vetiveria zizanoides*
Wild Indigo Root	*Baptisia tinctoria*
Wild Yam	*Dioscorea villosa*
White Willow Bark	*Salix alba*
Ylang Ylang	*Cananga odorata*
Yucca Root	*Yucca schidigera*

2.1.2 Chemistry

The chemicals present in essential oils are synthesized during normal development and may be classified as: (i) hydrocarbons of the general formula (C_5H_8)n-terpenes; (ii) oxygenated derivatives of these hydrocarbons; (iii) aromatic compounds having a benzoid structure; and (iv) compounds containing sulphur or nitrogen (FRIDGE, 2004). Chemically, the essential oils are composed of terpenoids and aromatic polypropanoids synthesized via the melvalonic acid pathway for terpenes and the shikimic acid pathway for aromatic polypropanoids (Simon, 1990). These pathways are schematically represented in Figure (2.1) (Ebbs, 2005).

Plants have been estimated to collectively synthesize more than 30 000 different terpenoids, which constitute the largest family of natural products, exceeding in number the alkaloids and phenylpropanoids combined. Terpenoids have many useful applications in the manufacture of foods, industrial compounds, and pharmaceuticals. They are synthesized from the condensation, in a head to tail fashion, of 5-carbon isoprene (or hemiterpene) units. Major terpenoid classes include mono-, sesqui-, and diterpenes, which are mostly secondary metabolites, as well as tri- and tetraterpenes, which are generally primary metabolites (Broun and Somerville, 2001). However, the vast majority are secondary metabolites, such as the volatile constituents of essential oils. Monoterpenes are the primary constituents of many essential oils.

Until recently, it was thought that the synthesis of terpenoids in higher plants was by a cytosolic route that is derived from mevalonate. However, during the past few years it has become clear that plants also use a parallel plastid pathway that converts pyruvate and glyceraldehyde3- phosphate to 1-deoxyxyulose5-phosphate (DXP), which is metabolized in a series of steps to isopentenyl diphosphate and dimethylallyl diphosphate, the common precursors of all terpenoids. Plants use the mevalonate-dependent pathway to synthesize

sesquiterpenes and triterpenes, whereas other major terpenoids are derived from the 1-deoxy-D-xylulose-5-phosphate synthase (DXPS) pathway. Because discovery of the plastidial route in plants is relatively recent, little is known of the mechanisms that limit flux through the DXPS pathway (Broun and Somerville, 2001).

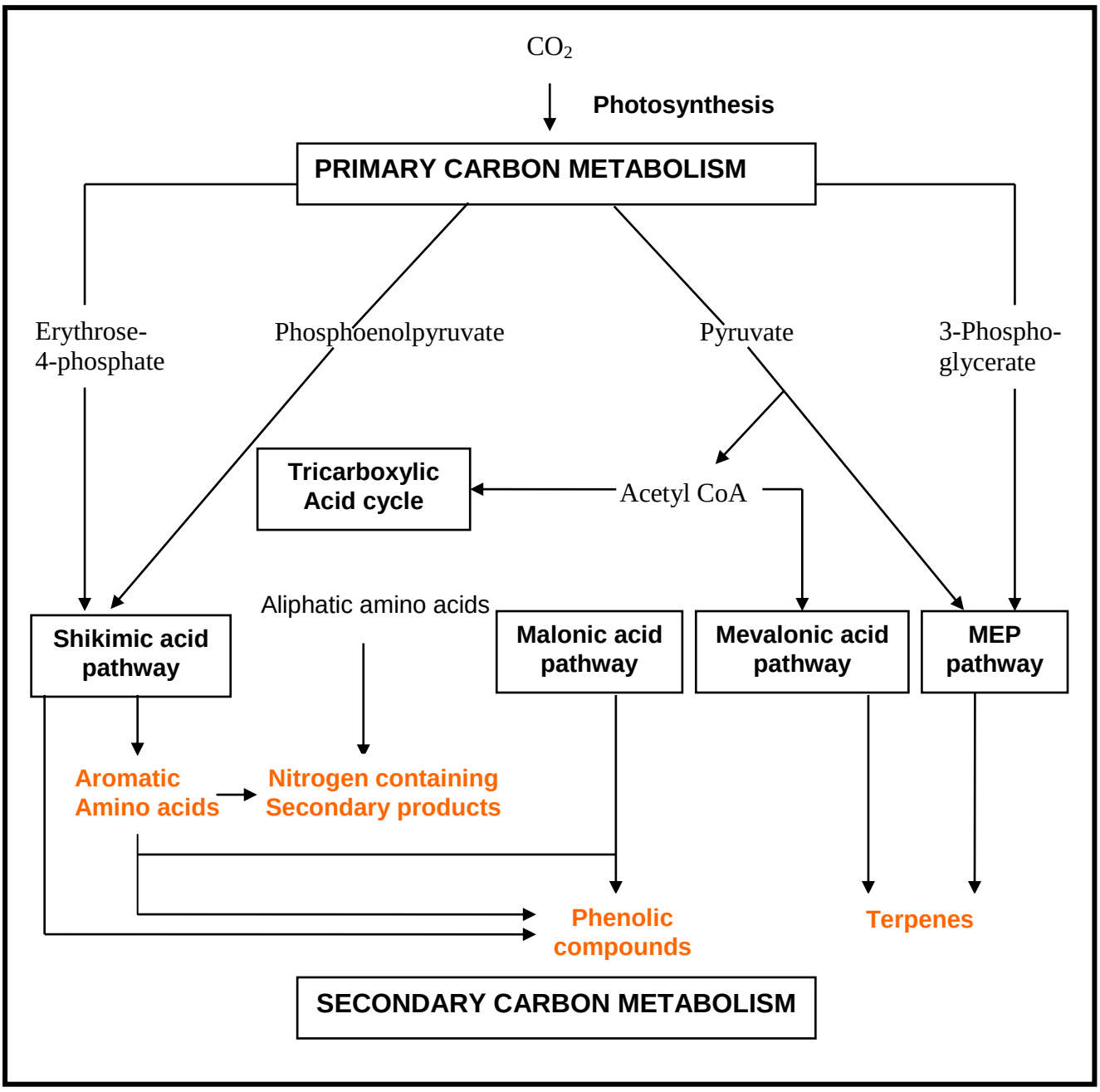


Figure 2.1: Metabolic pathways for terpenes and aromatic polypropanoids

2.1.3 Functions of phytochemicals

Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. It is well-known that plants produce these chemicals to protect themselves but recent research has demonstrated that they can protect humans against diseases (Top Cultures, 2006a). Phytochemicals have discrete bio-activities towards animal biochemistry and metabolism and are being widely examined for their ability to provide health benefits. Phytochemicals could provide health benefits as: (i) substrates for biochemical reactions; (ii) cofactors of enzymatic reactions; (iii) inhibitors of enzymatic reactions; (iv) absorbents/ sequestrants that bind to and eliminate undesirable constituents in the intestine; (v) ligands that agonize or antagonize cell surface or intracellular receptors; (vi) scavengers of reactive or toxic chemicals; (vii) compounds that enhance the absorption and/or stability of essential nutrients; (viii) selective growth factors for beneficial gastrointestinal bacteria; (ix) fermentation substrates for beneficial oral, gastric or intestinal bacteria; and (x) selective inhibitors of deleterious intestinal bacteria. Such phytochemicals include terpenoids, phenolics, alkaloids and fibre (Dillard and German, 2000). Some of these important compounds, their plant source and a few of their many properties are listed in Table 2.2 (Duke, 1998).

Essential oils do not provide an energy source for the plant since they remain in the leaves in plants that lose their leaves, whilst carbohydrate stores are moved into the stem before the leaves drop (Rose, 2005). It is generally assumed that compounds emitted from flowers serve to attract and guide pollinators (Reinhard *et al*., 2004). Many floral volatiles have anti-microbial or anti-herbivore activity (deMoraes *et al*., 2001; Friedman *et al*., 2002; Hammer *et al*., 2003), and so could also act to protect valuable reproductive parts of plants from enemies. The release of volatiles from vegetative organs following herbivore damage seems to be a general property of plant species. These substances have been demonstrated to serve

as indirect plant defences (Dudareva *et al*., 2004). The possibility that these substances also act in plant-plant communication has been discussed (Arimura *et al*., 2000; Dicke and Bruin, 2001; Engelberth *et al*., 2004). Herbivore-induced volatiles could have physiological roles within the plant, with their release being a consequence of their volatility and membrane solubility. Like isoprene, some herbivore-induced monoterpenes and sesquiterpenes have the potential to combine with various reactive oxygen species (Hoffmann *et al*., 1997; Bonn and Moortgat, 2003), and so could protect against internal oxidative damage (Delfine *et al*., 2000; Loreto *et al*., 2004).

Table 2.2: Plant sources and properties of some important phytochemicals

Compound Name	Compound Class	Plant Source	Properties
1, 8-Cineole	Oxygenic monoterpene	*Artemisia salsoloides*	Antibacterial Anti-inflammatory Antiseptic Fungicide Perfume
Alpha-Cadinol	Oxygenic sequiterpene	*Mentha longifolia* (L.) Huds.	Acaricide Anti-mite Pesticide
Alpha-Humulene	Sesquiterpene	*Syzygium aromaticum* (L.)	Anti-tumour Perfumery
Alpha-Pinene	Monoterpene	*Pinus insularis* Endl.	Anti-acne Anti-pneumonic Expectorant Insecticide Tranquilizer
Beta-Phellandrene	Monoterpene	*Piper cubeba* L. f.	Expectorant Fungicide Perfumery
Cis-Ocimene	Monoterpene	*Apium graveolens* L.	Antibacterial Anti-staphylococcic Fungicide
Delta-3-Carene	Monoterpene	*Pinus roxburghii* Sarg.	Anti-inflammatory Dermatitigenic Flavour Insectifuge Irritant
Aromadendrene	Sesquiterpene	*Eucalyptus leucoxylon* F. Muell.	Antiseptic Cancer-Preventive Pesticide
Camphor	Oygenic monoterpene	*Cinnamomum camphora* (L.)	Allelopathic Anti-acne Cancer-Preventive Cosmetic Insect repellent
Carvone	Oygenic monoterpene	*Carum carvi* L.	Candidistat CNS-Stimulant Fungistat Insecticide Nematicide
Citral	Oygenic monoterpene	*Zingiber officinale* Roscoe.	Anti-allergic Broncho-relaxant Herbicide Perfumery Sedative
Estragole	Oygenic monoterpene	*Agastache foeniculum* (Pursh) Kuntze.	Calcium-antagonist Carcinogenic DNA-Binder Hypothermic Insecticide

Table 2.2 Continued

Compound Name	Compound Class	Plant Source	Properties
Eugenol	Oygenic monoterpene	*Syzygium aromaticum* (L.) Merr. & L. M. Perry.	Acaricide Anti-genotoxic Anti-tumour Larvicide Trypsin-Enhancer
Farnesol	Oxygenic sesquiterpene	*Cymbopogon parkeri* Stapf.	Anti-leukemic Anti-melanomic Apoptotic Pheromonal Trichomonicide
Linalool	Oygenic monoterpene	*Mentha longifolia* (L.) Huds.	Acaricide Anti-lymphomic Hypnotic Larvicide Pro-oxidant
Myrcene	Monoterpene	*Pimenta racemosa* (Mill.) J. W. Moore.	ACE-Inhibitor Analgesic Anti-mutagenic Fungicide Myorelaxant
Neral	Oygenic monoterpene	*Zingiber officinale* Roscoe.	Antibacterial Anti-spasmodic Pesticide Termiticide
Perillaldehyde	Oygenic monoterpene	*Cymbopogon nardus* (L.) Rendle.	Antibacterial Anti-proliferative Antiseptic Fungicide Vibriocide
Terpinen-4-ol	Oygenic monoterpene	*Glycyrrhiza glabra* L.	Allelopathic Anti-asthmatic Bacteriostatic Diuretic Spermicide
Terpinolene	Monoterpene	*Pastinaca sativa* L.	Anti-feedant Antioxidant Flavour Fungicide Perfumery

2.2 METHODS USED TO TEST PHYTOCHEMICALS

Essential oils are subjected to both qualitative and quantitative tests to determine their purity, activity (Butterworth, 2004; Vankar, 2004) and safety. The characteristics of an essential oil are based on:

- Sensory evaluation: the appearance, clarity, colour, odour and viscosity of the oil

- Chemical characteristics: peroxide value, iodine value, free fatty acid content and chromatographic profile of the oil

- Biological characteristics: these include antimicrobial tests, anti-parasitic tests, anti-oxidative potential, anti-inflammatory activity

- Safety Characteristics: toxicity and mutagenicity

2.2.1 Sensory evaluation

Sensory evaluation of oils is considered a critical test of quality. The first steps of essential oil testing usually begins with sensory evaluation, since this prevents wasting precious time and money on more expensive analytical procedures and can identify inferior oils quickly. The viscosity, colour, clarity and odour of an essential oil can help to identify a poor quality oil right at the outset, provided you know precisely what to look for. Sensory odour evaluation of edible oils is governed by three factors, namely, medium in which the oil is dispersed, temperature of oil during testing and panel training for unambiguous understanding of descriptors (Rajalakshmi and Narasimhan, 1996). The information on the sensory profile of an oil could facilitate other useful studies that would help draw a picture of what makes the oil unique from other oils. Although sensory analysis is one of the most

sensitive methods available, it is not practical for routine analyses and generally lacks reproducibility. The scoring by taste or odour panelists may vary greatly from laboratory to laboratory. Chemical and physical methods developed therefore attempt to improve reproducibility, sensitivity, and quantitativeness (Gray, 1978).

2.2.2 Chemical characteristics

Many instrumental and chemical methods have been proposed over the years to evaluate the quality and stability of vegetable oils. GC-MS is mostly used to provide a chromatographic profile of the constituents of essential oils. Every individual component of the essential oil can be identified by the time at which the peak elutes on the trace. The data produced can then be compared to an established 'profile' or 'fingerprint' for that particular essential oil to finally determine the purity of the oil. Analysis of volatiles by gas chromatography is closely related to flavour evaluation and is therefore the most suitable method for comparison with the results of sensory panel tests (Frankel, 1993). The oxidative stability of an oil can be assessed by determining its peroxide value, induction period, % free fatty acids and iodine value.

2.2.2.1 Peroxide value

One of the most widely used tests for oxidative rancidity, peroxide value, is a measure of the concentration of peroxides and hydro peroxides formed in the initial stages of lipid oxidation. Milli equivalents of peroxide per kg of fat are measured by titration with iodide ion. Peroxide values are not static and care must be taken in handling and testing samples. It is difficult to provide a specific guideline relating peroxide value to rancidity. High peroxide values are a definite indication of a rancid fat, but moderate values may be the result of depletion of peroxides after reaching high concentrations.

2.2.2.2 Induction period (IP)

The induction period (IP) is measured as the time required to reach an endpoint of oxidation corresponding to either a level of detectable rancidity or a sudden change in the rate of oxidation (Frankel, 1993; Presa-Owens *et al*., 1995). Measurements of IP under standard conditions are generally used as an index of antioxidant effectiveness. For practical purposes however, predictions of oxidative stability in foods and oils based on measurements of IP should be related to measured product shelf life (Frankel, 1993). Accelerated oxidation tests should be calibrated for each formulation, and the conditions used should be maintained as close as possible to those under which the food is stored (Frankel, 1993).

2.2.2.3 Free Fatty Acids (FFA)

Free acids in a fat (or fat extracted from a sample) can be determined by titration. The FFA value is then expressed as % of a fatty acid common to the product being tested. Frequently, values are expressed as % oleic acid for tallows or soybean oils. For coconut oils or other oils that contain high levels of shorter chain fatty acids, FFA may be expressed as % lauric acid. It may also be useful to know the composition of the FFA present in a sample to identify their source and understand the cause of their formation. Extracts of samples can be analyzed for free fatty acid profiles when this information is required.

2.2.2.4 Iodine Number

While not a specific measure of fat stability, iodine number measures can indicate the potential of a fat to be oxidized. The method measures the reaction of iodine with double bonds of unsaturated fatty acids. Fats with a greater number of double bonds provide more sites for oxidation. Because other factors can influence fat stability, iodine number is not

useful by itself for predicting fat stability. Iodine number of oils (Table 2.3) may vary from 8-200 depending on the unsaturation (Alsberg and Taylor, 1928).

Table 2.3: Iodine number of oils and fats

Fat or Oil	Iodine number
Linseed oil	173 - 201
Tung Oil	170.6
Menhaden oil	139 - 173
Whale oil	121 - 146.6
Soy bean oil	137 - 143
Sunflower oil	119 - 135
Corn oil	111 - 130
Cottonseed oil	108 - 110
Sesame oil	103 - 108
Rapeseed oil	94 - 102
Peanut oil (arachis)	83 - 100
Olive oil	79 - 88
Horse oil	71 - 86
Lard	46 - 70
Palm oil	51.5 - 57
Milk fat	26 - 50
Beef tallow	38 - 46
Mutton tallow	35 - 46
Cacao butter	32 - 41
Palm kernel oil	13 - 17
Coconut oil	8 - 10

2.2.3 Bioassays

Robust bioassays and targeted collections of compounds and extracts for testing *in vitro* antibacterial, antifungal and anti-parasitic bioassays are designed for rapid screening of large numbers of products or extracts. They are simple, easy to implement and produce results quickly and preferably at low cost. Compounds or extracts with a specific activity at a non-toxic dose, so-called "hits", are then further evaluated in secondary or specialized *in vitro* bioassays and in animal models to define "lead" status (Verkman, 2004). Currently there are some guidelines for: (i) plant selection; (ii) extraction of essential oil; and (iii) compound

handling and storage. There are also standard protocols for testing: (i) antibacterial activity; (ii) anti-parasitic testing; and (iii) cyto-toxicity testing.

2.2.3.1 Selection of plants

Four standard approaches are available for selecting plants (Fabricant and Farnsworth, 2001):

(1) random selection followed by chemical screening - secondary metabolites containing various antimicrobial substances (e.g. alkaloids, isothiocyanates) are selected;

(2) random selection followed by antimicrobial assays - all available plant parts are collected, irrespective of prior knowledge and experience. This methodology is expensive and laborious and depends heavily on the panel of test pathogens and the 'activity' criteria used;

(3) follow-up of antimicrobial activity reports - exploits the vast resource of published reports on antimicrobial activities; and

(4) follow-up of ethnomedical or traditional uses of plants against infectious diseases - oral or written information on the medicinal use of a plant forms the basis for selection and focused evaluation.

2.2.3.2 Extraction Procedures

Irrespective of the adopted plant collection strategy, a critical step is the processing of the plant material. Plant extracts are prepared by maceration or percolation of fresh green plants or dried powdered plant material in water and/or organic solvents. For hydrophilic compounds, polar solvents such as methanol, ethanol or ethyl-acetate are used. For extraction of more lipophilic compounds, dichloromethane or a mixture of

dichloromethane/methanol (1:1) are used. In some instances, extraction in hexane is used to remove chlorophyll. It is also important to consider in the ethno-medical approach the need to prepare the extract as described by the traditional healer in order to mimic as closely as possible the traditional 'herbal' drug (Cos *et al*., 2006). To detect active substances present in very small quantities in the extracts, the sample is concentrated, extracted and evaporated at low temperatures so as not to destroy any thermo-labile constituent. Of the many extraction schemes that have been published, the method proposed by Mitscher *et al*. (1972) and later adapted by Ieven *et al*. (1979) can be considered as a practical standard since it offers a logical, low-cost, feasible and highly performing starting approach (Figure 2.2).

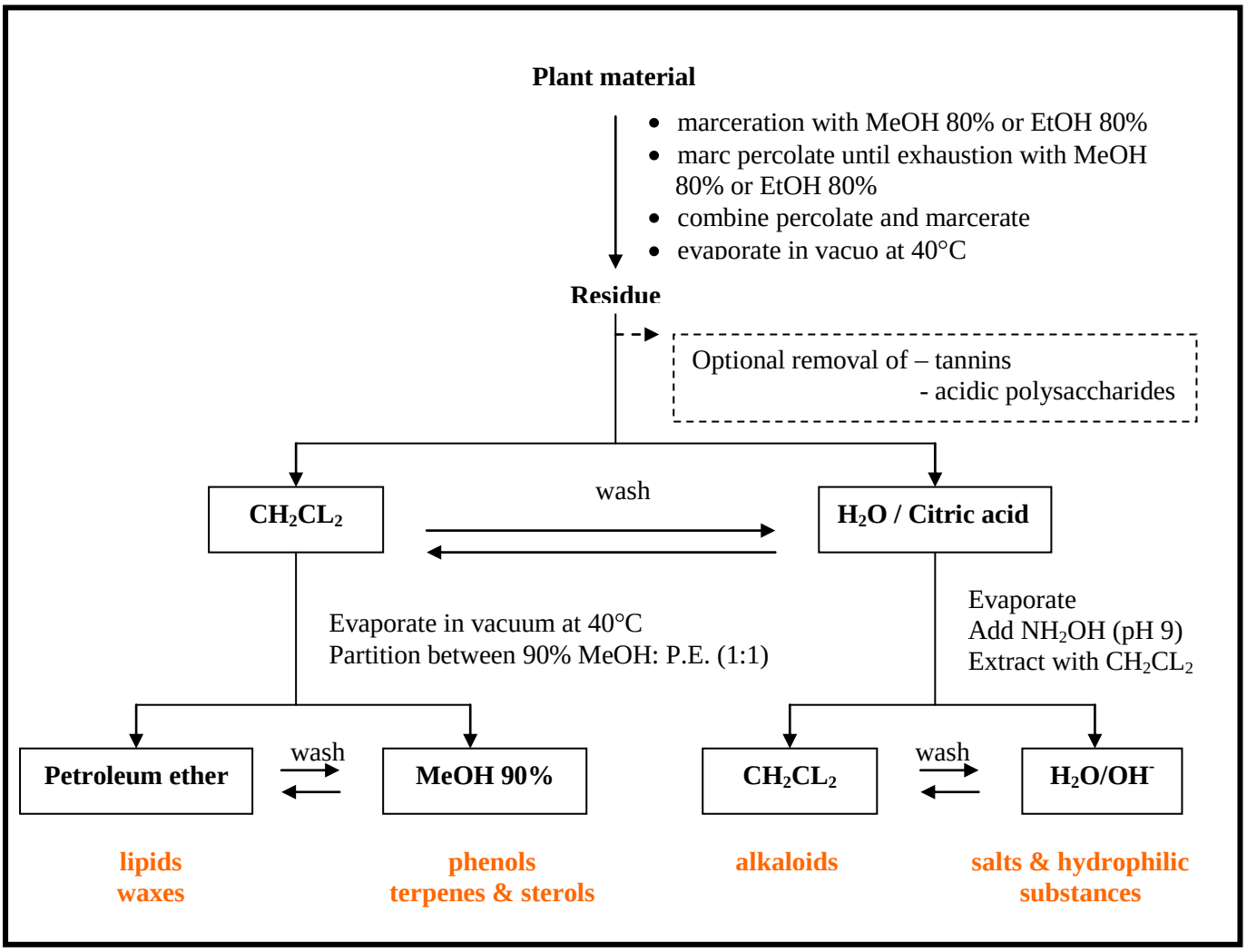


Figure 2.2: Standard scheme for preparation of plant extracts for biological screening

2.2.3.3 Selection of the appropriate bioassay

Different screening approaches are available to identify the primary pharmacological activity in chemical and/or natural products. The screening option will largely depend on the specific nature of the disease being targeted and on the availability of practical and biologically validated laboratory models. As illustrated in Figure 2.3, four levels of screening can be identified: (i) *In silico*; (ii) *In vitro* assays that target an enzyme, receptor or a gene; (iii) *In vitro* assays that target the whole organism; and (iv) *In vivo* assays that target animal models. The most rewarding strategy is to opt for models that remain as close as possible to the final target, i.e. the patient (Cos *et al*., 2006)

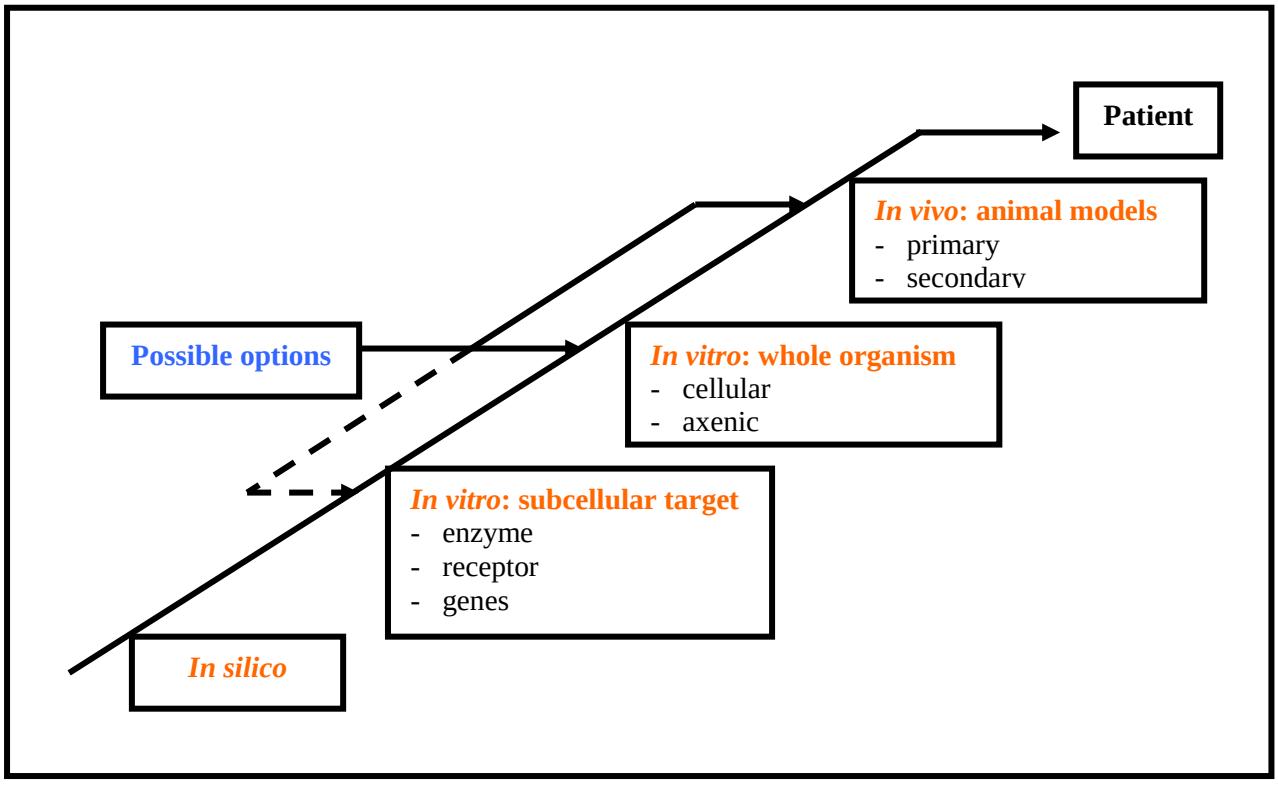


Figure 2.3: General approaches in (anti-infective) drug screening

2.2.3.4 Compound handling and storage

Frequently used solvents to make up test compound solutions include dimethyl sulfoxide (DMSO), methanol and ethanol. The latter two, however, have the disadvantage of rapid evaporation whereby the stated concentration of stock solutions cannot be maintained. Solutions in 100% DMSO have become the standard and are prepared at adequate strength ("master plates" at 20 mM or (xg/ml for pure compounds and extracts, respectively). Added advantages of stock solutions in 100% DMSO are: (i) elimination of microbial contamination, thereby reducing the need for sterilisation by autoclaving or other strenuous methods, which affect the quality of the test sample; and (ii) good compatibility with test automation and integrated screening platforms, assuring for example good solubility during the serial dilution procedures. Of importance when using DMSO is to remember that DMSO is toxic for cells and many micro-organisms, and to avoid later interference in the biological test systems, the in-test concentration of DMSO should not exceed 1% (Verkman, 2004). A practical recommendation for storage of compounds or extracts is either without solvent for long term storage or in 100% DMSO at -20°C with minimal exposure to freeze-thaw cycles and humidity.

2.2.3.5 Standard biological assays recommended

Antimicrobial activity of natural extracts and pure compounds can be detected by observing the growth response of various micro-organisms to samples that are placed in contact with them. The antibacterial and antifungal test methods are classified into three main groups: (i) diffusion; (ii) dilution; and (iii) bio-autographic methods. A fourth and upcoming test method is the conductimetric assay, detecting microbial growth as a change in the electrical conductivity or impedence of the growth medium (Sawai *et al*., 2002).

2.2.3.5.1 Agar diffusion methods

In the diffusion technique, a reservoir containing the test compound at a known concentration is brought into contact with an inoculated medium and the diameter of the clear zone around the reservoir (inhibition diameter) is measured at the end of the incubation period. In order to enhance the detection limit, the inoculated system is kept at lower temperature for several hours before incubation to favour compound diffusion over microbial growth, thereby increasing the inhibition diameter. Different types of reservoirs can be used, such as filter paper discs, stainless steel cylinders placed on the surface and holes punched in the medium. The hole-punch method is the only suitable diffusion technique for aqueous extracts, because interference by particulate matter is much less than with other types of reservoirs. To ensure that the sample does not leak under the agar layer, fixed agar is left on the bottom of the hole (Cole, 1994). The small sample requirements and the possibility to test up to six extracts per plate against a single micro-organism are specific advantages (Hadacek and Greger, 2000).

2.2.3.5.2 Dilution methods

In the dilution methods, test compounds are mixed with a suitable medium that has previously been inoculated with the test organism. It can be carried out in liquid as well as in solid media and growth of the micro-organism can be measured in a number of ways. In the agar-dilution method, the Minimal Inhibitory Concentration (MIC) is defined as the lowest concentration able to inhibit any visible microbial growth. In liquid or broth-dilution methods, turbidity and redox-indicators are most frequently used. In general, dilution methods are appropriate for assaying polar and non-polar extracts or compounds for determination of MIC and Minimal Bactericidal Concentration (MBC) / Minimal Fungicidal Concentration (MFC) values. Using redox indicators or turbidimetric endpoints, dose-

response effects allow calculation of IC_{50}- and IC_{90}-values, which are the concentrations required to produce 50 and 90% growth inhibition (Cos *et al*., 2006).

2.2.3.5.3 Bio-autographic methods

Bio-autography localizes antimicrobial activity on a chromatogram using three approaches: (i) direct bio-autography, where the micro-organism grows directly on the thin-layer-chromatographic (TLC) plate; (ii) contact bio-autography, where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact; and (iii) agar-overlay bio-autography, where a seeded agar medium is applied directly onto the TLC plate (Hamburger and Cordell, 1987; Rahalison *et al*., 1991). Despite high sensitivity, its applicability is limited to micro-organisms that easily grow on TLC plates.

2.2.3.6 Recommendations on antibacterial and antifungal screening

The choice of test organisms depends on the specific purpose of the investigation. In a primary screening, drug-sensitive reference strains are preferably used and should represent common pathogenic species of different classes. Various combinations are possible, but the panel should at least consist of a Gram-positive and a Gram-negative bacterium. A small set of reference fungi is used for primary screening and includes *Trichophyton mentagrophytes* and *Epidermophyton floccosum* as representatives of the dermatophytes as well as *Aspergillus niger* and *Fusarium solani* as opportunistic filamentous fungi. The proposed *in vitro* laboratory procedures and test designs for bacteria and fungi (Cos *et al*., 2006) are shown (Table 2.4).

2.2.3.7 Anti-parasitic assays

Contrary to the antibacterial and antifungal test systems that are based on common test conditions and endpoints, bioassays for parasites are more exclusive since they tend to be highly species-specific (Maes *et al*., 2004). The primary screening model should be as sensitive as possible to enable it to pick-up weakly active compounds, but at the same time also recognize and eliminate false-positives. To improve on the performance of the *in vitro* models, the following design options should be considered: (i) use of well-characterized drug-sensitive parasite strains; and (ii) use of sensitive endpoint reading techniques. The proposed *in vitro* laboratory procedures and test designs (Table 2.4) can be implemented in laboratory settings with limited technical resources (Cos *et al*., 2006).

2.2.3.8 Cyto-toxicity assays

Cyto-toxicity on host cells is a very important criterion for assessing the selectivity of the observed pharmacological activities and must always be included in parallel. Although many cell types can theoretically be used for that purpose, MRC-5 cells are given as an example. They are cultured in MEM medium, supplemented with 20 mM l-glutamine, 16.5 mM $NaHCO_3$ and 5% fetal calf serum. Assays are performed at 37°C and 5% CO_2 in 96-well tissue culture plates with confluent monolayers. After 4-7 days incubation, cell proliferation and viability is assessed after addition of Alamar BlueT^M or resazurin (McMillian *et al*., 2002). After 4 h at 37°C, fluorescence is measured (550 nm excitation, 590 nm emission).

2.2.3.9 Antioxidant Assays

A desirable method for evaluating the antioxidant activity of a compound should be rapid, reproducible, should require small amounts of chemicals and should not be influenced by the

physical properties of the compound (Marco, 1968). A plethora of different antioxidant assays is available and, because results rely on different mechanisms, they strictly depend on the oxidant/antioxidant models employed and on lipophilic/hydrophilic balance (Frankel *et al*., 1994). A single-substance/single-assay produces relative results and it is perceived as a reductive approach whenever a phytocomplex is involved. Therefore, a multiple-test and a simultaneous chemical characterization must be taken into account whenever assays of essential oils are performed to allow a balance between the sensory acceptability and functional properties. Chemical assays are based on the ability to scavenge synthetic free radicals, using a variety of radical-generating systems and methods for detection of the oxidation end-point. 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)- or 1,1-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging methods are common spectrophotometric procedures for determining the antioxidant capacities of plant components. These assays have been applied to determine the antioxidant activity of food, wine and plant extracts and pure components (Awika *et al*., 2003; van den Berg *et al*., 2000; Yu *et al*., 2002). Unfortunately, results from these methods often do not correlate with the ability of a compound to inhibit oxidative deterioration of foods.

Table 2.4: Proposed panel of test organisms for antibacterial, antifungal and anti-parasitic *in vitro* screening

Screen	Species	BSL[a]	Culture	CO_2[b]	Reference Drug
Bacteriology (Gram positive, Gram negative, Acid-fast)					
Gram positive cocci	*Staphylococcus aureus*	2	Axenic in Mueller Hinton	No	A broad selection of antibiotics is available e.g. penicillin, ampicillin, norfloxacin, doxycyclin, kanamycin
Spore-forming Gram positive rods	*Bacillus cereus* *Bacillus subtilis*				
Enterobacteriaceae –non-encapsulated	*Escherichia coli*				
Enterobacteriaceae – encapsulated	*Klebsiella pneumoniae*				
Non-Enterobacteriaceae	*Pseudomonas aeruginosa*				
Acid-fast bacteria	*Mycobacterium sp.*	2/3	Axenic or cellular	No	e.g. Rifampicin
Mycology (Yeast and Fungi)					
Yeast	*Candida albicans*	2[c]	Axenic in Sabouraud	No	Several broad-spectrum anti-fungals are available e.g. amphotericin B, miconazole, ketoconazole, flucytosine
Dermatophytes	*Trichophyton mentagrophytes* *Epidermophyton floccosum*				
Opportunistic filamentous fungi	*Aspergillus niger* *Fusarium solani*				
Parasitology					
Malaria	*Plasmodium falciparum*	3*	Cellular (human RBC)	d	e.g. Chloroquine, artemether
Leishmaniasis	*Leishmania donovani*	3*	Cellular (Macrophages)	Yes	e.g. Amphotericin B, sodium stibogluconate
Sleeping sickness	*Trypanosoma brucei*	2	Axenic HMI-medium	Yes	e.g. Suramin, melarsoprol

[a] BSL: biosafety level according to the European directive 2000/54/EC. Micro-organisms classified in BSL 3* may present a limited risk of infection for workers because they are not normally infectious by the airborne route.
[b] 5% CO_2 incubator.
[c] *Fusarium solani* is an opportunistic pathogen.
[d] Micro-aerophilic atmosphere (4% CO_2, 3% O_2, and 93% N_2

CHAPTER THREE: EXTRACTION AND CHEMICAL COMPOSTION OF *C. VALIDUS*

3.1 INTRODUCTION

The genus *Cymbopogon* (Sprengel.) belongs to the family Poaceae, its members occur abundantly in tropics and subtropics with unrestricted distribution ranging from mountains and grasslands to arid zones. While all plants synthesize some amount of aromatic compounds in their different parts, some plants including the so called aromatic grasses belonging to the genus *Cymbopogon* accumulate such large amounts of them in leaves, inflorescences, and/or in roots that these can be obtained as hydro-distilled essential oils. On account of their diverse uses in pharmaceuticals, cosmetics, food and flavour, and agricultural industries, *Cymbopogon* is cultivated (medicultured) on a large scale, especially in the tropics and subtropics. There is a large worldwide demand for the essential oils of *Cymbopogon* species.

Cymbopogon validus (Stapf) Stapf ex Burtt Davy (Figure 3.1) is commonly known as Giant Turpentine Grass, Reuse Terpentynegras (Afrikaans) and African Bluegrass in South Africa. This grass species has been described as a tufted perennial with culms up to 2.4 m tall; inflorescence a false panicle consisting of groups of paired racemes, each partially enclosed by a leaf-like spathe; spikelets in pairs, one member sessile and the other pedicellate, slightly shorter and awnless; glabrous leaf blade with a distinct midrib and a rough leaf margin; lower leaf sheaths short and hairy; ligule a conspicuous membrane. *C. validus* is usually found in mountainous grassland and in the high-rainfall parts of South Africa. It grows in vleis, wet sites, along roads and on the margins of tree communities and prefers stony slopes with loamy soil (Van Oudshoorn, 1999). In eastern and southern Africa *C. validus* is used as a durable thatch. It is highly unpalatable and utilized only in the young stage, when little other

grazing is available. The essential oil of *C. validus* has been used as an astringent skin toner and anti-ageing preparation for men and has anti-fungal and antiseptic properties. Its volatile oil and dedoction are often used traditionally as an anti-rodent, fermifuge, emetic, anti-infective, anti-plasmodic or to treat morning sickness (Chagonda *et al*., 2000).

Figure 3.1: *Cymbopogon validus*

There exists considerable ethno-botanical and pharmacological knowledge associated with the use of *Cymbopogon* materials. Lemongrass, palmarosa-and citronella-essential oils are the main raw material products of the cultivated *Cymbopogons*. Three species of *Cymbopogon* namely *citratus, flexuosus*, and *pendulus* yield lemongrass oil of commerce, used as flavouring agents, and in perfumery and pharmaceutical industry and as a natural precursor of semi-synthetic vitamin A. Palmarosa, the oil of *Cymbopogon martinii* finds usage in the perfumery, cosmetic and flavouring industries. Citronella Java (*Cymbopogon winterianus*) yields citronella oil employed in the manufacture of soap, pharmaceuticals, perfumery, cosmetic and flavouring agents (Kumar *et al*., 2000). The predominant

compounds, properties and traditional uses of the essential oils of *C. citratus* (Amer and Melhorn, 2006b; Koba *et al*., 2004; Lertsatitthanakorn *et al*., 2006; Mishra and Dubey, 1994; Nguefack *et al*., 2004; Spring, 1989), *C. giganteus* (Adjanohoun *et al*., 1985; Adjanohoun *et al*., 1986; Alitonou *et al*., 2006; Kimbi and Fagbenro-Beyioku, 1996), *C. martinii* (Duarte *et al*., 2007; Dubey and Luthra, 2001; Oussalah *et al*., 2006; Prashar *et al*., 2003; Rao *et al*., 2005), *C. nardus* (Burfield and Reekie, 2005; Janton *et al*., 1999; Koba *et al*., 2004; Lertsatitthanakorn *et al*., 2006) and *C. winterianus* (Amer and Melhorn, 2006a; de Mendonca *et al*., 2005; Duarte *et al*., 2007; Oussalah *et al*., 2006) are given (Table 3.1). Neral, a major constituent of *Cymbopogon citratus* oil, is known to possess antibacterial properties (Iwu, 1993). The essential oil from *C. martini* comprises mainly of geraniol, which is used as a flavourant and fragrant (Duke, 1998). Citronellal, found in *C. nardus* has fungicidal properties.

The only information available on the chemical composition of *C. validus* oil is work carried out by Chagonda *et al*. (2000). They reported the chemical composition of the essential oil from the aerial parts of wild and cultivated *C. validus* (Staph), found in Zimbabwe. The predominant compounds found in the oil from plants collected in the wild were myrcene (23.1-56.6%), (Z)-β-ocimene (10.3-11.5%), camphene (5.2-6.0%), (E)-β-ocimene (4.7-5.3%), ∝-pinene (4.2-5.4%), geraniol (3.4-8.3%) and linalol (3.2-3.7%). Cultivated mature plants contained myrcene (11.6-20.2%), (Z)-β-ocimene (6.0-12.2%), borneol (3.9-9.5%), camphene (3.3-8.3%), ∝-pinene (3.0-6.6%) and geraniol (1.7-5.0%). The oil from young nursery crop/seedlings (20-30 cm high) comprised of myrcene (20.6%), geraniol (17.1%), germacrene-D-4-ol (8.3%), (Z)-β-ocimene (6.8%), linalol (4.5%) and geranyl acetate (4.5%).

Essential oil quality and stability are the main factors that influence its acceptability and market value (Gan *et al*., 2005). Oxidative stability is one of the most important indicators of

the keeping quality of vegetable oils (Tan and Che Man, 2002). In general, the term rancidity has been used to describe the mechanisms by which lipids alter in the presence of oxygen or air (Hamilton, 1989). Today, rancidity in processed food is becoming increasingly important as manufacturers require longer shelf-lives and because of public awareness of nutritional issues (Rossell, 1989).

As soon as a food, feed, or ingredient is manufactured, it begins to undergo a variety of chemical and physical changes. Oxidation of lipids is one common and frequently undesirable chemical change that may impact flavour, aroma, nutritional quality, and, in some cases, even the texture of a product. The chemicals produced from oxidation of lipids are responsible for rancid flavours and aromas. Vitamins and other nutrients may be partially or entirely destroyed by highly reactive intermediates in the lipid oxidation process. Oxidized fats can interact with proteins and carbohydrates causing changes in texture. For most products, though, predicting and understanding oxidation of lipids is necessary to minimize objectionable flavours and aromas arising from fat rancidity.

Since the chemistry and biological activity have important implications, this chapter deals with the chemical characteristics of the oil. Hydro-distillation was chosen as the method for essential oil extraction from *C. validus* and its plant organs (flower-heads, leaves, culms and rhizomes). The oxidative stability of *C.validus* oil was determined by the rancimat induction period, peroxide and iodine values, percentage free fatty acids and cholesterol, carried out at the Food and Cosmetic Technology Company in Durban, KwaZulu-Natal. The chemical composition of the essential oils from *C. validus* and its plant organs was established by GC-MS, carried out at Umgeni Water Laboratories in Pietermaritzburg, KwaZulu-Natal.

Table 3.1: Predominant compounds, properties and uses of *Cymbopogon* species

Essential oil					
Species	**Common name**	**Distilled Part**	**Major Compounds (%)**	**Properties**	**Traditional uses**
Cymbopogon citratus	Lemongrass	Herb grass	Geranial (43.15) Neral (31.36) Myrcene (10.65) Geraniol (5.47)	Antibacterial Antifungal Anti-inflammatory Antioxidant	Antiseptic Carminative Diuretic Food preservative Pyretic
Cymbopogon giganteus	Unknown	Leaves	trans-p-1(7),8-menthadien-2-ol (22.3) cis-p-1(7),8-menthadien-2-ol (19.9) trans-p-2,8-menthadien-1-ol (14.3) cis-p-2,8-menthadien-1-ol (10.1)	Anti-inflammatory Antioxidant	Treats headaches, colds, skin disorders and conjunctivitis Strong effect against chloroquine resistant *Plasmodium* sp.
Cymbopogon martinii	Palmarosa	Herb grass	Geraniol (80) Geranyl acetate (9)	Antibacterial Antifungal Flavourant Perfumery Mosquito repellent	Antiseptic Insect repellent
Cymbopogon nardus	Ceylon Citronella	Herb grass	Citronellal (30.54) Geraniol (23.93) Elemol (12.04) Citronellol (7.65)	Anti-inflammatory Antimicrobial Antioxidant Mosquitocidal	Insect repellent
Cymbopogon winterianus	Java Citronella	Herb grass	Citronellal (34) Geraniol (21) Citronellol (11)	Antibacterial Larvicidal	Antiseptic Insect repellent Mosquito repellent

3.2 METHODOLOGY

3.2.1 Plant material

Fresh specimens (10 kg fresh weight) of *Cymbopogon validus* were collected by a botanist, Professor Baijnath during March - June 2005 from Durban, KwaZulu-Natal. The plant specimen was identified and deposited in the Ward herbarium (University of KwaZulu-Natal). Botanical identification of the plant was made by using local floral keys.

3.2.2 Isolation of essential oil

The plant organs were separated from the fresh plant material on the same day. These were dried in an extractor oven at 25°C for 3-5 days. The dried plant organs were then crushed to a coarse powder using an industrial grinder (Retsch Gmbh, West Germany) and stored at room temperature until further use. The essential oil was extracted using a hydro-distillation method.

One hundred grams of plant material was hydro-distilled for 4 hours in a modified Clevenger-type apparatus (Stahl, 1969). The apparatus for this technique is outlined (Figure 3.2). The powdered plant material was placed in a 2L round-bottom flask (A) containing approximately 1.3L of tap water. Heat (B) was applied to the flask and the volatile oil was carried with the steam to a cold condensor (C) where the volatile oil and steam returned to their liquid states. A separator (D), attached to the condensor received the oil and water mixture. Generally, the lighter oil rises to the top of the separator and can be drawn off from time to time as the layer builds up. The water, which contains traces of slightly soluble fractions of the oil is drawn off continuously from the bottom of the separator and is returned to the flask containing the plant material. The essential oil collected was dried over

anhydrous sodium sulphate, weighed and stored in a sealed vial covered with aluminium foil 4ºC.

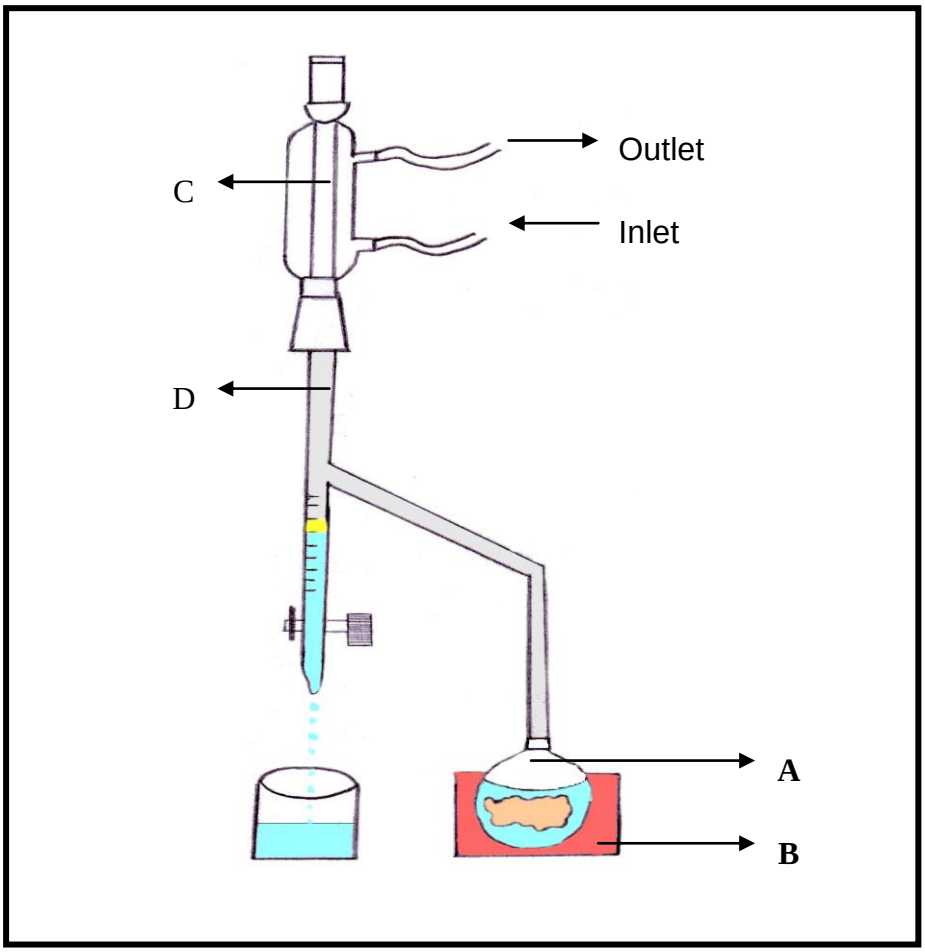


Figure 3.2: Lab-scale apparatus for hydrodistillation of *C. validus*.
(A) -– round-bottom flask, **(B)** – heating mantle, **(C)** – condenser, **(D)**) – separator

The percentage yield of essential oil was determined using the formula described by Rao *et al.* (2005) where the amount of essential oil recovered (g) was determined by weighing the oil after moisture was removed:

$$\text{Percentage yield (\%)} = \frac{\text{Amount of essential oil recovered (g)}}{\text{Amount of plant material distilled (100g)}} \times 100$$

3.2.3 Sensory evaluation

The essential oil from *C. validus* was evaluated on its colour, clarity and odour. The colour and clarity of the oil were evaluated visually. Three graduate students from the Department of Biotechnology were asked to make an evaluation of the oil based on its odour. Odour grading was based upon a five-point intensity scale: 1 = no perception; 2 = weak; 3 = medium; 4 = strong and 5 = extreme.

3.2.4 Rancimat induction period

Stability was expressed as the oxidation induction time (h), with the use of the Rancimat 679 apparatus (Metrohm AG, Herison, Switzerland). A flow of air (20L/h) was bubbled through the oil (2.5 g) heated at 120°C. The chart speed was 1 cm/hour.

Prior to the use of the Rancimat, it was imperative that all glassware was thoroughly cleaned as per the operating instructions in the 679 Rancimat operating manual. Contamination of the glassware could have contributed considerably to any discrepancies found in results obtained. A 2.5 g sample of standard sunflower oil was weighed accurately into each of the reaction vessels. The sample was then weighed into the reaction vessel. The vessel was then placed in the heating block of the wet section. Sixty millilitres of de-ionized water was measured into the measuring vessel, containing the electrodes. The measuring vessel was also placed in the wet section. All parts were connected to the apparatus as per the operating instructions, and the test was carried out until the endpoint of the sample was reached, with a maximum limit of 48 hours allowed.

Processing and evaluation of the recorded induction periods are performed in the 679 Rancimat automatically. For this particular exercise, sample sizes were very small, therefore

2.5 g of standard sunflower oil was added to the test samples in order to make up the required mass. The induction period of the sunflower oil only was subtracted from the induction period obtained for the test sample plus sunflower oil.

3.2.5 Peroxide value

The peroxide value (meq/kg of oil) was measured by titration with 0.002N sodium thiosulphate, using starch as an indicator.

Five grams of sample were weighed into a 250 ml conical flask, to which 250 ml of (glacial acetate: chloroform; 2:1 parts) solvent and 1 ml of potassium iodide (4:3 parts) solution was added. This was then mixed thoroughly and placed in the dark for 1 minute. Thirty five millilitres of distilled water was added to the mixture and titrated with 0.002N sodium thiosulphate, using starch (1%) as an indicator. A blank determination was also run. The peroxide value was calculated using the formula:

$$\text{Peroxide value} = \frac{1000 \times [\text{vol.sample (ml)} - \text{vol.blank (ml)} \times \text{normality Thiosulphate}]}{\text{Weight of sample (g)}}$$

The peroxide value is expressed in milliequivalents of peroxide oxygen per Kg sample.

3.2.6 Iodine value

This method determines the acidity of the sample under test conditions. Sample sizes and weights of oils and fats normally used are shown (Appendix 1).

The oil sample (0.15 g) was dissolved in 15ml of carbon tetrachloride. To this, 250 ml of Wijs and 10 ml of mercuric acetate were added, and placed in a dark cupboard for 3 minutes. Once removed from the dark, 20 ml of potassium iodine and 80 ml deionised water were

added. The solution was titrated with sodium thiosulphate until a pale yellow colour was visible and a starch indicator was added until the grey colour was removed. The blank determination was titrated at the same time. The sample (125 ml) was filtered (Filter paper No. 41), then heat–filtered, stirred and removed when the temperature reached 130°C. Once cooled, the sample was sealed in a sample bottle (+/- 100 ml) and allowed to cool further to +/- 25°C in a water bath. The sample bottle was then placed in a freezer (0°C) for 51/2 hours. Once removed from the freezer, the oil was checked for fat crystals; the oil should be crystal clear. The iodine value was calculated using the formula:

Iodine value = [Vol.blank (ml) –Vol.sample (ml)] x 12.69 x normality Thiosulphate

3.2.7 Percentage Free fatty Acids

Free fatty acids, as oleic acid percentages in oil samples, were determined using an alkali titration method. Fifty grams of oil were dissolved in 50 ml of neutralized methylated spirits (Appendix 1). Two drops of phenolphalein indicator were added to the sample and this was then heated on a hot plate only enough to produce a well emulsified sample on shaking. The sample was titrated with 0.1M sodium hydroxide until a faint pink-coloured end point was reached and which remains for only 30 seconds. The volume of sodium hydroxide used in the titration determined the percentage (i.e. g per 100 g) of free fatty acids in the sample by using the conversion table (Appendix 1). The result is expressed in terms of oleic acid. Free fatty acids were calculated using the formula:

$$\% \text{ Free fatty acids} = \frac{\text{Vol.NaOH (ml) x normality NaOH x 28.2 (Oleic acid)}}{\text{Weight of sample (g)}} \times 100$$

3.2.8 Percentage cholesterol

For cholesterol standard preparation, 0.1 g of cholesterol was dissolved in chloroform in a 100ml volumetric flask and made up to mark. Increasing volumes of standard, 100 μg to 400 μg (100 μg = 0.1 ml) were transferred into test tubes. For sample preparation, 0.2 g of sample was dissolved in chloroform in a 100 ml volumetric flask and made up to the mark. One millilitre of sample was transferred into a test tube. Both the standard and sample solutions were evaporated to dryness under nitrogen.

Once the standard and sample were dried, 6 ml of acidic acetic anhydride solution (Appendix 1) was added to the test tubes. The solutions were mixed vigorously and allowed to stand at room temperature for half an hour. After the duration had passed a turquoise colour developed. The absorbance of the standard and sample solutions was measured spectro-photometrically at 620 nm against the blank. The cholesterol concentration of the sample was extrapolated from a linear calibration curve. Percentage cholesterol was calculated using the equations:

$$\text{(\%) Cholesterol 'as is basis'} = \frac{\text{concentration of cholesterol (μg) 100 x 100}}{\text{weight of sample x 1000 x 1000}}$$

$$\text{Mg/100 g Cholesterol} = \frac{\text{\% cholesterol x weight of sample x 1000}}{100}$$

$$\text{(\%) Cholesterol 'as per fat content'} = \frac{\text{mg/100 g cholesterol}}{1000}$$

3.2.9 Gas chromatography / mass spectroscopy analysis

The composition of the volatile constituents of the essential oils from *C. validus* and its plant organs i.e. flower-heads, leaves, culms and rhizomes was established by GC-MS.

Five drops of the oil samples were dissolved in 1mL dichloromethane, diluted and analyzed. One microlitre of the oil sample was injected into the HP 6890 series Gas Chromatograph interfaced to a 5973 Mass Selective Detector (MSD) and controlled by HP Chemstation software (version b.02.05, 1989-1997). The chromatographic separation was achieved using a DB-5 MS capillary column (30.0 m x 250 μm x 0.25 μm). The column stationary phase comprised of 5%-Diphenyl-95%-Dimethylpolysiloxane. The operating GC conditions was an initial oven temperature of 50°C, then programmed to 300°C at the rate of 10°C/minute and then kept constant at 300°C for 3 minutes. The injector and detector temperatures were set at 250°C, injected volume was 1μl and nitrogen was used as the carrier gas. The identification of oil components was done by comparison of retention indices and mass spectra reported in the literature, as well as the Wiley275 spectral library.

3.3 RESULTS AND DISCUSSION

3.3.1 Essential oil yields

The essential oil collected from all the plant organs of *C. validus* was less dense than water and exhibited a pale yellow colour. The relative amount (g/100g powdered material) of essential oil extracted from the plant organs presented in Figure 3.3 varied from 0.05 to 1.23%. The greatest concentration of essential oil was obtained from the flower-heads and rhizomes, 1.23 and 1.12% respectively.

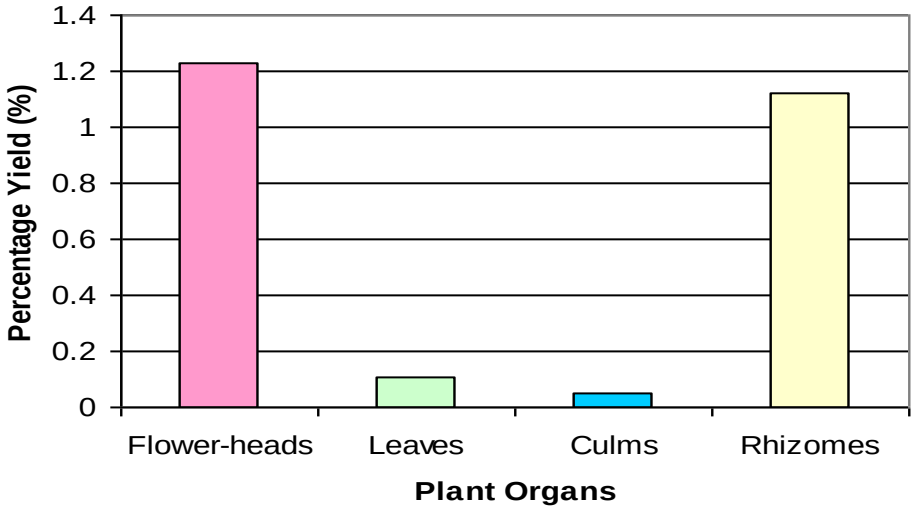


Figure 3.3: Percentage essential oil yields from plant organs

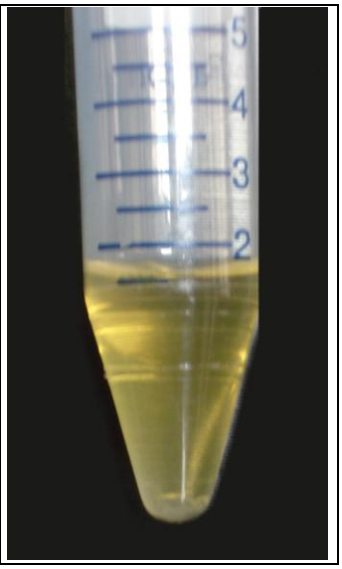
Of the plant organs in scented species, flowers produce the most diverse and the highest amount of volatile compounds, which peak when the flowers are ready for pollination. Vegetative tissue also releases small quantities of volatile organic compounds, which could be induced by mechanical damage or by herbivore- or pathogen infection (Loughrin *et al*., 1994; Pare and Tumlinson, 1997; Arimura *et al*., 2004). Volatile emission in flowers and accumulation in leaves increase during the early stages of organ development (when leaves

are young and not fully expanded or when flowers are ready for pollination) and then either remaining relatively constant or decreasing over the lifespan of the organ. (Bouwmeester *et al*., 1998; Dudareva and Pichersky, 2000; Gershenzon *et al*., 2000). This correlates to our study which revealed that the essential oil was concentrated in the flower-heads of *C. validus*.

3.3.2 Sensory Evaluation

The results of the sensory evaluation carried out on the essential oil from *C. validus* is shown in Table 3.2. The oil was pale yellow in colour and appeared to be slightly murky. Ratings on the odour intensity of the oil were unanimous. All three students recorded the intensity of the odour as being strong. The oil remained liquid at room temperature and its odour was terpentine-like.

Table 3.2: Sensory evaluation of *C. validus* oil

Characteristics	**Observation**	
Colour	Pale yellow	
Clarity	Slightly murky	
Odour	Turpentine-like	
Odour intensity	Strong	

3.3.3 Chemical indices

The stability of the oil was measured by its peroxide value, iodine value, percentage FFA, percentage cholesterol and rancimat IP. The results obtained for *C. validus* essential oil is shown in Table 3.3.

Table 3.3: Chemical indices of *C. validus* essential oil

Chemical Indices	**Results**
Peroxide Value (meq/kg)	60.56
Iodine Value	84.55
% Free Fatty Acids	0.19
% Cholesterol	3.03
Induction Period (hrs)	0

An important test for oxidative rancidity, is its peroxide value which measures the concentration of peroxides and hydro-peroxides formed in the initial stages of lipid oxidation. Fresh oils have been shown to have peroxide values lower than 10 mg/g oil and oils become rancid when the peroxide value ranges from 20.0 to 40.0 mg/g oil (Pearson, 1976). Ojeh (1981) reported that oils with high peroxide values are unstable and easily become rancid (having a disagreeable odour). Essential oil from *Cymbopogon citratus* contains peroxide values (meq/kg) between 7.3 to 59.3 depending on the rancidity of the oil (Bankole and Joda, 2004). The oil from *C. validus* had a peroxide value of 60.56 meq/kg suggesting that the oil is very susceptible to oxidation and thus unstable. Furthermore, the oil's Rancimat induction period was zero indicating that the oil is highly susceptible to oxidation.

The iodine value indicates the number of double bonds and this provides an indication of the sites for oxidation. Tan *et al*. (2002) has set the iodine value of the oils inside the interval

range of 9.37–145 g of I_2/100 g oil. In this study the iodine value was 84.55, also indicating that the oil is highly susceptible to oxidation.

The percentage FFA is also an indication of hydrolytic rancidity. Free fatty acids can stimulate oxidative deterioration of oils by enzymatic and/or chemical oxidation to form off-flavour components (Akintayo and Bayer, 2002). The % FFA in *C. validus* oil was 0.19%. In the tropics, where vegetable oils are the most common dietary lipids, it has been shown that it is desirable that the FFA content of cooking oil lies within limits of 0.0–3.0% (Bassir, 1971; Onyeiki and Acheru, 2002). The low percentage of FFA and high peroxide and iodine values clearly indicate pro-oxidation of the oil. The pro-oxidation of the oil could be related to the extraction process, i.e. hydro-distillation, where excessive heat could have affected the oil properties and may have induced partial alteration of the majority of minor constituents that have many functional, anti-oxidative and pro-oxidative effects (Espin *et al*., 2000; Koski *et al*., 2002; Tasioula-Margari and Okogeri, 2001).

Cholesterol, reported to be present in small amounts in vegetable oil (Kochhar, 1983), was detected in *C. validus* oil. Plant sterols have been suggested to have dietary significance and to protect vegetable oils from oxidative polymerisation during heating at frying temperatures (Gordon and Magos, 1983).

3.3.4 Chemical composition

Gas chromatography/mass spectroscopy analysis of the essential oil samples showed that the occurrence and amount of volatile compounds present in each of the tested organs of the plant varied. This can be seen from the total ion chromatograms of the oil samples (Figures 3.4-3.8). The essential oil samples comprised of hydrocarbons, mainly terpenes (monoterpenes, sesquiterpenes and diterpenes), oxygenated terpenes, alcohols, aldehydes,

carboxylic acids, esters, fatty acids and ketones. Identification of the volatile compounds from *C. validus*, the flower-heads, leaves, culms and rhizomes was made by comparing the mass spectra and retention times. Those compounds exhibiting a 95% or more qualitative match to the spectral library are highlighted Table 3.4-3.8.

The similarities of the chemical composition of the different parts of *C. validus* are presented in the terpenoid contents. It was found that *C. validus* essential oil contained the least number of volatile compounds (28), whilst the essential oil obtained from the flower-heads of *C. validus* contained the most (58). Of the twenty eight volatile compounds separated and identified from the essential oil of *C. validus*, monoterpenes accounted for 17.86%, sesquiterpenes accounted for 46.43%, oxygenated monoterpenes accounted for 3.57% and oxygenated sesquiterpenes for 3.57%. Sesquiterpenes present in *C. validus* oil included (+)-cycloisosativene, alpha-cubebene, alpha-copaene and beta-cubebene (Table 3.4). The essential oil from the flower-heads comprised of 18.97% monoterpenes, 20.69% sesquiterpenes, 12.07% oxygenated monoterpenes and 6.90% oxygenated sesquiterpenes. Monoterpene constituents such as allo-ocimene, gamma-terpinene, citronellal, geraniol, isoterpinolene, limonene, linalool and sabinene were present (Table 3.5). The leaf essential oil consisted of fifty six compounds of which 19.64% were monoterpenes, 14.29% sesquiterpenes, 10.71% oxygenated monoterpenes, oxygenated 3.57% sesquiterpenes and 1.79% diterpenes. Monoterpene constituents present in the leaf oil included alpha-pinene, beta-myrcene, camphene, citronellal, citronellol, para-cymene and tricyclene (Table 3.6). Fifty six compounds were also identified from the culm essential oil, consisting of 19.64% monoterpenes, 12.50% sesquiterpenes, 10.71% oxygenated monoterpenes, 7.14% oxygenated sesquiterpenes, 1.79% diterpenes and 1.79% oxygenated diterpenes. Monoterpene constituents were predominant in the oil from the culms. This included compounds such as (+)-2-carene, alpha-pipene, camphene, decyl aldehyde, geraniol, limonene and sabinene (Table 3.7). The essential oil from the rhizomes comprised of

31.11% monoterpenes, 13.33% sesquiterpenes, 13.33% oxygenated monoterpenes and 4.44% oxygenated sesquiterpenes. In the rhizome oil monoterpene constituents included alpha-pinene, alpha-terpipene, beta-myrcene, camphene, citronellal, geraniol and trans-beta-ocimene (Table 3.8). Although the oil from the plant organs contained a higher amount of monoterpenes constituents, more than 50% of *C. validus* oil comprised of sesquiterpene constituents. This contradicts the findings previously reported on *C. validus* oil and the essential oil from other *Cymbopogon* species (Alitinou *et al.*, 2006; Chagonda *et al*., 2000; Oussalah *et al*., 2006). Chagonda *et al*., (2000) found that the oils from wild and cultivated *C. validus* predominantly comprised of monoterpenes.

Volatile compounds exhibiting a 95% or more qualitative match to the spectral library are summarized in Table 3.9. From Table 3.9, it was possible to highlight those compounds common to all plant organs. The predominant compounds present in all plant organs were mainly monoterpene constituents namely, Alpha-Cubebene, Camphene, Citronellal, Geraniol, Limonene, Palmitic Acid and Sabinene. However, of the above mentioned compounds only Alpha-Cubebene and Limonene were present in *C. validus* essential oil. Monoterpene constituents were also reported to be predominant in other *Cymbopogon* oils (Khanuja *et al.*, 2005). Camphene was previously reported to be predominant in *C. validus* oil (Chagonda *et al*., 2000; Oussalah *et al*., 2006). Koba *et al.* (2004) and Duarte *et al.* (2005) found high amounts of citronellal in *C. nardus* and *C. winterianus* oils. Geraniol was reported as a predominant compound in the essential oil from *C. validus* (Chagonda *et al*., 2000), *C. citratus* (Cimanga *et al*., 2002), *C. nardus* (Koba *et al*., 2004), *C. winterianus* (Oussalah *et al*., 2006) and *C. martinii* whose oil comprised of 84% geraniol (Rao *et al*., 2005). Cimanga *et al.* (2002), Alitinou *et al.* (2006) and Kanko *et al.* (2004) found that limonene was one the predominant constituents of the oils from *C. citratus*, *C. giganteus* and *C. nardus* respectively. The chemical structures and properties of the predominant

compounds present in *C. validus* oil are shown in Table 3.10. Palmitic acid, a fatty acid, is used in manufacturing soaps and lubricants and was found to have antioxidant properties. Citronellal, an oxygenic monoterpene was reported to have antibacterial, fungicidal, insecticidal, nematicidal and mosquitoe repellency properties. The monoterpene hydrocarbon, Geraniol, has many medicinal properties such as anti-inflammatory, antiseptic and cancer-preventative properties and is also widely used in the flavour and fragrance industry (Duke, 1998; Mahalwal and Ali, 2002; Omolo *et al*., 2004). Oussalah *et al*. (2006) and Koba *et al*. (2004) reported that citronellal comprised more than 30% of the essential oils from *Cymbopogon winterianus* and *Cymbopogon nardus*, both of which were found to have antimicrobial and insecticidal properties. The oil from *Cymbopogon martinii* predominantly comprises of geraniol (Oussalah *et al*., 2006), this compound is prized in the perfumery industry (Kumar *et al*., 2000).

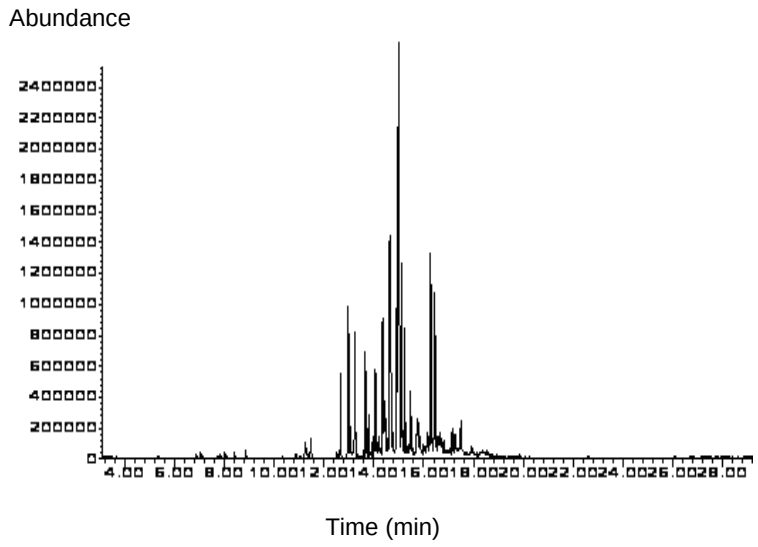


Figure 3.4: Chromatograph of *C. validus* essential oil

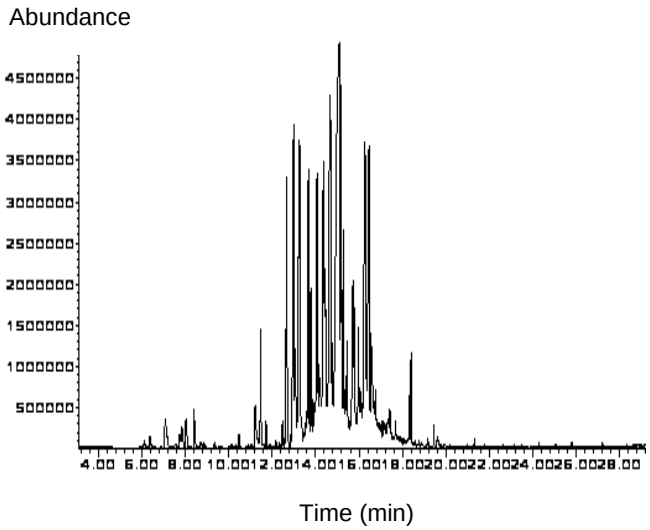


Figure 3.6: Chromatograph of leaf essential oil

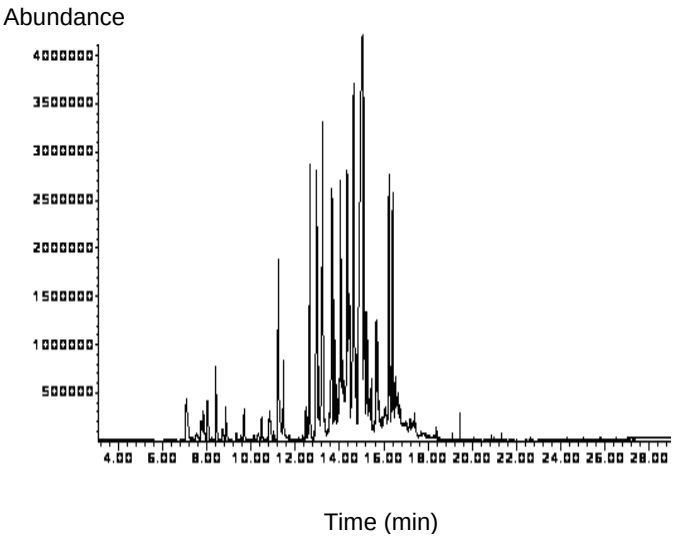


Figure 3.5: Chromatograph of flower essential oil

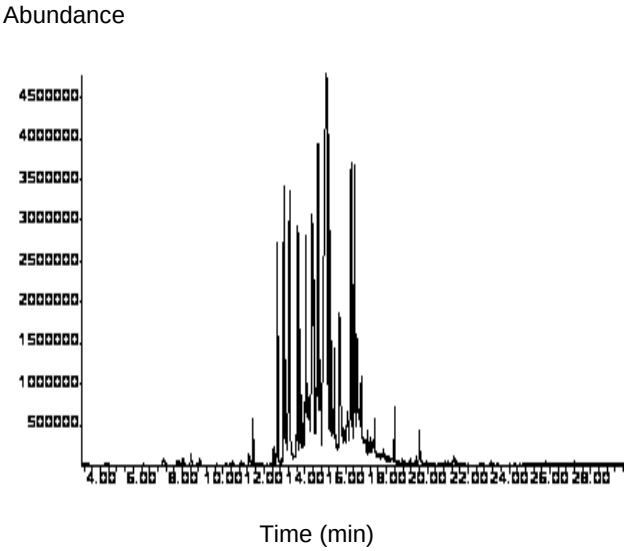


Figure 3.7: Chromatograph of culm essential oil

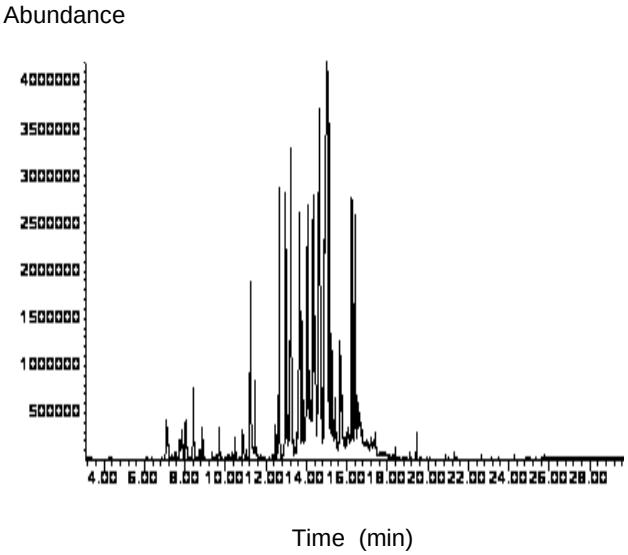


Figure 3.8: Chromatograph of rhizome essential oil

Table 3.4: Chemical composition of *Cymbopogon validus* essential oil

Compounds	Library Match Quality (%)	Retention time (min)
Ethenyl-Benzene	91	5.35
Myrcene	90	7.04
Limonene	**98**	**7.72**
Beta. -Ocimene	83	7.83
4-Nonanone	83	8.40
Alpha. -Terpinolene	**95**	**8.86**
Alloocimene	86	9.35
Geraniol	90	11.24
4-Undecanone	86	11.47
Undecane	83	11.85
Citronellyl butyrate	91	12.58
Alpha. -Cubebene	**98**	**12.67**
(+)-Cycloisosativene	**99**	**12.97**
Alpha. -Copaene	**99**	**13.06**
Beta. -Selinene	80	13.25
Alloaromadendrene	83	13.37
(-)-Isosativene	94	13.57
Germacrene d	93	13.66
Beta. -Cubebene	**95**	**13.79**
1,3,5-dimethyl-benzene	83	13.86
Trans-.beta.-Farnesene	93	14.00
Epi-bicyclosesquiphellandrene	89	14.19
Delta.-Cadinene	91	14.35
Alpha.-Muurolene	89	14.43
Beta.-Selinene	93	14.56
Naphthalene	**98**	**15.11**
Alpha.-Calacorene	94	15.25
6-Methoxy-1-acetonaphthone	83	16.17
Cis-Farnesol	91	17.25

Table 3.5: Chemical composition of flower essential oil

Compounds	Library Match Quality (%)	Retention time (min)
Alpha.-pipene	**97**	**6.11**
Camphene	**98**	**6.38**
1-Limonene	94	6.52
Sabinene	**95**	**6.79**
6-Methyl-5-hepen-2-one	91	6.99
Myrcene	**96**	**7.07**
Isoterpinolene	**95**	**7.53**
4-Nitrotoluene	**95**	**7.66**
Limonene	**99**	**7.74**
Trans-.Beta.-Ocimene	**95**	**8.03**
Gamma.-Terpinene	**97**	**8.23**
4-Nonanone	91	8.45
Terpinolene	**98**	**8.73**
Linalool	**97**	**8.89**
Cis-Rose Oxide	91	9.06
Allo-Ocimene	**98**	**9.38**
Citronellal	**98**	**9.71**
4-methyl-1-3-Cyclohexen-1-ol	86	10.14
Decyl Aldehyde	91	10.49
Citronellol	**97**	**10.90**
Geraniol	**95**	**11.40**
4-Undecanone	94	11.55
1-Bornyl Acetate	**95**	**11.78**
Alpha.-Cubebene	**97**	**12.76**
Clovene	**95**	**12.87**
Cyclosativene	**99**	**13.05**
Calarene	87	13.40
Trans-Caryophyllene	91	13.48
Germacrene d	91	13.79
Epi-Bicyclosesquiphellandrene	91	13.87
Epizonaren	83	14.16
Isolongifolene	90	14.21
Alpha.-Gurjunene	94	14.55
Delta.-Cadinene	93	14.75
Hedycaryol	91	15.26
Spathulenol	**97**	**15.66**
Junipene	93	15.71
Caryophyllene oxide	94	15.74
1,4-dimethyl-7-Azulene	**97**	**16.88**
(Z, E)-Farnesal	93	17.41
Cis-Jasmone	83	17.52
3-Methoxyphenyl-4-methylcyclohexanone	80	18.13
Dimethyl-1, 2-dicyano-3-methyl-1, 2-cyclopropanedicarboximidate	90	18.26
(Z)-2,3-dihydro-1H-cyclonona [def] biphenylene	83	18.31
6,10,14-trimethyl-2-Pentadecanone	87	18.41
Citronellyl propionate	91	19.12
Neryl acetate	86	19.47
Palmitic acid	**99**	**19.62**
Palmitic Aldehyde	86	20.14
Neryl propionate	90	20.40
Geranyl propionate	80	21.20
Geranyl Isovalerate	91	21.33
Tetradecanal	86	22.01
Nonadecane	91	22.64
n-Heptadecane	80	23.47
Pentacosane	**95**	**24.27**
2-methyl-3-phenyl-1H-Indole	80	24.95
Hentriacontane	91	25.79
Tetradecanal	86	22.01

Table 3.6: Chemical composition of leaf essential oil

Compounds	Library Match Quality (%)	Retention time (min)
Tricyclene	**96**	**5.92**
Alpha.-pinene	**97**	**6.13**
Camphene	**98**	**6.39**
Sabinene	**97**	**6.80**
beta.-Myrcene	**97**	**7.08**
Alpha.-Terpinene	93	7.54
Para-cymene	**95**	**7.67**
Limonene	**98**	**7.75**
Trans-.beta-Ocimene	91	7.87
1-Cyclopropane	94	8.05
gamma.-Terpinene	86	8.23
4-Nonanone	91	8.44
Terpinolene	**98**	**8.74**
Linalool	91	8.88
Citronellal	**98**	**9.70**
Decyl aldehyde	91	10.49
Citronellol	**98**	**10.90**
Neral	84	11.04
Geraniol	**95**	**11.27**
4-Undecanone	94	11.53
(-)-Bornyl acetate	**99**	**11.75**
Geranic acid	90	12.19
Citronellyl propionate	91	12.59
Alpha.-Cubebene	**95**	**12.74**
Clovene	91	12.86
(+)-Cycloisosativene	**98**	**13.06**
Isocaryophyllene	**95**	**13.49**
Germacrene d	86	13.81
Valencene	94	13.84
Delta.-Cadinene	91	14.22
Epi-bicyclosesquiphellandrene	90	14.87
Caryophyllene oxide	**95**	**15.77**
T-Cadinol	80	16.77
1,4-dimethyl-7-azulene	**97**	**16.99**
3,4'-Difluoro-4-methoxybiphenyl	83	17.11
Myristic acid	90	17.68
Neophytadiene	**99**	**18.39**
6,10-dimethyl-2-Undecanone	86	18.48
Geranyl propionate	89	19.49
Palmitic acid	**99**	**19.73**
Octadecanal	87	20.16
Heneicosane	83	20.87
2-Nonadecanone	87	20.94
2-N-Nonylaldehyde-2-hendecenoic acid	93	21.47
11-decyl-Docosane	90	21.77
Hexadecyl-Oxirane	91	22.01
Neryl acetate	90	23.06
Neryl propionate	91	23.28
1-Dotriacontanol	83	23.43
Eicosane	87	23.47
Pentacosane	94	24.28
Heneicosane	80	25.05
Heptacosane	93	25.79
Geranyl butyrate	91	26.17
Tetradecanal	86	26.80
Hexatriacontane	91	27.21
(Z)14-Tricosenyl formate	91	28.33
Neryl propionate	91	23.28

Table 3.7: Chemical composition of culm essential oil

Compounds	Library Match Quality (%)	Retention time (min)
Hexamethyl-Cyclotrisiloxane	83	4.27
Alpha.-pipene	**96**	**6.11**
Camphene	**97**	**6.38**
Sabinene	**95**	**6.79**
6-Methyl-5-hepten-2-one	90	6.99
Myrcene	**95**	**7.07**
1-Phellandrene	87	7.34
Alpha.-terpipene	91	7.53
1-methyl-2-Benzene	**97**	**7.66**
Limonene	**99**	**7.73**
Eucalyptol	91	7.77
Cis-Ocimene	83	7.84
7-methyl-4-Octanone	80	8.42
(+)-2-Carene	**98**	**8.73**
Linalool L	**95**	**8.87**
Citronellal	**96**	**9.69**
Alpha.-Terpineol	83	10.34
Decyl aldehyde	91	10.48
Geraniol	**95**	**11.25**
4-Undecanone	**95**	**11.50**
(-)-Bornyl acetate	**99**	**11.73**
Alpha.-terpinolene	**95**	**12.49**
Alpha.-Cubebene	**97**	**12.73**
Clovene	93	12.86
Cyclosativene	**99**	**13.06**
Germacrene d	86	13.79
Calarene	80	13.93
Delta.-Cadinene	91	14.15
Alpha.-Cadinol	94	14.53
Cadina-1,4-diene	94	15.52
Caryophyllene oxide	87	15.76
10-epi-.gamma.-eudesmol	93	15.79
1,4-dimethyl-7-azulene	**96**	**16.99**
(Z,E)-Farnesal	83	17.48
6,10,14-trimethyl-2-Pentadecanone	93	18.46
Pentadecanoic acid	**95**	**18.64**
Neophytadiene	89	18.80
Geranyl butyrate	87	19.47
Palmitic acid	**99**	**19.82**
Neryl propionate	86	20.42
Phytol	91	21.12
Linoleic acid	91	21.34
Oleic acid	94	21.40
Tricosane	91	22.64
Propanoic acid	90	23.06
Butanoic acid	90	23.28
Hexatriocontane	86	23.47
Pentacosane	**95**	**24.28**
Phthalic acid	91	24.71
Hexadecanal	80	25.32
Heptacosane	**97**	**25.79**
Tetratetracontane	83	26.50
Hexadecyl-Oxirane	90	26.79
Hentriacontane	90	27.20
(Z)14-Tricosenyl formate	94	28.33
11-decyl-Docosane	91	28.80

Table 3.8: Chemical composition of rhizome essential oil

Compounds	Library Match Quality (%)	Retention time (min)
Hexanal	86	3.91
Tricyclene	91	5.91
Alpha.-Pinene	**96**	**6.12**
Camphene	**98**	**6.38**
Sabinene	**95**	**6.80**
beta.-Myrcene	**97**	**7.06**
Alpha.-terpipene	**96**	**7.53**
1-methyl-2-Benzene	**95**	**7.66**
Limonene	**99**	**7.73**
Trans-.beta.-Ocimene	**95**	**8.02**
.beta.-Phellandrene	81	8.22
4-Nonanone	87	8.43
Terpinolene	**98**	**8.72**
Allo-Ocimene	87	9.34
Citronellal	**98**	**9.69**
Terpinen-4-ol	94	10.14
Alpha.-Terpineol	90	10.34
Decyl Aldehyde	91	10.48
6-Octen-1-ol	**97**	**10.89**
Neral	91	11.03
Geraniol	**95**	**11.24**
4-Undecanone	91	11.48
Exobornyl acetate	86	11.72
Germacrene b	94	12.36
Alpha.-Terpinene	**95**	**12.49**
Alpha.-Cubebene	**95**	**12.69**
(+)-Cycloisosativene	**99**	**13.04**
(1H) Benzocycloheptene	83	13.16
Germacrene d	87	13.78
delta.-Cadinene	80	14.14
Alpha.-Selinene	86	15.81
Beta.-Eudesmol	89	16.88
Trans,trans-Farnesal	87	17.47
1,4-dimethyl-7-Azulene	93	17.99
1-Naphthalenol	87	18.20
Citronellyl acetate	80	19.13
Geranyl Butyrate	91	19.46
Palmitic acid	**99**	**19.74**
Butanoic acid, 3,7-dimethyl-6-octenyl ester	91	21.01
Linoleic acid	**98**	**21.36**
9,12-Octadecadienoic acid	**99**	**21.47**
Ethyl 9-Octadecanoate	93	21.52
2-methyl-3-phenyl-1H-Indole	80	24.95
Nonadecane	81	25.79
Neryl 2-methylpropanoate	91	26.17
1-methyl-5-Cyclohexene	83	27.44

Table 3.9: Chemical compounds exhibiting a 95% or greater spectral match and are common to all plant organs

Whole Plant		Flowers		Leaves		Culms		Rhizomes	
Compound	**RT**	**Compound**	**RT**	**Compound**	**RT**	**Compound**	**RT**	**Compound**	**RT**
				Tricyclene	5.92				
		Alpha-Pipene	6.11	Alpha-Pinene	6.13	Alpha-Pipene	6.11	Alpha-Pinene	6.12
		Camphene	**6.38**	**Camphene**	**6.39**	**Camphene**	**6.38**	**Camphene**	**6.38**
		Sabinene	**6.79**	**Sabinene**	**6.80**	**Sabinene**	**6.79**	**Sabinene**	**6.80**
		Myrcene	7.07	Beta-Myrcene	7.08	Myrcene	7.07	Beta-Myrcene	7.06
		Isoterpinolene	7.53					Alpha-terpipene	7.53
		1-methyl-4-Benzene	7.66			1-methyl-2-Benzene	7.66	1-methyl-2-Benzene	7.66
				Para-cymene	7.67				
Limonene	**7.72**	**Limonene**	**7.74**	**Limonene**	**7.75**	**Limonene**	**7.73**	**Limonene**	**7.73**
		Trans-Beta-Ocimene	8.03					Trans-Beta-Ocimene	8.02
		Gamma-Terpinene	8.23						
Alpha-Terpinolene	8.86	Terpinolene	8.73	Terpinolene	8.74	(+)-2-Carene	8.73	Terpinolene	8.72
		Linalool	8.89			Linalool L	8.89		
		Allo-Ocimene	9.38						
		Citronellal	**9.71**	**Citronellal**	**9.70**	**Citronellal**	**9.69**	**Citronellal**	**9.71**
		Citronellol	10.90	Citronellol	10.90			6-Octen-1-ol	10.89
		Geraniol	**11.40**	**Geraniol**	**11.27**	**Geraniol**	**11.25**	**Geraniol**	**11.24**
						4-Undecanone	11.50		
		1-Bornyl-Acetate	11.78	(-)-Bornyl-Acetate	11.75	(-)-Bornyl-Acetate	11.73		
						Alpha-Terpinolene	12.49	Alpha-Terpinene	12.49
Alpha-Cubebene	**12.76**	**Alpha-Cubebene**	**12.76**	**Alpha-Cubebene**	**12.74**	**Alpha-Cubebene**	**12.73**	**Alpha-Cubebene**	**12.69**
		Clovene	12.87						
Alpha-Copaene	13.06	Cyclosativene	13.05	(+)-Cycloisosativene	13.06	Cyclosativene	13.06	(+)-Cycloisosativene	13.04
Beta-Cubebene	13.79			Isocaryophyllene	13.49				
Naphthalene	15.11								
				Caryophyllene oxide	15.77				
		Spathulenol	15.66						
		1,4-dimethyl-7-Azulene	16.88	1,4-dimethyl-7-Azulene	16.99	1,4-dimethyl-7-Azulene	16.99		
				Neophytadiene	18.39				
						Pentadecanoic acid	18.64		
		Palmitic acid	**19.62**	**Palmitic acid**	**19.73**	**Palmitic acid**	**19.82**	**Palmitic acid**	**19.74**
								Linoleic acid	21.36
								9,12-Octadecadienoic acid	21.47
		Pentacosane	24.27			Pentacosane	24.28		
						Heptacosane	25.79		

Table 3.10: Structures and properties of compounds exhibiting a 95% or greater spectral match and are common to all plant organs

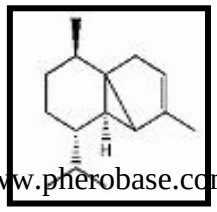
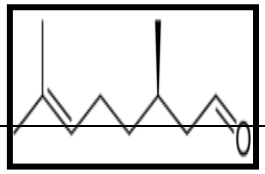
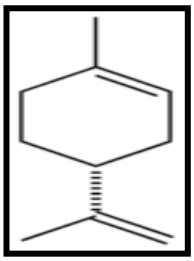
Compound Name	Properties
Alpha.-Cubebene  www.pherobase.com	**Sesquiterpene** Insecticide (Orme and Kegley, 2006)
Camphene http://en.wikipedia.org/	**Monoterpene** Allelopathic, Antibacterial, Antifungal, Antioxidant, Expectorant, Flavour, Insectifuge, Mosquito repellency, Spasmogenic (Duke, 1998; FAO, 1994; Omolo *et al*., 2004)
Citronellal  http://en.wikipedia.org/	**Oxygenic Monoterpene** Acaricide, Allergenic, Antibacterial, Antioxidant, Antiseptic, Anti-staphylococcic, Anti-streptococcic, Candidicide, Embryotoxic, Flavour, Fungicide, Insectifuge, Irritant, Mosquito repellency, Motor-Depressant, Nematicide, P450-2B1-Inhibitor, Perfumery, Sedative, Teratogenic (Duke, 1998; Mahalwal and Ali, 2002; Omolo *et al*., 2004)
Geraniol http://en.wikipedia.org/ 	**Oxygenic Monoterpene** Allergenic, Anthelmintic, Antibacterial, Anti-cancer (Pancreas), Anti-cariogenic, Anti-inflammatory, Anti-melanomic, Antioxidant, Anti-salmonella, Antiseptic, Antis-pasmodic, Anti-tubercular, Anti-tumour, Anti-tumour (Pancreas), Ascaricide, CNS-Stimulant, Cancer-Preventive, Candidicide, Embryotoxic, Emetic, Expectorant, Flavour, Fungicide, Herbicide, Insectifuge, Insectiphile, Mosuitoe repellency, Mycobactericide, Nematicide, Perfumery, Sedative, Trichomonicide (Baylac and Racine, 2003; Duke, 1998; Omolo *et al*., 2004; Top Cultures; 2006b)

Table 3.10 continued

Compound Name	Properties
Limonene  http://en.wikipedia.org/	**Monoterpene** AChE-Inhibitor, Acaricide, Allelochemic, Allergenic, Anti-acetylcholinesterase, Anti-asthmatic, Antibacterial, Anti-cancer, Anti-feedant, Anti-flu, Anti-inflammatory, Anti-lithic, Anti-lymphomic, Anti-mutagenic, Anti-obesity, Antioxidant, Antiseptic, Anti-spasmodic, Anti-tumour, Anti-tumour (Breast), Anti-tumour (Pancreas), Anti-tumour (Prostate), Antiviral, Apoptotic, Bronchoprotectant, Cancer-Preventive, Candidistat, Chemopreventive, Detoxicant, Enterocontractant, Expectorant, Flavour, Fungiphilic, Fungistat, GST-Inducer, Herbicide, Histaminic, Immunomodulator, Insecticide, Insectifuge, Irritant, Lipolytic, Mosquito repellent, Myorelaxant, Nematicide , NO-Genic, Ornithine-Decarboxylase-Inhibitor, Ozone-Scavenger, P450-Inducer, Photosensitizer, Sedative, Transdermal (Duke, 1998; Njenga and Viljoen, 2006; Omolo *et al*., 2004)
Palmitic acid http://en.wikipedia.org/	**Fatty Acid** 5-alpha-reductase-inhibitor, Anti-alopecic , Anti-androgenic, Anti-fibrinolytic, Antioxidant, Flavour, Hemolytic, Lubricant, Hypercholesterolemic, Nematicide, Propecic, Soap (Duke, 1998)
Sabinene http://en.wikipedia.org/	**Monoterpene** Antibacterial, Anti-helicobacter, Antiseptic, Anti-ulcer, Perfumery (Duke, 1998)

3.4 SUMMARY OF FINDINGS

The essential oil is concentrated in the flower-heads and rhizomes of *C. validus* indicated by its percentage yields, 1.23 and 1.12% respectively. *C. validus* produced a slightly murky, pale-yellow coloured oil that had a strong, turpentine-like smell and remained liquid at room temperature. The oxidative stability of this oil was determined by its peroxide value (60.56 meq/kg), iodine value (84.55), % FFA (0.19), % cholesterol (3.03) and IP (negative). These results indicate that the oil is highly susceptible to oxidation. The main chemical constituents of the oil are alpha-cubebene, camphene, citronellal, geraniol, limonene, palmitic acid and sabinene.

CHAPTER 4: ANTIMICROBIAL PROPERTIES

4.1 INTRODUCTION

It has long been recognised that some essential oils have antimicrobial properties (Boyle, 1955; Deans *et al.*, 1992; Piccaglia *et al.*, 1993) and these have been reviewed in the past (Shelef, 1983; Nychas, 1995) as have the antimicrobial properties of spices (Shelef, 1983). A relatively recent enhancement of interest in 'green' consumerism has lead to a renewal of scientific interest in these substances (Nychas, 1995; Tuley de Silva, 1996). This antimicrobial activity could act as a chemical defence against plant pathogenic diseases. Pathogens can readily penetrate at wound sites caused, for example, by herbivores. Wounding of leaves which are covered with volatile oil glands results in the rupture of glands causing the oil to flow over the wound. The existence, therefore, of antimicrobial activity in the oil, would be of considerable benefit to the plant (Svoboda and Hampson, 1999).

Although the antimicrobial properties of essential oils and their components have been reviewed in the past (Koedam, 1977a; 1977b; Shelef, 1983; Nychas, 1995), the mechanism of action has not been studied in great detail (Lambert *et al*., 2001). Considering the large number of different groups of chemical compounds present in essential oils, it is most likely that their antibacterial activity is not attributable to one specific mechanism but that there are several targets in the cell (Skandamis *et al*., 2001; Carson *et al.*, 2002). An important characteristic of essential oils and their components is their hydrophobicity, which enables them to partition in the lipids of the bacterial cell membrane and mitochondria, disturbing the structures and rendering them more permeable (Knobloch *et al*., 1986; Sikkema *et al*., 1994). Leakage of ions and other cell contents can then occur (Oosterhaven *et al*., 1995; Gustafson *et al*., 1998; Helander *et al*., 1998; Cox *et al*., 2000; Lambert *et al*., 2001; Skandamis *et al*.,

2001; Carson *et al*., 2002; Ultee *et al*., 2002). Although a certain amount of leakage from bacterial cells may be tolerated without loss of viability, extensive loss of cell contents or the exit of critical molecules and ions will lead to death (Denyer and Hugo, 1991).

Most studies investigating the action of whole essential oils against food spoilage organisms and food borne pathogens agree that, generally, essential oils are slightly more active against Gram-positive than Gram-negative bacteria (Canillac and Mourey, 2001; Cimanga *et al*., 2002; Delaquis *et al*., 2002; Demetzos and Perdetzoglou, 2001; Harpaz *et al*., 2003; Juliano *et al*., 2000; Lambert *et al*., 2001; Marino *et al*., 1999; Marino *et al*., 2001; Mendoza-Yepes *et al*., 1997; Negi *et al*., 1999; Pintore *et al*., 2002; Ruberto *et al*., 2000; Senatore *et al*., 2000; Smith-Palmer *et al*., 1998). That Gram-negative organisms are less susceptible to the action of antibacterials is perhaps to be expected, since they possess an outer membrane surrounding the cell wall (Ratledge and Wilkinson, 1988), which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering (Vaara, 1992). However, not all studies on essential oils have concluded that Gram-positives are more susceptible (Wilkinson *et al*., 2003); *A. hydrophila* (gram-negative) appears in fact to be one of the most sensitive (Stecchini *et al*., 1993; Hao *et al*., 1998a; 1998b; Wan *et al*., 1998). In another study no obvious difference between Gram-positives and Gram-negatives was measured in the susceptibility after 24 h, but the inhibitory effect was more often extended to 48 h with gram-negative than with gram-positive organisms (Quattara *et al*., 1997; Quattara *et al*., 2001).

It was postulated that individual components of essential oils exhibit different degrees of activity against gram-positives and gram-negatives (Dorman and Deans, 2000) and it is known that the chemical composition of essential oils from a particular plant species can vary according to the geographical origin and harvesting period. It is therefore possible that variation in composition between batches of essential oils is sufficient to cause variability in

the degree of susceptibility of Gram-negative and Gram-positive bacteria (Burt, 2004). Of the gram-negative bacteria, Pseudomonads, and in particular *P. aeruginosa*, appear to be least sensitive to the action of essential oils (Cosentino *et al*., 1999; Dorman and Deans, 2000; Lis-Balchin *et al*., 1999; Paster *et al*., 1990; Pintore *et al*., 2002; Ruberto *et al*., 2000; Senatore *et al*., 2000; Tsigarida *et al*., 2000; Wilkinson *et al*., 2003).

Generally, essential oils produced from herbs harvested during or immediately after flowering possess the strongest antimicrobial activity (McGimpsey *et al*., 1994; Marino *et al*., 1999). Enantiomers of essential oil components have been shown to exhibit antimicrobial activity to different extents (Lis-Balchin *et al*., 1999). The composition of essential oils from different parts of the same plant can also differ widely. For example, the oil obtained from the seeds of coriander (*Coriandrum sativum* L.) has a quite different composition to the oil of cilantro, which is obtained from the immature leaves of the same plant (Delaquis *et al*., 2002). The chemical compounds of essential oils, more specifically terpenoids and phenolic compounds have been associated with the antimicrobial activity of essential oils. However, there is evidence that essential oils are more strongly antimicrobial than is accounted for by the additive effect of their major antimicrobial components; minor components appear, therefore to play a significant role (Paster *et al*., 1995). It is also suggested that a complex oil presents a greater barrier to pathogen adaptation than would a more simple mixture of monterpenes. This theory is well documented in the detailed study of *Myricia gale* volatile oil and its inhibitory properties against a broad spectrum of fungal species (Carlton *et al*., 1992; Svoboda *et al*., 1998). The complicated mixtures of monoterpenes and sesquiterpenes in the whole oil represented the strongest barrier to fungal infection (Svoboda and Hampson, 1999).

The aim of this study was to investigate the antimicrobial activity of *C. validus* against several common bacteria and fungi.

4.2 METHODOLOGY

The antimicrobial activity of the essential oil was carried out by evaluating the bactericidal effect and minimum inhibitory concentration (MIC) on selected bacteria and fungi in a petri dish using the filter paper disk and agar disk diffusion method (National Committee on Clinical Laboratory Standards, 1997). The bacterial and fungal cultures were obtained from the Microbiology Laboratory, Department of Biotechnology, Durban University of Technology.

4.2.1 Preparation of bacterial cultures

For the bacterial assay, ten bacterial cultures, *Bacillus cereus* (DBT_F), *Bacillus stearothermophilus* (DBT_Q), *Esherischia coli* (DBT_L), *Klebsiella oxytoca* (DBT_AM), *Micrococcus sp*. (DBT_AR), *Pseudomonas aeruginosa* (DBT_D), *Proteus mirabilis* (DBT_ O), *Salmonella typhimurium* (DBT_AF), *Staphylococcus aureus* (DBT_E) and *Staphylococcus epidermis* (DBT Q) were used. The cultures were maintained on Tryptone Soya Agar plates and were recovered for testing by growth in Tryptone Soya Broth for 24 hours. The concentration of bacterial cells was adjusted to match McFarland Standard 0.5 (Matasyoh *et al*., 2007) which corresponds to 10^8 CFU/ml.

4.2.2 Preparation of fungal cultures

Two yeast cultures, *Candida albicans (*DBT_AB*)* and *Saccharomyces cerevisiae (*DBT_R*)*, as well as seven moulds *Aspergillus flavus (*DBT_AE*)*, *Cladosporium sp (*DBT_AS*)*, *Fusarium verticilloides (*DBT_AT*)*, *Geotrichum sp (*DBT_AA*)*, *Penicillium sp (*DBT_AC*)* and *Rhizopus sp (*DBT_Y*)* and *Trichoderma sp (*DBT_AU*)* were inoculated on Sabourand Dextrose Agar plates. The yeast cultures were recovered for testing by growth in Sabourand Dextrose Broth for 24 hours. The moulds were incubated at 28°C for 4 to 7 days until

sporulation. The spores were collected in 10 ml sterile distilled water, counted in a Neubauer counting chamber and the concentration adjusted to 10^6 spores/ml.

4.2.3 Determination of antimicrobial activity

A suspension of the test bacteria and fungi was spread on Mueller Hinton Agar and Sabourand Dextrose Agar plates respectively. Filter paper disks (6 mm in diameter) were individually impregnated with five microlitres of the oil samples (essential oil and 1:1 dilution) and then placed on the inoculated plates for 2 hours at 4°C following which they were incubated at 37°C for 24 hours for bacteria and 30°C for 48 hours for fungi. DMSO (5 μl) served as the negative control, whilst Ciprofloxacin (5 μg per disk) and Amphotericin B (5 μg per disk) served as a positive control for bacteria and fungi respectively. Each test was carried out in duplicate using two individually impregnated filter paper disks. At the end of the incubation time the positive antibacterial and antifungal activities were established by the presence of measuarble zones of inhibition. The antimicrobial activity was recorded as the width (in millimetres, diameter of the disc included) of the zone of inhibition after incubation. The MIC was taken as the lowest concentration that inhibited growth after incubation.

4.3 RESULTS AND DISCUSSION

The zones of inhibition and MIC of *C. validus* essential oil for the test Gram positive and Gram negative bacteria are shown in Table 4.1. The essential oil inhibited the growth of all the Gram positive bacteria tested and showed no activity against any of the Gram negative bacteria. The oil showed more or less similar activity against all Gram positive bacteria (MIC 0.0625 vol/vol), with *Micrococcus sp*. being the most sensitive organism. No activity was found against any of the fungi tested (Table 4.2). All the essential oil samples screened, displayed poor or no activity against bacteria and fungi compared to their respective controls (Ciprofloxacin and Amphotericin B).

Table 4.1: Zones of inhibition and MIC values for test bacteria

Organism	Zones of Inhibition (mm)[a]			MIC (vol/vol)
	Essential Oil	(Oil:DMSO) 1:1	Ciprofloxacin[b]	
B. cereus	**9**	**8**	**27**	**0.0625**
B. stearothermophilus	**9**	**8**	**36**	**0.0625**
E. coli	0	0	39	n/a
K. oxytoca	0	0	38	n/a
P. aeruginosa	0	0	25	n/a
P. mirabilis	0	0	39	n/a
S. typhimurium	0	0	37	n/a
S. aureus	**8**	**6.5**	**30**	**0.0625**
S. epidermis	**9**	**8**	**38**	**0.0625**
Micrococcus sp.	**10**	**10**	**28**	**0.0625**

a Diameter of zone of inhibition including disc diameter of 6 mm

b Tested at a concentration of 5 µg/disk

n/a Not applicable

Table 4.2: Zones of inhibition for test fungi

Organism	Zones of Inhibition (mm)[a]		
	Essential Oil	(Oil:DMSO) 1:1	Amphotericin B[b]
Aspergillus flavus	0	0	9.5
Candida albicans	0	0	25
Cladosporium sp	0	0	10
Fusarium moniliforme	0	0	23
Geotrichum sp.	0	0	10
Penicillium sp	0	0	16
Rhizopus sp.	0	0	10.5
Saccharomyces cerevisiae	0	0	26
Trichoderma sp.	0	0	10

a Diameter of zone of inhibition including disc diameter of 6 mm

b Tested at a concentration of 5 μg/disk

The essential oil evaluated in this study has a great variety of phytochemicals that could be considered as responsible for a larger or smaller part of the antimicrobial activity. Although they usually occur as complex mixtures, their activity can generally be accounted for in terms of their major monoterpenoid components. Research into the antimicrobial actions of monoterpenes suggests that they diffuse into and damage cell membrane structures (Sikkema *et al*., 1995).

The antibacterial activity of the oil could be due to the presence of ∝-terpineol (Carson and Riley, 1995; Cosentino *et al*., 1999), ∝-pinene (Chalchat *et al*., 2000; Oladimeji *et al*., 2001), β-pinene (Oladimeji *et al*., 2001), γ-terpene (Oladimeji *et al*., 2001), p-cymene (Carson and Riley, 1995; Chalchat *et al*., 2000; Cosentino *et al*., 1999), caryophyllene oxide (Ulubelen *et al*., 1994), eucalyptol (Mourey and Canillac, 2002; Viljoen *et al*., 2003), geraniol (Zakarya *et*

al., 1993), limonene (Chalchat *et al*., 2000), linalool (Carson and Riley, 1995; Cosentino *et al*., 1999; Zakarya *et al*, 1993), neral (Zakarya *et al*., 1993) and terpinen-4-ol (Barel *et al*., 1991), all of which have been reported to exhibit antimicrobial activity.

The essential oil of *C. validus* displayed poor antimicrobial activity which contradicts the findings previously reported on other *Cymbopogon* species. The essential oils of *C. citratus, C. flexosus, C. martinii, C. nardus and C. winterianus* (Oussalah *et al*., 2006) exhibited activity against both Gram positive and Gram negative bacteria. Antifungal activity of *C. citratus* essential oil has been previously reported by Nguefack *et al.* (2004) and Sacchetti *et al.* (2005). Nevertheless, the oil's antibacterial activity could be due to its predominant compounds, i.e. camphene, citronellal, geraniol, limonene and sabinene (Table 3.10, Section 3.3.4).

Evaluation of the antimicrobial activity of essential oils is quite problematic due to many factors such as composition and solubility of the oil, the source of the test organism used and the quantity of the active compound in the essential oil. The test method used for assessing the antimicrobial activity is often the disc diffusion method, which is highly dependant on H_2O solubility and ability of test components to diffuse through the agar. Thus it would be expected that hydrophobic components such as the essential oils would show less activity (Kamatou *et al*., 2005). Also, the reduced activity against Gram negative bacteria has been adduced to the impervious cell envelope possessed by these organisms, which makes them characteristically resistant to many antibacterial agents (Lamikanra, 1999).

4.4 SUMMARY OF FINDINGS

The essential oil of *C. validus* was found to be active against the growth of Gram positive bacteria and inactive against the growth of Gram negative bacteria as well as fungi. The antibacterial activity of the oil could be due to the presence of the predominant compounds, namely camphene, citronellal, geraniol, limonene and sabinene (Table 3.10, Section 3.3.4). These results do not support the usage of the oil as an antifungal agent.

CHAPTER 5: ANTIOXIDANT AND ANTI-INFLAMMATORY PROPERTIES

5.1 INTRODUCTION

Infectious and inflammatory diseases are among those using traditional remedies, especially aromatic plants or volatile extracts obtained from them. Aromatherapy utilizes a number of essential oils in the treatment of inflammatory disorders (Alexander, 2001). The inflammatory processes involve reactive oxygen species started by leucocyte activation. This involves a series of events in which the metabolism of arachidonic acid plays an important role. It can be metabolized by the cyclooxygenase (COX) pathway to prostaglandins and thromboxane A_2, or by the lipoxygenase (LOX) pathway to hydroperoxy-eicosatetraeonic acids. For this reason, the *in vitro* inhibition of soybean LOX and a combination of this assay with an evaluation of the radical scavenging activity by the 1,1-diphenyl-1-picrylhydrazyl (DPPH) method (Mellors and Tappel, 1966) constitutes a good indication on the potential anti-inflammatory activity of a drug.

A large range of essential oils is reported to have anti-inflammatory effects. Clove (*Syzygium aromaticum*) essential oil has exhibited anti-inflammatory effects *in vivo*, inhibiting both LOX and COX pathways of the metabolism of arachidonic acid (Saeed and Gilani, 1994). A variety of essential oils has been found *in vitro* to significantly inhibit an enzyme of inflammation, 5-LOX (Baylac and Racine, 2003); not all findings agreeing with anecdotal reports of anti-inflammatory effects. Lavender oil and eugenol (major components of clove oil) have been found to inhibit allergic inflammatory responses *in vitro* and *in vivo* in animals (Kim and Cho, 1999; Kim *et al*., 1997); while eugenol and ginger essential oil have been found to have significant anti-inflammatory activity in rats (Sharma *et al*., 1994). Other components of essential oils have been found to have anti-inflammatory actions; α-

pinene in a rat model (Martin *et al*., 1993), and β-caryophyllene, both *in vivo* (Martin *et al*., 1993; Tambe *et al*., 1996), and *in vitro* (Tanaka *et al*., 2001). Components of tea tree (*Melaleuca alternifolia*) oil have been found to have anti-inflammatory effects *in vitro* (Brand *et al*., 2001; Hart *et al*., 2000), while in human volunteers administration of the whole oil has been found to reduce histamine-induced skin inflammation (Koh *et al*., 2002). Anti-inflammatory activity has also been found for 1,8-cineole, a major component of eucalyptus and other essential oils, *in vitro* (Juergens *et al*., 1998) and in vivo in an animal model (Santos and Rao, 2000).

It has also been found that eucalyptus and myrtle essential oils attenuated leucocyte activation by scavenging hydroxyl radicals indirectly produced by leucocyte degranulation, thereby interfering with inflammatory processes by acting as antioxidants. Therefore, screening of antioxidant properties may provide important information about the potential activity of a drug on inflammatory processes (Grabmann, 2005).

Recently there has been an increase in the use of plants as sources of natural antioxidants for the scavenging of free radicals (Njenga and Viljoen, 2006). An antioxidant can be defined as "any substance that in small quantities, normally in concentrations much lower than the oxidizable materials to be protected, is able to prevent or greatly delay its oxidation" (Beirao and Bernado-Gil, 2005). The reason that antioxidants are important to human physical well being comes from the fact that oxygen is a potentially toxic element since it can be transformed by metabolic activity into more reactive forms such as superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radicals, collectively known as active oxygen. These molecules are formed in living cells by various metabolic pathways. The hydroxyl radical is very reactive as it combines with almost all molecules found in living cells. Proteins, lipids, carbohydrates and DNA in living cells represent oxidizable substrates. The secondary events include changes in membrane structure, permeability and fluidity, lysosomal destabilization

and stimulation of apoptosis. Lipid peroxidation finally leads to loss of membrane function and integrity leading to cell necrosis and death. Hydroxyl radicals can also react with bases in the DNA and cause mutations. Oxygen free radicals appear to be an important factor in chronic inflammatory joint disease such as rheumatoid arthritis. Single oxygen can also be generated in the lens of the eye and contribute to the development of cataracts. Superoxide and hydrogen peroxide can stimulate growth in a variety of malignant mammalian cell types. They may have an important role as extracellular messengers for cell growth and viability (Svoboda and Hampson, 1999)

Essential oils and their components are gaining increasing interest because of their relatively safe status, their wide acceptance by consumers, and their exploitation for potential multi-purpose functional use (Ormancey *et al*., 2001; Sawamura, 2000). Traditional uses of *C. validus* indicate that it is used for anti-ageing preparations. Hence, the aim of this study was to determine the antioxidant activity of *C. validus* using a modified version of the DPPH assay (Lourens *et al*., 2004) and its anti-inflammatory potential with 5-LOX assay (Lertsatitthanakorn *et al*., 2006; Baylac and Racine, 2003).

5.2 METHODOLOGY

5.2.1 Determination of antioxidant activity

5.2.1.1 Sample Preparation

Stock solutions of essential oil from *C. validus* were diluted to final concentrations in DMSO. Two hundred microlitres of a 96.2 mM DPPH in methanol solution were added to 50 μl of sample solution of different concentrations and allowed to react at room temperature.

5.2.1.2 DPPH radical scavenging assay

Radical scavenging activity of plant extracts against stable DPPH (2, 2-diphenyl-2-picrylhydrazyl hydrate) was determined spectrophotometrically. When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in colour (from deep violet to light yellow) were measured at 518 nm on a UV/visible light spectrophotometer (Varion Cary 1E UV- Visible spectrophotometer). Methanol (200 μl) plus essential oil solution (50 μl) was used as a blank, whilst DPPH solution (200 μl) plus methanol (200 μl) was used as a negative control. The positive control was DPPH solution (200 μl) plus 50 μl Rutin. Each test was carried out in duplicate and the average absorbance values were converted into the percentage antioxidant activity, using the equation:

Scavenging capacity % = 100 - [(Ab of sample -Ab of blank) x 100/Ab of control]

5.2.2 Determination of anti-inflammatory activity

5.2.2.1 Reagents

Soybean lipoxygenase (EC. 1.13.11.12) was purchased from Fluka whereas Nordihydroguaiaretic acid (NDGA) and Linoleic acid were obtained from Sigma Chemical Co; potassium phosphate buffer 0.1 M, pH 9 was prepared with analytical grade reagents purchased from standard commercial sources. Deionized water was used for the preparation of all solutions.

5.2.2.2 Determination of 5- lipoxygenase activity

Lipoxygenase is known to catalyse the oxidation of unsaturated fatty acids containing 1–4 diene structures. The conversion of linoleic acid to 13- hydroperoxy linoleic acid was followed spectrophotometrically by the appearance of a conjugate diene at 234 nm. Nordihydroguaiaretic acid, a known inhibitor of soybean LOX, was used as a reference drug. Comparisons were made with NDGA and with the essential oils sandlewood (*Amyris balsamifera)* and tea tree oil (*Melaleuca alternifolia).* The reaction was initiated by the addition of aliquots (50 µl) of a soybean LOX solution (prepared daily in potassium phosphate buffer 0.1M pH 9.0 in a sufficient concentration to give an easily measurable initial rate of reaction to 2.0 ml of sodium linoleate (100 µM) in phosphate buffer; the enzymatic reactions were performed in absence or in presence of inhibitor and their kinetics were compared. The inhibitors were dissolved in DMSO in such a manner that an aliquot of each (30 µl) yielded a final concentration of maximum 100 ppm in each assay. The initial reaction rate was determined from the slope of the straight line portion of the curve and the percentage inhibition of the enzyme activity was calculated by comparing with the control (using 30 µl of DMSO alone instead of 30 µl of the inhibitor solution). Each inhibitor

concentration was tested in triplicate and the results averaged; the concentration (ppm) of the essential oil that gave 50% inhibition (IC_{50}) was calculated from the outline of the inhibition percentages as a function of the inhibitor concentration

5.3 RESULTS AND DISCUSSION

5.3.1 Antioxidant activity

The anti-radical scavenging capacity of *C. validus* essential oil was investigated and compared to that of Rutin. Rutin is a phenolic antioxidant and has been demonstrated to effectively scavenge superoxide radicals. The percentage antiradical scavenging capacity of the essential oil samples together with Rutin is shown in Figure 5.1. The diluted essential oil sample notably reduced the concentration of DPPH free radicals, with an efficacy slightly lower than that of Rutin. Rutin reduced 92.29% of free radicals, whilst the essential oil sample (0.1 v/v) reduced 83.13%. The essential oil was not effective at low concentrations and scavenging action over 0.01 v/v (25.54%) possessed no activity.

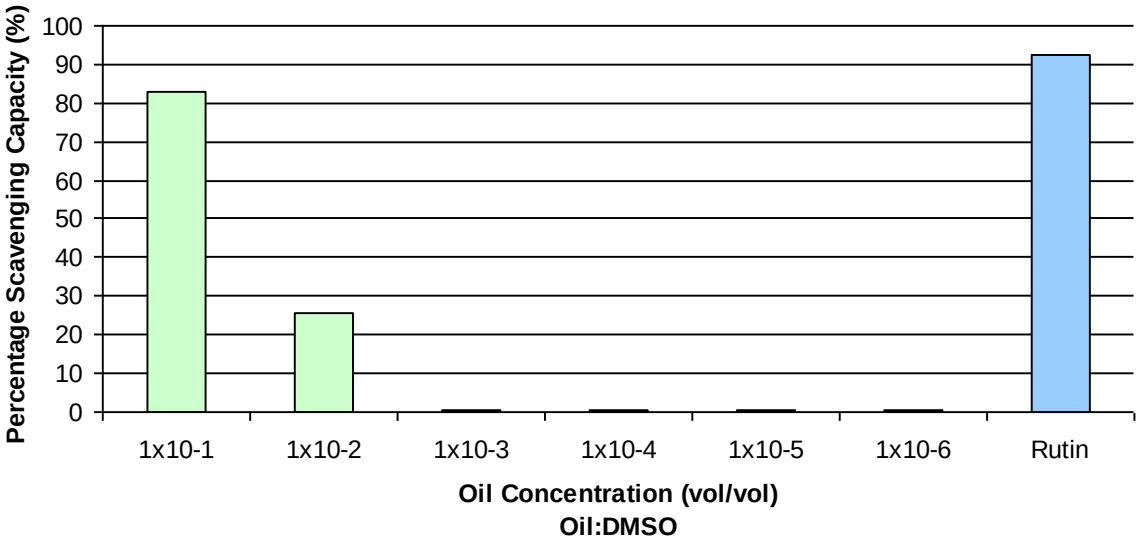


Figure 5.1: Scavenging capacity of the essential oil compared to Rutin

Although there are a plethora of methods for measuring free radical scavenging activities, the DPPH assay was chosen since it can determine the activity of both hydrophilic and lipophilic species, and also enabled the comparison of the antioxidant property of *C. validus* to other known useful essential oils. A comparison of the *in vitro* antioxidant activity of *C. validus* to 11 other essential oils (Sacchetti *et al*., 2005), and to that of a frequently used reference, *Thymus vulgaris* essential oil (Mantle *et al*., 1998) is shown in Table 5.1. The % scavenging capacity of *C. validus* oil was greater than that of all the other oils tested, including the reference oil, *Thymus vulgaris*.

Table 5.1: Free radical-scavenging activity percentage of 11 essential oils evaluated by the DPPH assay and comparison with that of the reference (*Thymus vulgaris*) essential oil

Plant Species	**% Free radical scavenging capacity**
Thymus vulgaris	**75**
Zingiber officinale	53
Thymus citriodora	22
Rosmarinus officinalis	67
Psidium guajava	22
Piper crassinervium	45
Pinus radiata	20
Eucalyptus globulus	12
Cymbopogon citratus	60
Curcuma longa	62
Cupressus sempervirens	22
Cananga odorata	67
Cymbopogon validus	**83**

Many compounds such as eucalyptol (Tomaino *et al*., 2005), citronellal (Mahalwal and Ali, 2002; Tomaino *et al*., 2005), geranial (Mahalwal and Ali, 2002), linalool (Dorman *et al*., 1995; Tomaino *et al*., 2005), neral (Tomaino *et al*., 2005), para-cymene (Dorman *et al*., 1995), terpinolene (Ruberto and Baratta, 2000), and terpinen-4-ol (Mahalwal and Ali, 2002;

Masuda *et al*., 1993) have been demonstrated to possess *in vitro* antioxidant properties and these compounds are present in *C. validus* essential oil.

The high antioxidant activity of *C validus* could be due the presence of geraniol (Figure 5.2), citronellal (Figure 5.3) and camphene (Figure 5.4), the main chemical constituents in *C. validus* oil (Table 3.10, Section 3.3.4). Their mechanism of action could be due to their chemical structures as reported by other authors. In literature, Shahidi and Wanasundara (1992) found that the main antioxidant activity from the essential oil may be due to the ability of the alcohol, phenolic and aldehydric compounds in the oil donating hydrogen to free radicals and thus stopping the chain reaction of lipid oxidation at the initial step. The studies of Ruberto and Baratta (2000) on the antioxidant activities of 98 pure essential oils showed that sesquiterpene hydrocarbons exerted a low, if any, antioxidant effect, while monoterpene hydrocarbons showed a significant protective effect, with several variants due to the different functional groups.

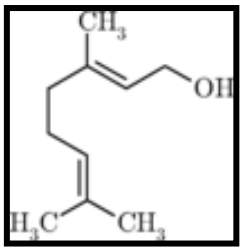


Figure 5.2: Geraniol

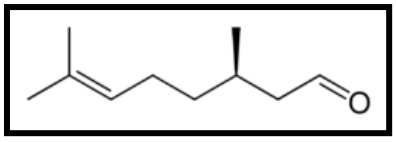


Figure 5.3: Citronellal

Figure 5.4: Camphene

The recent reports of the antioxidant levels in *Cymbopogon citratus* (IC_{50} 27 µl/ml) (Lertsatitthanakorn *et al.*, 2006), *Cymbopogon giganteus* (IC_{50} 1,18 mg/ml) (Alitonou *et al*., 2006) and *Cymbopogon nardus* (IC_{50} 2 µl/ml), (Lertsatitthanakorn *et al.*, 2006) are in-keeping with the results we found i.e. *C. validus* ($0.01 < IC_{50} < 0.1$ vol/vol).

5.3.2 Anti-inflammatory activity

5-Lipoxygenase (5-LOX) converts arachidonic acid into leukotrienes, which are mediators of inflammatory and allergic reactions (Werz and Steinhilber, 2005). Reduced IC_{50} values suggest the better inhibitory actions on 5-LOX and hence a greater anti-inflammatory activity. In this study, NDGA, a non-specific LOX inhibitor, was used as a positive control. The percentage inhibition of 5-LOX activity by essential oil (ppm) along with NDGA (ppm) are given in Table 5.2. The concentration (ppm) that gave 50% enzyme inhibition (IC_{50}) for the essential oils was calculated. The IC_{50} value of *C. validus* was found to be 190 ppm, whereas *Amyris balsamifera* had 97.8 ppm and *Melaleuca alternifolia* gave 184.5 ppm. NDGA known inhibitor of soybean LOX had an IC_{50} value of 0.35 ppm. These results indicate that the essential oil from *C. validus* is 543.4 times less active than NDGA for the inhibition of soybean LOX activity. It has been reported that 1,8-cineole possesses anti-inflammatory activity (Juergens *et al*., 1998) and terpenes such as α-pinene and sesquiterpenes such as β-caryophyllene exhibited activity in the *in vitro* 5-LOX assay (Baylac and Racine, 2003). The presence of geraniol and limonene (Table 3.10, Section 3.3.4) may contribute to the activity of the oil.

Nevertheless, the LOX inhibitory activities shown by essential oil extracted from *C. validus* and the other two oils studied are similar to those evaluated by Baylac and Racine (2003) for essential oils presented as natural extracts with ‘‘significant potential anti-inflammatory activity’’. These findings were similar to the studies of Lertsatitthanakorn *et al.* (2006) and Alitonou *et al.* (2006) on *Cymbopogon nardus* (IC_{50} 0.15 µl/ml), and *Cymbopogon giganteus* (IC_{50} 40 ppm). Apart from inhibition of the 5-LOX enzyme, the anti-inflammatory action can also be attributed to the inhibition of COX enzyme (Lertsatitthanakorn *et al.*, 2006; Fiorucci *et al*., 2001).

Therefore, this preliminary study on the anti-inflammatory role of the essential oils could suggest an action via the inhibition of 5-LOX enzyme only. The other pathways of inflammatory responses are not elucidated and thus further *in vitro* and clinical studies are needed to establish the role of essential oil from *C. validus* as a potential anti-inflammatory agent.

Table 5.2: Inhibition of soybean LOX activity by essential oils compared to NDGA

Concentration	***Cymbopogan validus***	***Amyris balsamifera* (sandlewood)**	***Melaleuca alternifolia* (tea tree)**	**NDGA**
(ppm)	**Inhibition percentage[a]**	**Inhibition percentage[a]**	**Inhibition percentage[a]**	**Inhibition percentage[a]**
100	34.5	51.1	29.6	
200	52.1	63.8	54.2	
300	65.7	70.2	61.6	
400	73.4	70.1	61.2	
500	73.4	70.1	61.2	
0.1				18.2
0.2				42.5
0.3				64.1
0.4				78.4
0.5				91.0
IC_{50} (ppm)[b]	**190.2**	**97.8**	**184.5**	**0.35**

a Mean values obtained from experiments performed in triplicate.

b Mean value determined graphically.

5.3 SUMMARY OF FINDINGS

The anti-radical activity observed with *C. validus* together in combination with a significant anti-inflammatory effect could explain the interesting activities described for this botanical species in traditional medicine. This activity could be due to the presence of predominant compounds such as camphene, citronellal, geraniol and limonene (Table 3.10, Section 3.3.4).

CHAPTER 6: ANTI-MOSQUITO PROPERTIES

6.1 INTRODUCTION

Mosquitoes serve as vectors of several diseases like malaria, yellow fever, dengue fever, filariasis and encephalitis, causing serious health problems to humans (Kiran *et al.*, 2006; Erler *et al*., 2006). For instance, *Aedes aegyptii* is one of the mosquito species responsible for the transmission of both dengue fever and dengue haemorrhagic fever (Cheng *et al*., 2003). Constant applications of organo-phosphates such as temephos and fenthion and insect growth regulators such as diflubenzuron and methoprene are generally used for the control of mosquito larvae. Although effective, their repeated use has disrupted natural biological control systems and has led to outbreaks of insect species, which sometimes resulted in the widespread development of resistance, had undesirable effects on non-target organisms, and fostered environmental and human health concerns (Kiran *et al.*, 2006; Yang *et al*., 2002).

Researchers are now focused on finding alternative insecticides which will be effective, environmentally friendly, biodegradable, safer and cheaper than existing ones (de Paula *et al*., 2003; Erler *et al*., 2006). Many researchers reported that the phytochemicals derived from plant sources might be alternative agents for the control of mosquitoes, because they have larvicidal, pupicidal, adulticidal and repellent activity (Ansari *et al.*, 2000, Vahitha *et al.*, 2002, Jeyabalan *et al.*, 2003; Rajkumar and Jebanesan, 2004). The various synthetic products and devices designed to combat such vectors are not successful because of increased resistance developed by various mosquito species. Most of the mosquito control programs in Asia target the larval stage in their breeding sites with larvicides, because adulticides may only reduce the adult population temporarily (El Hag *et al*., 1999; 2001). Larvicides target larvae in the breeding habitat before they can mature into adult mosquitoes and disperse. Corbet *et al.* (2000) suggested that conventional larvicidals affect mosquito

larvae in one or more of three possible modes: (i) by physical flooding of the tracheal system; (ii) by toxicity (especially that of volatile components); and (iii) by interference with surface forces. Larvicides include the bacterial insecticides *Bacillus thuringiensis* and *Bacillus sphaericus*, the insect growth inhibitor methoprene and the organophosphate insecticide temephos. Mineral oils and other materials form a thin film on the surface of the water, which causes larvae and pupae to drown. Liquid larvicide products are applied directly to water using back-pack sprayers and truck- or aircraft-mounted sprayers. Tablet, pellet, granular and briquet formulations of larvicides are also applied by mosquito controllers to breeding areas. The application of easily degradable plant compounds is considered to be one of the safest methods of control of insect pests and vectors (Alkofahi *et al*., 1989).

Many plant products which have been used traditionally by human communities in many parts of the world against the vector species of insects (Jacobson, 1958) have been evaluated against mosquitoes. The use of repellent plants as sources of repellent blends has been widespread among different communities in Africa (Palsson and Jaenson, 1999a; 1999b, Seyoum *et al*., 2002a; 2002b). Two principal traditional uses have been documented: (i) production of fumes from plant materials placed on burning charcoal stove (Palsson and Jaenson, 1999a; Seyoum *et al*., 2002a); and (ii) hanging leafy branches near mosquito entry points (Kokwaro, 1993). A recent study has shown that fumes generated by burning foliage materials of different plants give varying levels of protection and that the traditional method can be substantially improved if direct contact of the plant material with burning charcoal is avoided by placing it on a hot plate above a stove (Seyoum *et al*., 2002a; Seyoum *et al*., 2003).

Plant essential oils in general have been recognized as an important natural resource of insecticides (Gbolade *et al*., 2000; Adebayo *et al*., 1999). Their lipophilic nature facilitates

them to interfere with basic metabolic, biochemical, physiological and behavioural functions of insects (Nishimura, 2001). The potential of plants as sources of essential oils or fumigants that are repellant to mosquitoes and other insect pests and vectors is well known (Snow *et al*., 1987; Thorsell *et al*., 1998). Essential oils from a large number of plants, including *Ocimum* spp. (Chokechaijaroenporn *et al*., 1994, Bhatnagar *et al*., 1993; Tawatsin *et al*., 2001), *Cymbopogon* spp. (Rutledge *et al*., 1983; Ansari and Razdan, 1995), *Eucalyptus spp.* (Collins and Brady, 1993), *Pelargonium citrosum* (Matsuda *et al*., 1996), *Artemisia vulgaris* (Hwang *et al*., 1985), *Lantana camara* (Dua *et al*., 1996; Seyoum *et al*., 2002a; 2002b), *Mentha piperita* (Ansari *et al*., 2000), Vitex rotundifolia (Grayson, 2000), *Curcuma* spp. (Pitasawat *et al*., 2003), *Tagetes* species (Perich *et al*., 1995), *Conyza newii*, *Plectranthus marrubioides*, *Tetradenia riparia*, *Tarchonanthus camphoratus, Lippia javanica* and *L. ukambensis* (Omolo *et al*., 2004), have been demonstrated to exhibit good repellent activities against mosquitoes.

A brief review of some of the scientific literature available on essential oils as mosquito repellents indicates that citronella oils from *Cymbopogon nardus* and *Cymbopogon winterianus* have been reported to be the most widely used insect repellents (Hermes and James, 1961). Tiwari *et al*. (1996) tested lemongrass oil against the housefly *Musa nebulo* and female mosquitoes of *Culex fatigans* and *Aedes aegypti* assessing the contact toxicity, fumigant action and repellency of the oil. The findings of the latter tests indicated that lemongrass oil was less efficient than dimethyl phthalate and protection lasted only 40 min as opposed to 300 min for dimethyl phthalate. In contrast to these findings, Ansari and Razdan (1995) studied the effects of *Cymbopogon martini, C. citratus, C. nardus* and *Cinnamomum camphora* against local Indian mosquito species (*Anopheles cujicifacies* and *C. quinquefasciatus*) by exposing the feet, forearms and faces of local volunteers from dawn till dusk, dosed with 1 ml of each of the oils. Repellency of each of the *Cymbopogon* oils lasted 11 h against *A. cujicifacies* and 6 - 7h against *C. quinquefasciatus*, (but less for the

Cinnamomum oil). The oils were found to be comparable in efficacy to dimethyl phthalate and dibutyl phthalate.

There are no studies on the insectidal, larvicidal and repellent activities of *C. validus* essential oil. Thus the aim of this investigation was to determine the potential of *C. validus* as a mosquitocidal agent. *Anopheles arabiensis* was used in this study because it is one of the major malaria vectors in South Africa. The tests were carried out at the Malaria Research Programme, Medical Research Council (MRC) in Durban.

6.2 METHODOLOGY

6.2.1 Sample preparation

The stock solution of essential oil from *C. validus* was used for the larvicidal and repellency assays. For the larvicidal assay, the stock solution of essential oil was diluted to final concentrations in DMSO. To ensure consistency amongst results, each oil sample had undergone a repeat trial whilst control assays were conducted to make certain the trial had been performed under controlled conditions.

6.2.2 Insecticidal Activity

Two hundred microlitres of the essential oil was sprayed onto a clean dry non-porous ceramic tile using a pre-calibrated Potter's Tower (Figure 6.1). The Tower was cleaned with acetone between each different sample application. The sprayed tiles were air-dried and assayed within 24 hrs of spraying. A standard bioassay cone was fixed in place over the sprayed tile and thirty blood-fed *Anopheles arabiensis* females (3-5 days old) were introduced into the cone (Figure 6.2). The mosquitoes were then observed for knockdowns

after thirty and sixty minute exposures. The test mosquitoes were thereafter removed from the bioassay cone and transferred to a holding cage containing a nutrient solution. After 24 hrs, the number of dead mosquitoes were recorded and percentage mortality calculated.

Figure 6.1: Potter's Tower

Figure 6.2: Bioassay cones containing *Anopheles arabiensis* females

6.2.3 Larvicidal Activity

One millilitre of the essential oil samples (tenfold serial dilutions) was added to polypropylene containers (10 cm x 10 cm) containing 250 ml of distilled water and 30 *Anopheles arabiensis* larvae (3^{rd} instar). A negative control was set up in which DMSO was added instead of the extract. The positive control was an organo-phosphate (Mostop) used by the malaria control programme as a larvicide. Each container was monitored for larval mortality (dead larvae were removed) at 24 hr intervals for seven days.

6.2.4 Repellent Activity

Repellency was assessed by topical application of the test essential oil to the skin of a rodent and subsequent exposure of the treated areas of skin to unfed female mosquitoes. The rodent *Mastomys coucha* was used as the test animal for the general screening of extracts for repellent activity. Ethical approval for the use of *Mastomys* in these trials has been sought from MRC's Ethics Committee for Research on Animals. Adult rodents were weighed individually, and injected intraperitoneally with sodium pentobarbital. The anaesthetized rodents were then shaved on the ventral surface and a drop of essential oil was applied to each of two rodent's abdomens.

Paper cups (500ml) were modified by replacing the base of the cup with mosquito netting held in place with a rubber band and covering the mouth of the cup with transparent plastic film. Thirty unfed 4-day old *Anopheles arabiensis* females were introduced into the cup and held in contact with treated ventral surface of each rodent (Figure 6.3). Mosquito activity was observed through the transparent film. After a period of three minutes the number of mosquitoes probing was recorded. The rodents were then returned to the animal facility and allowed to recover from the anaesthetic. Each rodent was monitored for 7 days for adverse reactions to the applied plant extracts. N, N-Diethyl-meta-Toluamide (DEET) served as a positive control.

Figure 6.3: The ventral surface of a rodent in contact with *Anopheles arabiensis* females

6.3 RESULTS AND DISCUSSION

In South Africa malaria is categorised as seasonal and unstable, with the mosquitoes *Anopheles arabiensis* and *Anopheles funestus* being the major malaria vectors (Maharaj *et al*., 2005). The growing resistance of mosquitoes to insecticides is a potentially serious challenge to malaria vector control (Gericke *et al*., 2002). Africa is currently using pyrethroids, carbamates, organochlorines and organophosphates for malaria vector control. However *Anopheles funestus* was found to be pyrethroid resistant (Gericke *et al*., 2002). This increased resistance, has lead the way for investigating botanical extracts for use in malaria vector control.

In our study, the oil exhibited adulticidal, larvicidal, and repellent activities against *Anopheles arabiensis.* In the adulticidal assay, the essential oil paralyzed 84.28% of the adult female mosquitoes after 30 minutes exposure and 92.23% after 60 minutes. However the percentage mortality determined after 24 hours was only 53.70%, compared to K orthrine, a commercial insecticide which exhibited 100% knockdowns at both time intervals and 100% mortality (Table 6.1). The initial repellent activity of the oil was as effective as DEET, both showing 100% repellency against adult mosquitoes (Table 6.1).

Results on percent mortality of larvae of *Anopheles arabiensis* at different oil dilutions and with respect to different time periods are presented (Table 6.2). The effect of the oil samples against *Anopheles arabiensis* larvae was compared to Mostop, a commercial organophoshate which showed 100% mortality within 24 hours. At the highest oil dose (10^{-1}), all the larvae showed irregular movement and died within 24 hours. No pupal or adult emergence was observed at this dose, while in the case of both negative controls, the larvae developed into pupae and then into adults by day 5. More than 50% of the larvae were killed by the oil

dilution (10^{-2}), with pupae forming on day 5. Adult mosquitoes were observed on day 6 with the remaining oil dilutions.

Table 6.1: The adulticidal and repellent effect of the oil against *Anopheles arabiensis*

Samples	Adulticidal (%)			Repellency (%)	
	%Knockdowns (30 minutes)	%Knockdowns (60 minutes)	%Mortality (24 h)	No. of bites	%Repelled
Essential Oil	**84.28**	**92.23**	**53.70**	**0**	**100**
K Orthrine	100	100	100	n/a	n/a
DEET	n/a	n/a	n/a	0	100

(n/a) – not applicable

Table 6.2: Larvicidal effect of the oil against *Anopheles arabiensis*

Sample	Day 0	Number of dead larvae						Total Deaths %
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	
10^{-1}	30	**30**	**n/a**	**n/a**	**n/a**	**n/a**	**n/a**	**100**
10^{-2}	30	12	2	1	2	0 (P)	0	56.67
10^{-3}	30	0	2	1	2	0 (P)	0 (A)	16.67
10^{-4}	30	0	1	1	1	0 (P)	0 (A)	10
10^{-5}	30	0	1	1	0	0 (P)	0 (A)	6.67
10^{-6}	30	0	0	0	0	0 (P)	0 (A)	0
DMSO	30	0	0	0	0	0 (P)	0 (A)	0
DH_20	30	0	0	0	0	0 (P)	0 (A)	0
Mostop	30	30	n/a	n/a	n/a	n/a	n/a	100

(P) – larvae that have reached the pupal stage

(A) – larvae that have reached the adult stage

(n/a) – not applicable

The repellency and larvicidal activity of *C. validus* oil is similar to that of other *Cymbopogon* oils. *Cymbopogon winterianus* oil showed 75.7%, 52.4% and 100% repellency against *Aedes aegyptii, Anopheles stephensi* and *Culex quinquefasciatus* respectively (Amer and Mehlhorn, 2006a). This essential oil also exhibited 100% larval mortality against *Aedes aegyptii* (de Mendonca *et al*., 2005). The essential oil from *Cymbopogon citratus* exhibited 70.3% repellency against *Aedes aegyptii* and 100% repellency against *Anopheles stephensi* and *Culex quinquefasciatus* (Amer and Mehlhorn, 2006a). However, the oil exhibited no effect on *Aedes aegyptii* larvae at one, two and twenty four hours exposure (Amer and Mehlhorn, 2006b).

Our study shows that *C. validus* oil has tremendous larvicidal and repellency potential and compared favourably with commercial Mostop and DEET, used as the positive controls. The exposure time is very important for the values of LC_{50} in the tested oil samples. In most cases, the LC_{50} values had an inverse relation with time; thus, they decreased after 24 hours exposure. Thus, very low concentrations of the oils led to high mortality rates. The anti-mosquito properties of the oil against *Anopheles arabiensis* could be due to the presence of its predominant compounds i.e. alpha-cubebene, camphene, citronellal, geraniol and limonene (Table 3.10, Section 3.3.4). Other compounds present in the oil such as alpha-pinene, citronellol, isoborneol, myrcene and terpinolene (Amer and Melhorn, 2006a; 2006b), have been reported to exhibit larvicidal and repellency activity against mosquitoes. With further investigation the oil could be considered as an alternative botanical larvicide and repellent.

6.4 SUMMARY OF FINDINGS

The essential oil from *C. validus* exhibited aduliticidal, larvicidal and repellent activities against *Anopheles arabiensis*. More than 50% of adult *Anopheles arabiensis* females and 100% *Anopheles arabiensis* larvae were killed within 24 hours exposure to the oil. When applied to the ventral surface of rodents, the oil exhibited 100% repellency against *An. arabiensis* females. These activities could be due to the predominant compounds present in the oil i.e. alpha-cubenene, camphene, citronellal, geraniol and limonene (Table 3.10, Section 3.3.4).

CHAPTER 7: SAFETY EVALUATION

7.1 INTRODUCTION

In the research work carried, it has been shown that *C. validus* has potential as a therapeutic arsenal. However, many plants are known to be toxic. For this reason, research is carried out in order to determine the toxicity and mutagenicity of *C. validus*.

Artemia salina L. (Artemiidae), the brine shrimp, is an invertebrate component of the fauna of saline aquatic and marine ecosystems. It plays an important role in the energy flow of the food chain (Sanchez Fortun *et al*., 1995) and it can be used in a laboratory bioassay in order to determine toxicity through the estimation of the medium lethal concentration (LC_{50} values) (Lewan *et al*., 1992), which have been reported for a series of toxins and plant extracts (Meyer *et al*., 1982). This method, which determines the LC_{50} value of the active compounds and extracts in saline medium in μg/mL (Massele and Nshimo, 1995), has been used in research on medicinal plants carried out in different countries in order to evaluate toxicity, gastro-protective action, and other biological actions, which in some cases have been related to pharmacological studies carried out for different chemical compounds (Mathews, 1995; Fumaral and Garchitorena, 1996), as a screening method mainly for products of plant origin.

The evaluation of the toxic action of plant extracts is indispensable in order to consider a treatment safe; it enables the definition of the intrinsic toxicity of the plant and the effects of acute overdose (Cáceres, 1996). Laboratory mice are sensitive to toxic substances occurring in plants. The administration of the extracts in increasing amounts enables the evaluation of the toxicity limits, and the test should be carried out in two ways, for three doses, and for both sexes, taking into account such factors as age, sex, weight, species, diet, and environmental conditions (Cáceres, 1996). Because there is currently a tendency to limit the

use of laboratory animals in toxicological tests (Yajes, 1997), and the brine shrimp is a crustacean whose larvae are sensitive to a variety of substances, the brine shrimp bioassay can be useful as a quick and simple test for predicting the toxicity of plant extracts and guiding their phytochemical fractionation (Cáceres, 1996). This test is used particularly in developing countries, where 85% of the population use medicinal plants in traditional therapy (Feroze, 1969). It has also been considered a bio-indicator of environmental contamination by traces such as arsenic, lead, copper, zinc, cadmium, mercury and selenium. Due to its commercial availability, *Artemia salina* L. is widely used in toxicological applications and research. Some authors say that there is no correlation between this bioassay and the toxicological effects in a whole animal (Sanchez *et al*., 1993); however, 20 plant extracts were toxicologically tested in this study using "*in vivo*" and "*in vitro*" methods, whose results showed a good correlation ($r = 0.85$ $p < 0.05$), suggesting that the brine shrimp bioassay is a useful alternative model. In toxicity evaluation of plant extracts by brine shrimp bioassay, an LC_{50} value lower than 1000 μg/ml is considered bioactive (Meyer *et al*., 1982).

Extensive scientific data on major constituents in essential oils have not revealed any results giving rise to safety concerns. Chronic studies have been performed on over 30 major chemical constituents (menthol, carvone, limonene, citral, cinnamaldehyde, benzaldehyde, benzyl acetate, 2-ethyl-1-hexanol, methyl anthranilate, geranyl acetate, furfural, eugenol, etc.) present in many essential oils. Even at these high intake levels, the majority of the constituents show no carcinogenic potential (Smith *et al*., 2005a). In addition to dose, the carcinogenic potential of some flavor ingredients are related to several factors including mode of administration, species and sex of the animal model, and target organ specificity. In the vast majority of studies, the carcinogenic effect occurs through a non-genotoxic mechanism in which tumours form secondary to pre-existing high-dose, chronic organ toxicity. Selected subgroups of structurally related substances (e.g., aldehydes, terpene

hydrocarbons) are associated with a single target organ and tumour type in a specific species and sex of rodent (i.e., male rat kidney tumours secondary to alpha-2u-globulin neoplasms with limonene in male rats) or using a single mode of administration i.e. fore-stomach tumours that arise due to high doses of benzaldehyde and hexadienal given by gavage (Smith *et al*., 2005b). There may well be constituents not yet studied which are weak non-genotoxic carcinogens at chronic high dose levels. However, because of the relatively low intake (Lucas *et al*., 1999) as constituents of essential oils, they are expected to be many orders of magnitude less potent than similar levels of aflatoxins, the polycyclic heterocyclic amines, or the polynuclear aromatic hydrocarbons.

The preclinical toxicological evaluation, carried out routinely in mice, is of great importance for validation of the traditional use of medicinal plants. There is currently a tendency to call for substituting the use of laboratory animals in toxicological tests, due to the high cost and the animal suffering caused by these tests. Alternative methods include procedures that could replace experiments carried out with animals, reduce the number of animals used in every test, or refine the existing methodology in order to reduce pain and stress, according to the "3 R principle" (Johnston and Rusche, 1997; Yajes, 1997). Such methods include the Ames test and Brine shrimp lethality assay.

The Ames test uses mutant strains of *Salmonella typhimurium,* mutant yeast cells *(Saccharomyces cerevisiae),* the common bacteria *Escherichia coli,* and a variety of both animal and human cell cultures. The brine shrimp lethality assay has been used as a tool for preliminary assessment of toxicity and it has been used for the detection of fungal toxins, plant extract toxicity, heavy metals, pesticides and cyto-toxicity testing of dental materials (Barahona and Sanchez-Fortun, 1999; Martinez *et al*., 1998; McLaughlin *et al*., 1991; Pelka *et al*., 2000).

These methods are useful tools for more easily assaying large numbers of compounds, natural or synthetic, for genotoxicity. However, these tests are of even less relevance to human consumption, as the whole range of innate degradation/excretion pathways are not at work and the results do vary from test method to test method. Yet, these tests play a large role in the 'banning' of suspect compounds (Rang *et al*., 1995). *In vitro* cell assay tests can be a useful screening tool for potentially carcinogenic compounds. The great sensitivity of such tests can show some genotoxicity for a wide variety of common compounds, both natural and synthetic.

7.2 METHODOLOGY

7.2.1 Brine Shrimp Assay

The brine shrimp assay (Meyer *et al*., 1982) is an *in vitro* test used for determining toxicity of the plant extracts in brine shrimp

7.2.1.1 Hatching the shrimp

Twenty five milligrams of class C *Artemia salina* eggs (Natures Petland, Durban, South Africa) were incubated in a hatching chamber with artificial salt water, at temperatures from 20 to 30°C. Artificial salt water consisted of 23 g NaCl, 11g $MgCl_2 \cdot 6H_2O$, 4 g Na_2SO_4, 1.3 g $CaCl_2 \cdot 2H_2O$ or $CaCl_2 \cdot 6H_2O$, 0.7 g KCl in 1000 ml distilled water. The pH was adjusted to 9.0 using Na_2CO_3 to avoid risk of death to the *Artemia* larvae by decrease of pH during incubation (Lewan *et al*., 1992). After 24 hours, 15 ml of yeast solution 0.06 % was added to the chamber for every litre of salt water in order to feed the larvae; 48 hours after the eggs were incubated, the larvae were extracted and counted using a Pasteur pipette.

7.2.1.2 Sample Preparation

The stock solution of essential oil was diluted to final concentrations in DMSO. Fifty microlitres of each dilution were impregnated on filter paper disks. The disks were dried at room temperature to allow evaporation of DMSO, leaving only the essential oil on the filter disks. For every oil sample, two concentrations (in duplicate) were tested in order to determine dose-response relationship, and a control group was set with the vehicle used for dilutions.

7.2.1.3 Bioassay

Every vial with sample contained 10 larvae of brine shrimp, including the control group, and was filled to 5 ml total volume with artificial salt water. A drop of yeast suspension (3 mg in 5 ml sea water) was added to each vial. The vials were then incubated at 27ºC for 24 hours. After 24 hours, dead larvae were counted and percentage death determined.

7.2.2 Mutagenicity Test

The objective of this assay was to evaluate the mutagenic potential of the oil by studying its effect on a histidine requiring strain of *Salmonella typhimurium* in the absence of a liver metabolising system. When the cultures are exposed to a mutagen some of the bacteria undergo genetic changes due to chemical interactions resulting in reversion of the bacteria to a non-histidine-requiring state. The reverted bacteria were then grown in the absence of exogenous histidine thus providing an indication of the potential of the chemical to cause mutation. *Salmonella typhimurium* TA 100 tester strain was used in this assay (Maron and Ames, 1983).

7.2.2.1 Preparation of stock cultures

From the frozen disc cultures of *S. typhimurium* TA 100 tester strain broth cultures were made. One disc culture was asceptically removed and inoculated into a 250 ml Erlenmeyer flask containing 25 ml of nutrient broth (Oxoid) and 78 µl of 10 mg/ml of Ampicillin. The culture was incubated at 37°C on a shaker at 150 rpm for 16 hours until an optical density of between 1.2 and 1.4 was obtained. One hundred microlitres of the culture was then transferred to a sterile test tube containing 2 ml of 0.05 mM histidine and 0.05 mM biotin top agar (Appendix 2). This was then vortexed and plated onto minimal glucose agar plates (Appendix 2) which were incubated at 37°C for 48 hours. Well-separated colonies were used for initial broth cultures.

7.2.3.2 Ames test procedure

Broth cultures of *S. typhimurium* were made by inoculating nutrient broth with the master plate colonies. These cultures were incubated on a rotary shaker (150 rpm) at 37°C for 24 hours. Tenfold serial dilutions of the essential oil were made using DMSO. Sodium azide (NaN_3), a highly mutagenic compound (Arenaz *et al*., 1989) was used as a positive control. NaN_3 was dissolved in DMSO to obtain concentrations of 5 µg/ml, 10 µg/ml and 20 µg/ml. Sterile distilled water and DMSO was used as the negative controls. Two plates were prepared for each concentration of test essential oil. In a sterile test tube 100 µl of bacterial culture, 50 µl of test essential oil and 2.9 ml of top agar were added. This was mixed and poured onto minimal glucose agar plates. Once the agar solidified, the plates were inverted and incubated for 48 hours at 37°C, after which the number of revertant colonies were counted and the mutant frequency determined. The mutant frequency is expressed as the quotient of the number of revertant colonies over the number of colonies in the negative control. This was expressed using the following formula:

Mutant frequency = Revertant number of colonies / Negative control

A mutagenic potential of a test compound is assumed if the mutant frequency is 2.0 or higher. A possible mutagenic potential is assumed if the quotient ranges between 1.7 and 1.9. No mutagenic potential is assumed if all quotients range between 1.0 (or lower) and 1.6.

7.3 RESULTS AND DISCUSSION

The cyto-toxic potential of *C. validus* essential oil was investigated using the Brine shrimp lethality assay and its mutagenic potential was evaluated using the Ames test. These results are presented in Table 7.1.

Table 7.1: Cyto-toxic potential of *C. validus* essential oil

Oil:DMSO Dilutions	**Brine Shrimp % Deaths**	**Ames Mutagenic Frequency**
10^{-1}	100	0
10^{-2}	100	0
10^{-3}	90	0
10^{-4}	40	0
10^{-5}	0	0
10^{-6}	0	0

As far as we know, the cyto-toxic effect of *C. validus* essential oil has not been assessed. Meyer *et al*. (1982) classified crude extracts into toxic (LC_{50} < 1000 ppm) and non-toxic (LC_{50} >1000 ppm), according to the levels required to attain the LC_{50} in the Brine shrimp assay. Based on this classification, *C. validus* oil was found to be toxic (LD_{50} < 1000 ppm). However, when diluted the oil was safe and the MIC was 0.0001 (vol/vol). These results are similar to the findings of *Cymbopogon nardus* and *Cymbopogon citratus* oils which were

found to be toxic (LD_{50} 118.92 ppm and 9.83 μg/ml respectively) to brine shrimp larvae (Brasileiro *et al*., 2006; Parra *et al*., 2001).

Toxicity of the oil to *S. typhimurium* was evaluated in a preliminary test carried out with TA 100 tester strain. The absence of revertant colonies at all essential oil dilutions suggest that the oil is not mutagenic. It has been demonstrated that *S. typhimurium* TA 100 tester strain detects base pair substitutions and most known mutagens (Maron and Ames, 1983) such as sodium azide (Figure 7.1), which can be seen by the presence of revertant colonies.

Figure 7.1: Revertant colonies in the presence of sodium azide (10 μg/ml)

7.4 SUMMARY OF FINDINGS

In our study, the essential oil from *C. validus* had a LC_{50} value < 1000 ppm in the brine shrimp lethality assay, classifying it as toxic. However, when diluted to concentrations less than 0.0001 (vol/vol), the oil was safe. The oil showed no mutagenic potential against *Salmonella typhimurium* TA 100 tester strain in the Ames test.

CHAPTER EIGHT: CONCLUSIONS

The essential oil from *Cymbopogon validus* is used as (i) an anti-ageing preparation, (ii) antifungal and antiseptic agent, (iii) anti-rodent, and (iv) to treat various ailments. A scientific investigation of the characteristics of this oil is necessary to validate its use and in turn improve the quality of healthcare.

The essential oil extracted from the plant organs of *C. validus* was found to be concentrated in the flower-heads and rhizomes as indicated by the oil yields. This information could be useful to those harvesting this grass species. A sensory evaluation revealed that the oil was slightly murky, pale yellow in colour, had a strong turpentine-like odour and was liquid at room temperature. The unpalatability of this grass species could be attributed to the oil's turpentine-like smell. The oil was also found to be susceptible to oxidation as revealed by its peroxide value, induction period, iodine value and percentage free fatty acids.

Predominant compounds exhibiting a 95% or greater qualitative spectral match were identified from chemical profiles of *C. validus* oil, as well as that of the plant organs. These oil constituents, namely alpha-cubebene, camphene, geraniol, limonene, myrcene, palmitic acid and sabinene were present in all plant organs. The importance of conducting studies on essential oils lies not only in the chemical characterization, but also in the possibility of linking the chemical contents with particular biological activities.

Our study showed that the oil possessed antibacterial activity only against the Gram positive bacteria tested namely *Bacillus, Micrococcus and Staphylococcus* species. This activity is suspected to be associated with the predominant compounds, namely camphene, citronellal, geraniol, limonene and sabinene, all of which have been previously reported to have

antibacterial activity. The oil showed no activity towards fungal growth. These results do not support the use of *C. validus* oil as an antifungal agent.

The oil exhibited significant free radical scavenging activity as well as anti-inflammatory properties. Predominant compounds associated with these activities include camphene, citronellal, geraniol and limonene. These results validate the use of the oil as an anti-ageing preparation.

This study indicated that *C. validus* oil possessed remarkable larvicidal and repellent activity against the malarial vector, *Anopheles arabiensis* and compared favourably with the commercially available larvicide and repellent, Mostop and DEET, respectively. These results could be useful in the search for safer and more effective natural extracts as larvicides. If *C. validus* oil is to be employed as an alternative mosquito repellent, further studies are necessary to evaluate how long the protective effect lasts. The adulticidal activity of the oil is notable. The anti-mosquito activity of the oil could be related to its predominant compounds i.e. alpha-cubebene, camphene, citronellal, geraniol and limonene.

The essential oil demonstrated significant lethality against brine shrimp larvae. Based on these results, it may be concluded that the oil is toxic at high doses. However, since data indicated that the toxicity of *C. validus* oil is dose dependant, the majority of adverse events can be avoided with the use of lower concentrations. In addition, the oil did not exhibit any mutagenic potential in the Ames test.

In conclusion, *Cymbopogon validus* essential oil has antibacterial, antioxidant, anti-inflammatory and anti-mosquito activities which may be associated with predominant compounds present in the oil. The oil is not toxic at low concentrations, nor is it mutagenic. Thus, the oil can be exploited as a multi-functional agent.

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APPENDICES

APPENDIX 1: SOLUTIONS FOR CHEMICAL TESTS

1. Test for iodine value

Sample sizes and weights of oils and fats normally used:

Iodine value	Sample Size (g)	Fats and Oils	Weight (g)
0-5	3.00	Sunflower oil	0.20
5-20	1.00	Groundnut oil	0.30
20-60	0.35	Hardened GNO M1, M2 and M3	0.35
60-80	0.25	Magarine blends	0.20
80-130	**0.15**	Palm Olein	0.35
130+	0.10	Hardened Palm Kernel	3.00

2. Test for % free fatty acids

Phenolphthalein indicator solution

Dissolve 1 gram of powdered phenolphthalein in 100 ml methylated spirits.

Neutralization of methylated spirits

Place +/- 3 drops of phenolphthalein indicator into a flask containing methylated spirits. Add sodium hydroxide (0.1M) to this solution drop by drop until the very first sign of pink remains.

Conversion table

The following factors apply to the different fatty acids:

Fatty Acid	Factor
Lauric acid	20.0
Palmitic aid	25.6
Stearic acid	28.4
Arachidic acid	31.3
Oleic acid	28.2
Linoleic acid	28.0
Linolenic acid	27.8

3. Test for % cholesterol

Acid acetic anhydride solution

Mix 100 ml acetic anhydride (chilled) with 5 ml concentrated sulphuric aid. Add 50 ml glacial acetic acid to the solution.

APPENDIX 2: SOLUTIONS AND MEDIA FOR AMES TEST

1. 0.5 mM histidine / 0.5 mM biotin solution for top agar

Ingredient	**per 250 ml**
D-Biotin	30.5 mg
L-Histidine	26.2 mg
Double distilled water (ddH_20)	250 ml

Dissolve biotin and histidine in warm ddH_20 and filter sterilize.

2. Top agar

Ingredient	**per 1000 ml**
Bacteriological Agar	6 g
Nacl	5 g
ddH_20	1000 ml

Microwave to dissolve the agar. Mix thoroughly and make 100ml aliquots in 250 ml glass bottles with screw caps. Autoclave at 120°C for 20 minutes. Cool the agar and tighten caps. Add 1/10 volume (10 ml) of the 0.5 mM histidine / 0.5 mM biotin solution to molten top agar. Mix thoroughly by swirling.

3. Vogel-Bonner medium E stock for minimal agar base

Ingredient	**per 1000 ml**
Warm ddH_20 (45°C)	670 ml
$MgSO_4.7\ H_20$	10 g
Citric acid monohydrate	100 g
K_2HPO_4	500 g
$NaHNH_4PO_4.4\ H_20$	175 g

Add salts in order indicated to warm water in a 2L flask placed on a magnetic stirring hot plate. Allow each salt to dissolve completely before adding the next. Adjust the volume to one litre. Distribute into two 1L glass bottles. Autoclave at 120°C for 20 minutes.

4. 40% glucose solution (autoclave sterile)

Ingredient	**per 1000 ml**
Glucose	400 g
ddH20	1000 ml

5. Minimal glucose agar plates

Ingredient	**per 1000 ml**
Bacteriological Agar	15 g
ddH_20	930 ml
Vogel-Bonner medium E stock	20 ml
40% glucose solution	50 ml

Add ddH_20 to agar in a 2L flask. Place a large magnetic stirring bar inside for later mixing and autoclave at 12°C for 20 minutes. When the agar has cooled add the Vogel-Bonner medium E stock and glucose solution. Stir the mix thoroughly. Pour 30 ml into each Petri plate.