The composition, geographical variation and antimicrobial activity of *Lippia javanica* (Verbenaceae) leaf essential oils

Sivanasen Subramoney

Supervisor – Dr. A. M. Viljoen,
Co-Supervisor – Mrs. S. F. van Vuuren
Department of Pharmacy and Pharmacology
Faculty of Health Sciences
University of the Witwatersrand
7 York Road
Parktown
Johannesburg
2193
South Africa

A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, in partial fulfilment of the requirements for the degree
of
Master in Science (Medicine) Pharmaceutical Affairs

Johannesburg, 2003

Declaration

I, Sivanasen Subramoney declare that this research report is my own work. It is being submitted for the degree Master of Science in Medicine (Pharmaceutical Affairs) in the University of the Witwatersrand, Johannesburg. This work has not been submitted before for any degree or examination at this or any other University.

.....

.....day of ....., 2003.

Dedication

To my family, for their undying love and support.

Abstract

Antimicrobial properties and geographical variation in essential oil composition of fever tea - *Lippia javanica* (Verbenaceae)

Lippia javanica is a widely spread woody shrub and the major traditional use is reflected in its vernacular name; fever tea and 'koorsbossie'. An infusion of the leaves is also used as a decongestant for colds and coughs. Infusions may also be used topically to treat scabies and lice. A preliminary study indicated that the essential oil chemistry varies dramatically both within and between natural plant populations. As the antimicrobial activity is directly related to the specific composition of the oil, the activity also fluctuates. *Lippia javanica* species from various locations in Southern Africa were collected for a study into the essential oil content and antimicrobial activity. The hydrodistilled essential oils were analysed by GC/MS and a cluster analysis was performed on the essential oil dataset. From sixteen samples (representing five natural populations), five chemotypes were identified;

1. a carvone and limonene rich type,
2. a myrcenone rich type,
3. a limonene and piperitenone rich type,
4. an ipsenone and myrcene rich type and
5. a linalool rich type.

The myrcenone and linalool chemotypes have been mentioned in the literature but the carvone, ipsenone and piperitenone chemotypes have not previously been reported.

The essential oil from *Lippia javanica* showed minimal activity against *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus cereus*, and no apparent activity against *Pseudomonas aeruginosa* in a disk diffusion assay. The oil did however show activity against *Candida albicans* and *Cryptococcus neoformans*, in the same assay.

Acknowledgements

- Dr A. M. Viljoen for his immense knowledge and guidance.
- Mrs S. F. van Vuuren for her kind assistance and support.
- Mr A. de Castro for supplying of plant material from Swaziland and his enthusiastic interest in the project.
- Prof. K. H. C. Başer, Drs Betül Demirci and Temel Özek from the Medicinal and Aromatic Plant and Drug Research Centre (TBAM), Anadolu University, Turkey, for allowing me to use their facilities to do the necessary chemical assays, and also for their kind hospitality in making my stay in Turkey a memorable one.
- Miss S. Govender and Mr J. Subramoney in assisting me to capture the raw data correctly and convert it into electronic copies.

Study Objectives

- To determine the essential oil variation both within and between natural plant populations.

- To document essential oil chemotypes of *Lippia javanica*.

- To study the antimicrobial activity of this widely used medicinal aromatic plant. This information would provide a scientific rationale for the use of *Lippia javanica* in traditional medicine.

- To investigate any correlation between antimicrobial activity and essential oil composition.

Table of Contents

Chapter 3 - Results **Page**

List of Tables

List of Figures

Chapter 1 - Introduction

"Medicinal plants are the backbone of traditional medicine." (Farnsworth 1994)

The subjects of ethnobotany and ethnopharmacology are based on the integration of information on plant material from different cultures. Traditional uses in conjunction with scientific rationale are employed in a search for new biologically active substances. The discovery of new active molecules had reached saturation point almost three decades ago, where synthetically derived molecules were manipulated and studied for possible medicinal uses. Now there is a sense of urgency in the discovery of new lead compounds due to the exhaustion of so many present day active molecules. A major reason, is the resistance of strains of microorganisms to a wide variety of medicinal regimens. A common example is the resistance of *Staphylococcus aureus* to penicillin antibiotics, more specifically, to Methicillin. The most important cause of resistance is the repeated use of a given antimicrobial for the same microorganism, which leads to reduced sensitivity to the antimicrobial by the microorganism. The microorganism achieves this through changes in its physical nature or metabolism (Cowan 1999, Essawi and Srour 2000). The advances in technology and the better understanding by researchers of disease initiation and progression brings us to the next major reason for the need for new chemical agents. At present, research is providing solutions to medical conditions that have eluded medical experts for many years. Conditions like cancer, Parkinson's disease and Alzheimer's disease are well researched now. Researchers understand patterns of disease progression now more than in the past, which puts them in a advantageous position to identify possible lead compounds in drug research. Presently, solutions to medical problems are being provided at an almost exponential rate!

Another reason in the search for new active molecules is that certain medicines have very narrow therapeutic windows between low efficacy and toxicity, for example drugs derived from the *Digitalis* species. These drugs, when administered to treat heart failure, have to be monitored regularly through analysis of serum concentrations. If the serum concentration is above the therapeutic range, toxicity could result in death. Conversely, if the serum concentration is below the therapeutic range, no clinical effect is observed. For

much of the third world, western medicine is not within reach as cost and availability play a major role. With no other option, people resort to herbal remedies to provide relief, if not a cure to the ailment (Rates 2001). The traditional healer is the first contact that these people have with the health sector. The traditional healer's knowledge is based on numerous years of experience, and trial and error. It is only recently, in the last decade, that modern science has found justifications for the use of many plants. In many cases, the therapy in question is a combination of various plants and plant parts.

The focus of research into plant and plant products exists for many reasons, one of which is the vast diversity of plant species on earth, with more than 250 000 species known to man. This represents a renewable resource if correctly conserved, due to the almost limitless ability of the plant to synthesize substances (Geissman 1963). Plants can thus be a valuable source of new biologically active molecules, because of the thousands of constituents they contain (Marsten and Hostettman 1999). Over and above this, plants have already provided us with many drugs. Examples include vincristine and vinblastine from *Catharanthus roseus*, atropine from *Atropa belladona* and morphine and codeine from *Papaver somniferum* (Rates 2001). An example of renewed interest in plant products is the use of *Artemisia* to produce quinine (Klayman 1985). Although chloroquine and its synthetic derivatives are successful agents in the treatment of malaria, quinine has lately been used more and more against *Plasmodium falciparum*, the causative agent of malaria. This is due to the significant loss in efficacy of chloroquine and its synthetic derivatives in the treatment of malaria, which in turn is due to the parasite developing resistance. There is such a great demand at present for this plant alkaloid, that extraction laboratories cannot satisfy the growing demand, since malaria is still a great health risk to people inhabiting and visiting tropical areas (Naranjo 1995). Much of the information obtained prior to plant research was at many times unsubstantiated. Now, with the advances in technology, it is possible to identify active compounds and suggest possible mechanisms of action based on the structure of the molecule. The sheer diversity in any one plant again reinforces the need for research into this field. It is through current research, that empirical knowledge is now being generated which can be extrapolated into the healthcare field.

Lippia – an overview

The genus *Lippia* (Houst.) belongs to the family *Verbenaceae*, which consists of approximately 200 herbs, shrubs and small trees, and which are often of an aromatic nature (Terblanché and Kornelius 1996). The species are distributed throughout South and Central America and Tropical and Southern Africa (van Wyk *et al.* 1997, Velasco-Negeureula *et al.* 1993). This genus is currently being extensively researched, with more than a quarter of the number of species being characterised by their chemical composition. Of note is the use of certain *Lippia* species in food preparations or as seasoning. Compadre *et al.* (1985) cited several species of the genus *Lippia* that were used as sweeteners, more specifically *Lippia dulcis* Trevir. This species was used widely as a sweetener prior to the introduction of sucrose (Naranjo 1995). It was only much later that (+)-hernandulcin was isolated from this plant, which proved to be a thousand times sweeter than sucrose (Pascual *et al.* 2001[b], Naranjo 1995). Another species of culinary importance is *Lippia graveolens* H.B.K., commonly known as Mexican oregano which is used in many savoury dishes (Lawrence 1984, Uribe-Hernández *et al.* 1992). Another plant from the genus *Lippia* with similar properties is *Lippia alba* (Mill) N.E. Brown. By far, this is the most studied species in the genus *Lippia*. This species is distributed throughout Florida, Texas and Central and South America (Pascual *et al.* 2001[b]). Its traditional uses are widely varied and stretch from being used as a culinary seasoning, to being used in the treatment of syphilis and gonorrhea (Pascual *et al.* 2001[b]). Studies not based on the traditional uses were conducted into the cytostatic properties of the essential oil from this plant (Lopez *et al.* 1979), and more recently into the action of the plant extract on neural pathways, (Kleuger *et al.* 1997). Flavonoid 4-sulphates were isolated from a butanolic fraction of this plant, as possible active compounds with neuropharmacological activity (Kleuger *et al.* 1997). de Barros Viana *et al.* (2000) conducted studies into the anticonvulsant activity of three chemotypes identified in this species. It was found from the data that when diazepam and the essential oil were used together, there was a potentiated effect, which suggests a possibly similar mechanism of action and indicates that citral, β-myrecene and limonene are the essential oil's active compounds. Costa *et al.* (1989), in a previous study, studied the action of two *Lippia alba*

extracts and their effect on neural pathways. However, the studies focused on the analgesic effects, where it was found that the extracts inhibited pain reflexes in mice to a certain extent (Costa *et al.* 1989). Most recently Pascual *et al.* (2001[a]), tested *Lippia alba* infusions on rat gastric mucosa, where they showed an antiulcerogenic activity against indomethacin induced gastric ulceration in the short term (1 day) and long term (5 days). Vale *et al.* (1999) looked at what behavioural effects in mice were produced from different chemotypes of *Lippia alba*. Their work showed that the EOII chemotype with major compounds citral and limonene showed the greatest effect in altering the behaviour of the mice.

Another *Lippia* species that is presumed to exist in different chemotypes is *Lippia javanica* (Burm.f.) Spreng (Chagonda *et al.* 2000, Pascual *et al.* 2001[b]). There is not much work presently being done on this species in terms of its essential oil composition or the identification of chemotypes. The plant occurs in Southern Africa (Figure 1.1.) and northwards into tropical Africa (van Wyk *et al.* 1997) where it occurs as an erect woody shrub approximately 2 metres in height (Figure 1.2.). Dense rounded heads of small yellowish-white flowers appear during the flowering season (van Wyk *et al.* 1997), refer to Figure 1.3. Its hairy leaves (Figures 1.4. and 1.5.) have compound veins and are highly aromatic with a strong lemon smell.

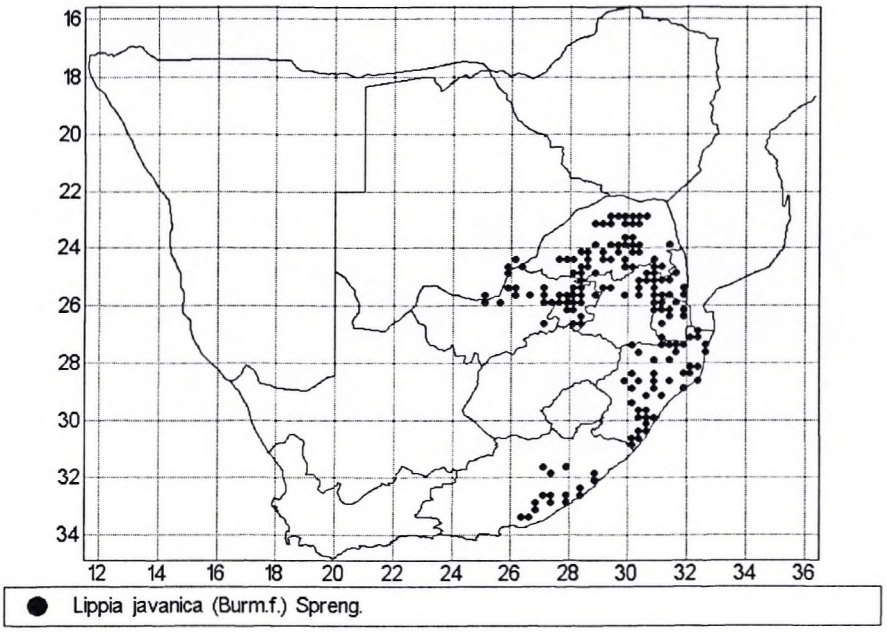


Figure 1.1. Geographical distribution of *Lippia javanica* in South Africa.

(A) (B)

Figure 1.2. (A) and (B) *Lippia javanica* in habitat.

Figure 1.3. Dense rounded heads of small yellowish-white flowers appear during the flowering season.

Figure 1.4. Scanning electron micrograph of the leaf surface of *Lippia javanica*, showing numerous bulb-shaped oil glands.

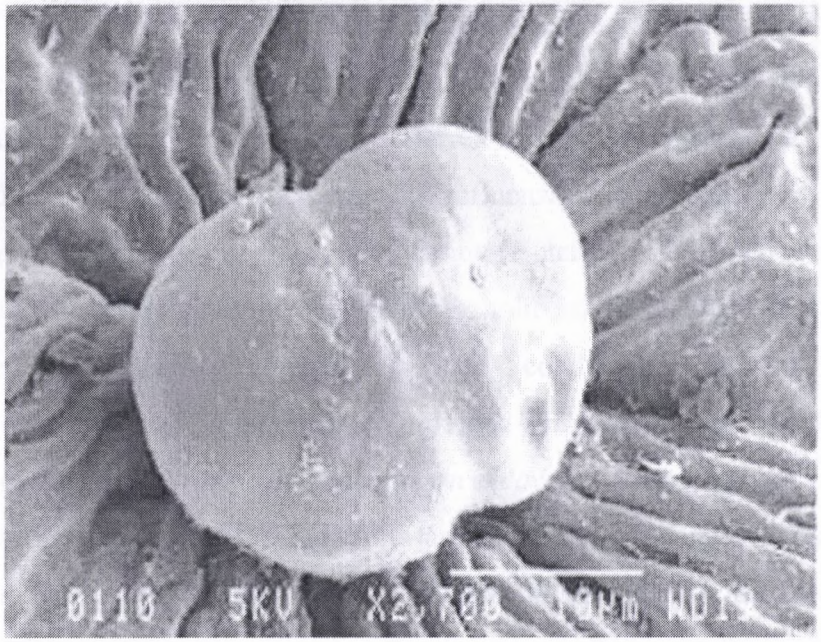


Figure 1.5. Scanning electron micrograph of the leaf surface of *Lippia javanica* showing one of many multi-cellular oil glands.

The plant is used extensively in traditional medicine by both lay people and traditional healers to treat minor ailments. Many of its uses relate to the plant or parts of it being used for respiratory disorders e.g. coughs or colds and also for skin infections or wounds. The traditional uses of *Lippia javanica* are summarised in Table 1.1. The leaves and stems are often used and in some cases the roots as well (van Wyk *et al.* 1997). Strong leaf infusions are made, which are commonly used as inhalants. The leaf infusions are also used topically for scabies and lice (van Wyk *et al.* 1997, Gelfand *et al.* 1985). More commonly, leaf and stem infusions are used as a tea, and this is taken to treat coughs, colds, fever and bronchitis (Smith 1966, Watt and Breyer-Brandwijk 1962, Hutchings 1996). The plant has also been used for bronchial ailments and influenza (Hutchings 1996). The Vhavenda people use leaf infusions as anthelmintics for respiratory and febrile ailments and as prophylactics against dysentry, diarrhoea and malaria (Mabogo 1990). Roots are used as antidotes for suspected food poisoning and for bronchitis and sore eyes (Hutchings 1996). *Lippia javanica* is commonly referred to as "fever tea" in English. This name is probably derived from its use as a weak infusion for fever symptoms. In Afrikaans it is referred to as "koorsbossie", which literally translated means "fever bush". In Zulu it is commonly referred to as "umsuzwane". Ethnobotanical literature documents its uses for fever and influenza in combination with leaves of *Artemisia afra* and it is drunk as a weak infusion (Hutchings 1996). The latest research into *Lippia javanica* was into the use of the ethanolic extract as a repellent to mosquito bites caused by *Anopheles arabiensis*. This study, conducted by Govere *et al.* (2000), showed that an extract of *Lippia javanica* provided 76.7% protection, which was higher than *Pelargonium reniforme* and *Cymbopogon excavatus* to which it was compared.

Prior to this, the extract was assessed for its larvicidal activity against *Anopheles gambiae s.s* mosquito larvae, where it proved to be less than half as effective as a larvicide with a LC_{50} of 125,34 x 10^3µg/ml compared to *Ocimum canum* (hoary basil) which had a LC_{50} of 54,94 x 10^3µg/ml (Lukwa 1994).

Major components in the essential oil were reported to be myrcenone, myrcene and (*E*)- and (*Z*)-tagetenone (Mwangi *et al.* 1991 and 1992, Fujita 1965, Terblanché and Kornelius 1996, Velasco-Negueruela *et al.* 1993).

Table 1.1. Traditional uses of *Lippia javanica* as summarised from ethnobotanical literature.

Plant Part	Preparation	Uses	References
Leaves	Crushed into paste	Febrile rashes; protection from dogs and crocodiles	Doke and Vilakazi 1972*
Leaves	Washes, poultices	Chest ailments	Roberts 1990*
Leaves	Cold infusions	Gangrenous rectitis	Bryant 1966*
Leaves and stems	Weak infusions	Coughs, colds and bronchial ailments	Smith 1895*, Neuwinger 2000
Leaves and stems, combined with *Artemisia afra*	Weak infusions	Coughs, colds, fevers and measles	Watt and Breyer-Brandwijk 1962*
Leaves	Infusions	Anthelmintics; respiratory and febrile ailments; prophylactics against dysentry, diarrhoea, malaria	Mabogo 1990*
Burnt roots	Powder	Applied to scarifications made around sprained joints, antidotes for suspected food poisoning, bronchitis, sore eyes	Hedberg and Staugard 1989*
Leaves and twigs	Infusions, as tea	Chest ailments, influenza, rashes, malaria, stomach problems and headaches	Hutchings and van Staden 1994*, Watt and Breyer-Brandwijk 1962*

* These references in the table are extracted from the reference Hutchings (1996).

Another study reported the components of *Lippia javanica* as caryophyllene, linalool and p-cymene (Neidlein and Staehle 1974). In a study conducted in Zimbabwe, Chagonda *et al.* (2000) reported variations in essential oil major compounds for *Lippia javanica* samples taken from the same location (in Zimbabwe) e.g. linalool, which had a range of between 1,7% and 27,0%. This variability of results will lead to contradicting reports and to disagreement between certain authors. It is remarkable that the phytochemistry of this widely used medicinal aromatic plant remains largely unexplored – the only chemical compounds that have hitherto received some attention were the essential oil compounds. Exploration of the phytochemistry of the plant, and the possible occurrence of falvonoids, triterpenoids, iridoids (as were found in other *Lippia* species) is beyond the scope of the present study, as the work is narrowly focused on the volatile compounds. It is the aim of this study to better understand the essential oil content of this plant and to elucidate the possible role of the volatile constituents in the traditional medicinal uses.

Chapter 2 - Materials and Methods

Chemical Aspects

Extraction of Essential Oils

Lippia javanica samples were collected in the 2000/2001 growing season from various sites in the wild. The collection data for these samples is summarised in Table 3.1. Fresh plant material was cut up and the essential oils were collected by hydro-distillation in 500 ml of water for approximately 3 hours. This process was carried out in a modified Clevenger-type apparatus (Figure 2.1.). The oil and some water were found in the collection arm of the apparatus and were separated through differences in miscibility.

Method for Thin Layer Chromatography (TLC)

The hydrodistilled essential oils were diluted with n-Hexane (1:7). The samples were applied to the silica plate (Merck) using a glass capillary tube (Figure 3.2.). The TLC plate was placed in a rectangular glass container. It was placed in an upright position, slightly slanted, with the reference base line slightly above the level of the mobile phase, toluene : ethyl acetate (93:7). Sufficient time was allowed to elapse for the mobile phase to reach the top of the chromatographic plate. The plate was then removed, and placed under UV light of wavelength 254 and 356 nm and viewed. The plate was sprayed with a vanillin-sulphuric acid spray reagent before it was placed into an oven at 100 °C and allowed to dry.

Method for Gas Chromatography (GC)

Analytical GC analysis was performed on a Shimadzu 17A gas chromatograph using the following parameters; Column: J&W-DB1 (30 m x 0.25 mm id., 0.25 μm film thickness), Temperatures: injection port 230 °C, column 60 °C for 1 min., 5 °C / min to 180 °C, 180 °C for 2 min., (total = 25 min.).

Method for Gas Chromatography combined with Mass Spectrometry (GC/MS)

The essential oils were analysed by GC/MS using a Hewlett-Packard G1800A GCD system. An Innowax FSC column (60 m x 0.25 mm diameter, with 0.25 μm film thickness) was used with Helium (0.8 ml/min) as the carrier gas. The oven temperature of the GC was maintained at 60 °C for 10 min and the programmed temperature to rise to 220 °C at a rate of 4 °C / min, and then kept constant at 220 °C for 10 min and again programmed to rise to 240 °C at a rate of 1 °C / min. Split flow was adjusted at 50ml / min. The injector and detector temperatures were 250 °C. Mass Spectra were taken at 70 eV. Mass range was from *m/z* 35 to 425. Kovats Indices for all compounds were determined. Relative percentage amounts of the separated compounds were calculated automatically from peak areas of the total ion chromatogram. A library search was carried out using the Wiley GC/MS Library and TBAM Library of Essential Oil Constituents. Relative percentage amounts were calculated from TIC by the computer.

Antimicrobial Assays

Antimicrobial assays were performed on the sixteen oil samples of *Lippia javanica*, with oil samples being tested against eleven organisms. The essential oils were tested for activity against *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739) and *Candida tropicalis* (clinical strain) in an initial disk diffusion assay. Selected samples, based on their activity in the disk diffusion assay, were then evaluated for activity against *Cryptococcus neoformans* (ATCC 90112) and *Aspergillus niger* (clinical isolate) in a second disk diffusion assay. These preliminary results were evaluated and the oil samples that showed the most promising activity were then evaluated for activity in a disk diffusion assay against another group of four bacteria: *Serratia odorifera* (ATCC 33132), *Bacillus subtilis* (ATCC 15244), *Bacillus cereus* (ATCC 11778) and *Staphylococcus epidermidis* (ATCC 2223). Of the samples that showed the most promising activity, six were chosen and further subjected to a microdilution assay for a minimum inhibitory concentration (MIC) assessment against the yeast, *Candida albicans* (ATCC 10231).

Method for Disk Diffusion Assay

The assay was performed on eight bacterial cultures and three fungal cultures. The growth medium used for the bacterial disk diffusion assays was Mueller-Hinton (Oxoid) agar and that for the fungi was Tryptone Soya (Oxoid) agar. Tryptone Soya agar was prepared by dissolving 40 g Tryptone Soya powder in 1000 ml sterile water. The Mueller-Hinton (Oxoid) agar was prepared by dissolving 38 g Mueller-Hinton powder in 1000 ml sterile water. A total of 200 ml was used for the preparation of every tray. A total of eleven trays were prepared for the bacterial and the fungal strains. Twenty to thirty microlitres of plant extract was placed onto disks. The quantities absorbed by these disks are fairly constant (Janssen *et al.* 1987). The agar plate is prepared by pouring 100 ml of molten Mueller-Hinton agar into a sterile glass-bottom tray. The molten agar is allowed to reach all the corners and edges of the glass-bottomed tray to allow for a level and even surface. This is the base layer, which is then allowed to cool. One millilitre of standard bacterial solution of inoculum (Mc Farlands standard) was incorporated into the remaining 100 ml of molten agar. This was then poured over the now cool base layer and allowed to solidify. A paper template marking off gridlines was placed underneath the glass-bottomed tray. The orientation of this template was noted in relation to the glass-bottomed tray. Disks of Whatman filter paper (size 6 mm) were impregnated with 25 µl of the oil to be analysed. The saturated disks containing the plant extract were carefully placed onto the squares correlating with the sample numbers, using sterile disposable needles. The trays were turned upside down and refrigerated at a temperature of 4 °C to allow the plant extracts to prediffuse into the agar surface. Thereafter, the trays were incubated for 24 hours and were kept at a temperature of 37 °C. The yeasts were incubated at a temperature of 25 °C for 48 hours and moulds for 7 days. After the incubation period, the trays were viewed against the paper template used earlier and the results were recorded in millimeters from the edge of the paper disk to the edge of the inhibition zone. The trays and glassware used during the procedure were sterilised by autoclaving. Plant extracts were transferred using disposable micro pipette tips, that were autoclave sterilised.

Method for Minimum Inhibitory Concentration

Samples that showed promising results in the disk diffusion assays were further subjected to a microdilution assay. These samples were tested against *Candida albicans*. The minimum inhibitory concentration is defined as the lowest concentration of sample that inhibited growth by way of visual inspection. After the incubation period, 40 µl of a ρ-iodonitrotetrazolium violet salt solution (INT) (Sigma) was placed into each well. The plate was then further incubated for a period of 10 – 30 minutes at 37 °C. The plate was viewed at 30 minutes, 60 minutes, 120 minutes and at 24 hours. Oil solutions were prepared using 51.2 µg of oil sample with 400 µl of filter-sterilised acetone to give a concentration of 64 µg / ml. These prepared oil solutions were brought to a laminar flow unit, where the rest of the manipulation of the micro-titre plate was done, excluding the addition of the fungal culture. Each well was first filled with 100 µl of sterile water. In row A (Figure 2.2.), 100 µl of oil/acetone extract was placed from each sample to be analysed, from well A1 up to well number A6. Serial dilutions of the oil samples were performed from wells A through to H as seen in Figure 2.2. For example, 100 µl of contents from well A1 was withdrawn after the contents were thoroughly mixed and released through the micropipette; and was transferred to well B1. This procedure was then repeated from well C1 onwards to well H1, with the exception that the 100 µl withdrawn from well H1, was discarded. This dilution method (Eloff 1998) was repeated in each column. One hundred microlitres of fungal culture (*Candida albicans*) was placed into each well. The micro-titre plate was incubated at 37 °C for 24 hours. A 0.02 mg/ml ρ-iodonitrotetrazolium violet (INT) solution was prepared after the 24 hour incubation period. Forty microlitres of this solution was added to each well and the micro-titre plate was incubated at 37 °C for 10 – 30 minutes. The plate was viewed after 30 minutes, 60 minutes, 120 minutes and 24 hours. Tetrazolium salts like INT above, are used to indicate biological activity because the colourless compound acts as an electron acceptor and is reduced to a coloured product by biologically active organisms (Eloff 1998). The contents of the well turned to red or purple if any bacterial growth was present.

Figure 2.1. Freshly collected plant material was cut up and the essential oil was collected by steam distillation using a Clevenger apparatus.

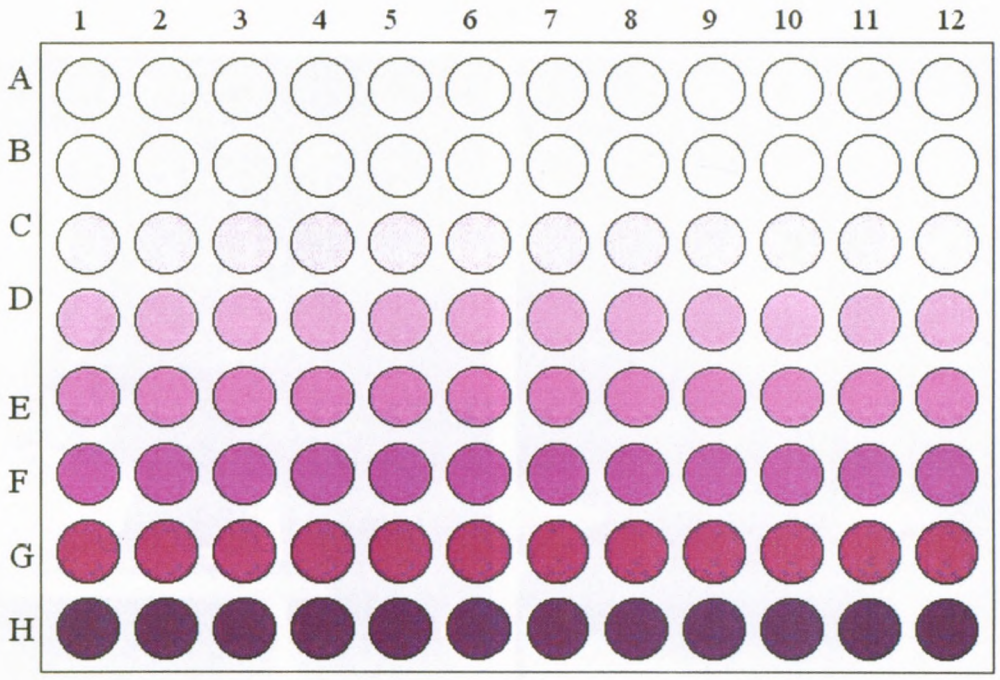


Figure 2.2. Diagram of micro-titre plate. Each circle represents one well.

(A)

(B)

(C)

(D)

Figure 2.3. (A)-(D) Plant samples were manipulated in a microbiology laboratory for the antimicrobial assays.

(A) and (B) Bacterial and fungal cultures were mixed prior to use. The agar trays were then seeded with these cultures, in preparation for the disk diffusion assays.

(C) The disks impregnated with the essential oil solutions, were then carefully placed onto the solidified agar surface, into the square allocated to the particular sample number.

(D) Preparation of a microtitre plate in the laminar flow unit for the microdilution assay.

Statistical Analysis

Generation of Cluster Analysis Data

Cluster analysis was carried out with the NTSYS-PC package version 2.00 (Rohlf 1997). The quantitative dataset (16 populations) and 98 compounds were analysed using standard clustering algorithms. A hierarchical cluster analysis was also performed on a qualitative (absence/presence) dataset.

Chapter 3 - Results

Essential Oil Yields

The relative percentage of oil yield per sample is shown in the graph in Figure 3.1. and the collection data for all samples studied, is given in Table 3.1. below.

Table 3.1. Collection data and essential oil yield of *Lippia javanica* analysed.

Sample Abbreviation	Locality	Yield*
SW1	Balakane (Swaziland)	0.48%
SW2	Balakane (Swaziland)	0.22%
SW3	Balakane (Swaziland)	0.38%
SW4	Balakane (Swaziland)	0.44%
SW5	Balakane (Swaziland)	0.34%
SW6	Balakane (Swaziland)	0.33%
N1	Nelspruit (Mpumalanga)	0.44%
N2	Nelspruit (Mpumalanga)	0.57%
N3	Nelspruit (Mpumalanga)	0.37%
W1	Warmbaths (Limpopo Province)	0.77%
W2	Warmbaths (Limpopo Province)	1.05%
W3	Warmbaths (Limpopo Province)	0.48%
LTP1	Long Tom Pass (Mpumalanga)	0.65%
LTP2	Long Tom Pass (Mpumalanga)	0.05%
LTP3	Long Tom Pass (Mpumalanga)	0.45%
F	Fairland (Gauteng)	0.20%

* Yield expressed on a wet weight basis

The samples are variable in essential oil yield ranging from 0.05% (LTP2) to 1.05% (W2). The samples collected from the Swaziland population (SW1, SW2, SW3, SW4, SW5 and SW6) produced an average oil yield of 0.37%. The Nelspruit population (N1, N2 and N3) produced an average oil yield of 0.46%. From the Warmbaths population (W1, W2 and W3), an average oil yield of 0.76% was obtained. The Long Tom Pass population (LTP1, LTP2 and LTP3) produced an average oil yield of 0.38%. Sample F from the Fairland population produced an oil yield of 0.20%.

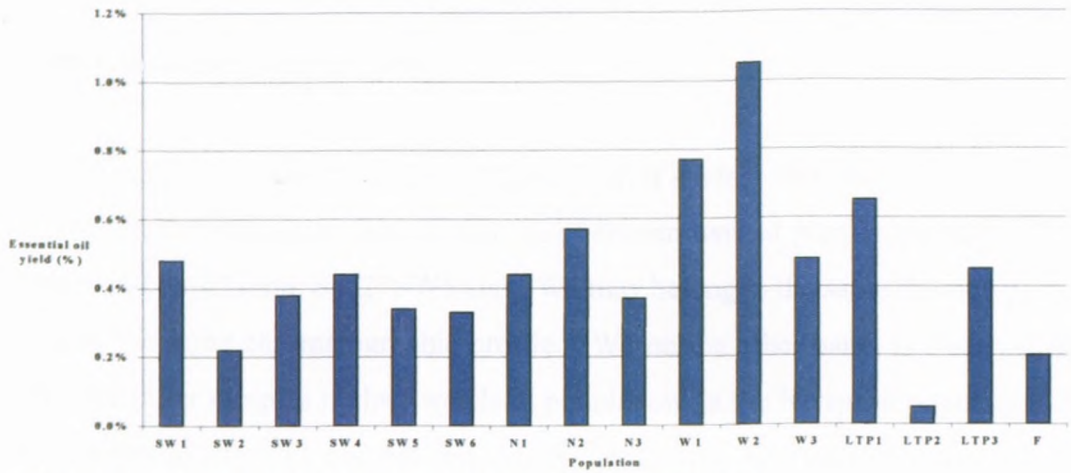


Figure 3.1. Graph showing relative percentage of oil yield.

(A) (B)

Figure 3.2. (A and B) TLC plates were spotted with the essential oil solutions at specific marked points on the reference base line.

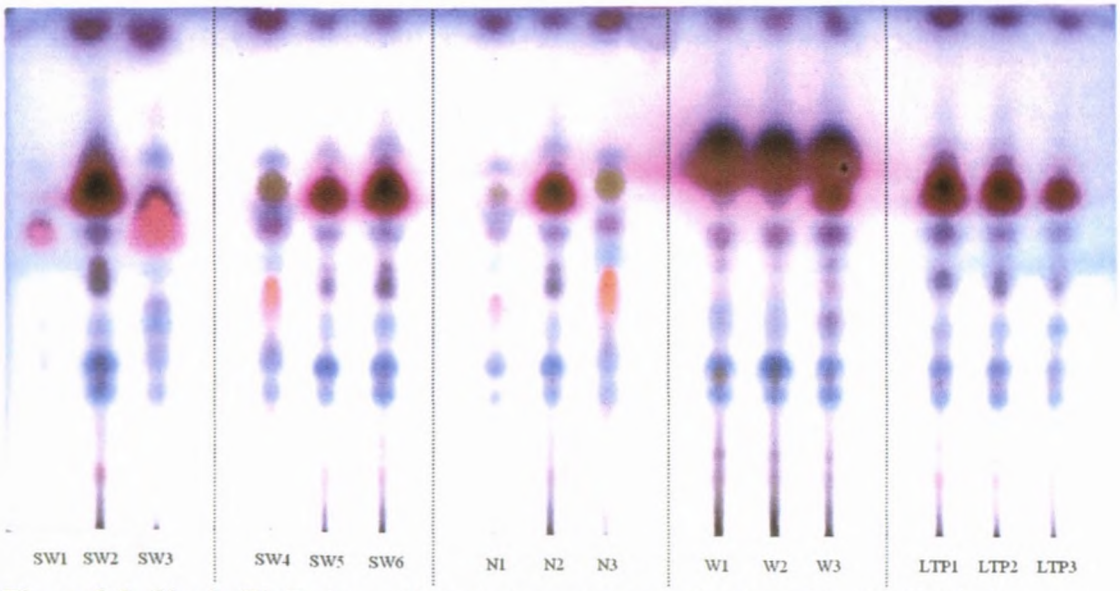


Figure 3.3. Single TLC plate of *Lippia javanica* samples (graphically divided).

Thin Layer Chromatography

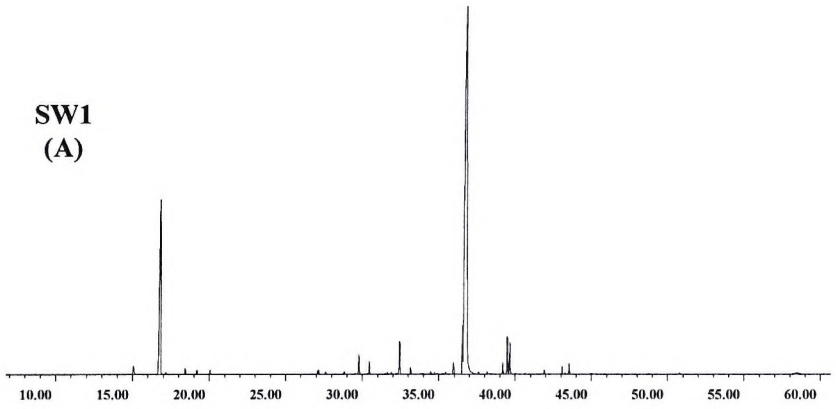
On examination of the TLC plate (Figure 3.3.), it is clear that there is variation in the essential oil composition both within and between natural plant populations. For the Swaziland population, SW2, SW5 and SW6 may belong to the same chemotype as they display the same chromatographic profile. SW4 on the other hand, is clearly different from the other samples in the Swaziland population. In the Nelspruit population, N2 is very different from N1 and N3. N1 and N3 look alike, with similar chromatographic profiles and they resemble SW4 very closely. N2 of this population resembles SW2, SW5 and SW6 of the Swaziland population. For the Warmbaths population, all three samples seem to be very similar with respect to chromatographic profiles. In the last population on the TLC plate above, Long Tom Pass, all three samples have the same chromatographic profile that bears a close similarity to SW2, SW5, SW6 and N2. From the four populations analysed by thin layer chromatography, it is apparent that there are at least four distinct types *of Lippia javanica*. The variation in chemical composition seems to be random, in the sense that different individuals in the same population and geographical area yield different chemical compounds in different quantities. The TLC results clearly indicated chemotypic variation. Although a crude and less-sensitive method, these results were valuable as they indicated the need to further investigate the essential oils by more sensitive chromatographic methods.

Gas Chromatography/Mass Spectrometry Data

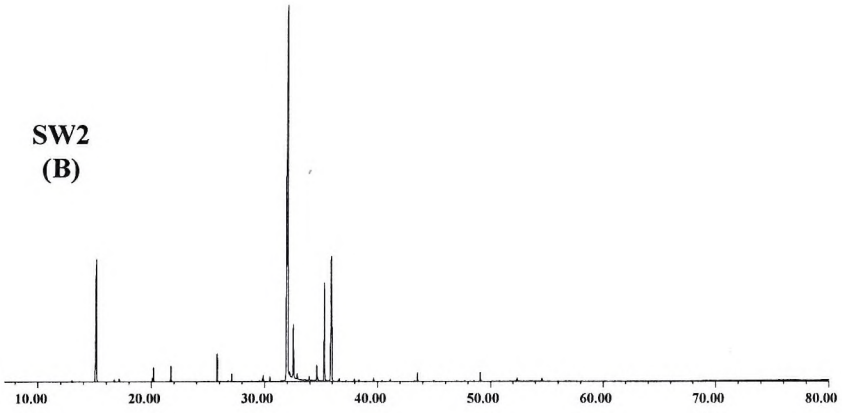
Swaziland population:

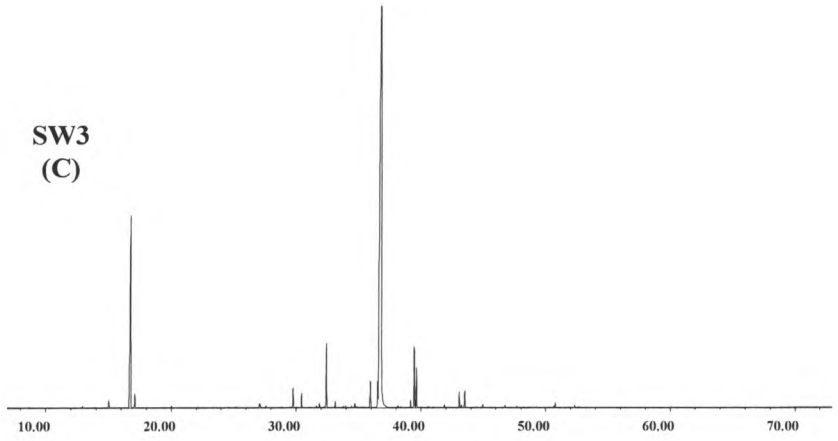
Plant material was collected from six individual plants (in a 10 m radius) in Balakane (Swaziland). The GC profiles and essential oil composition for each of the six collections are shown below.

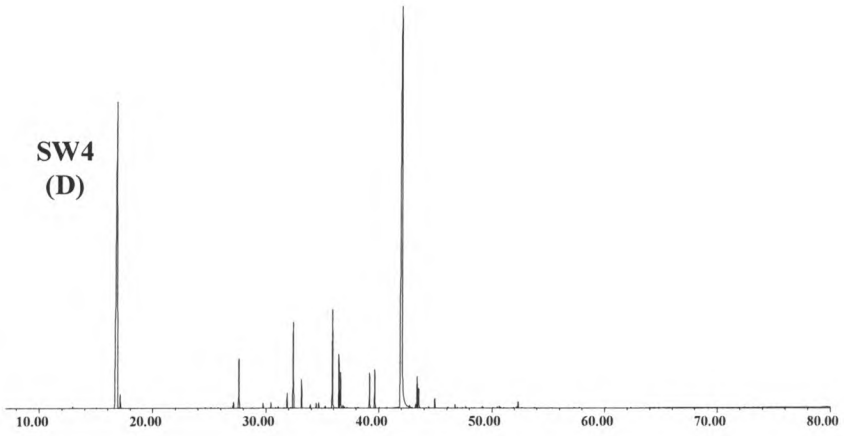
SW1
(A)

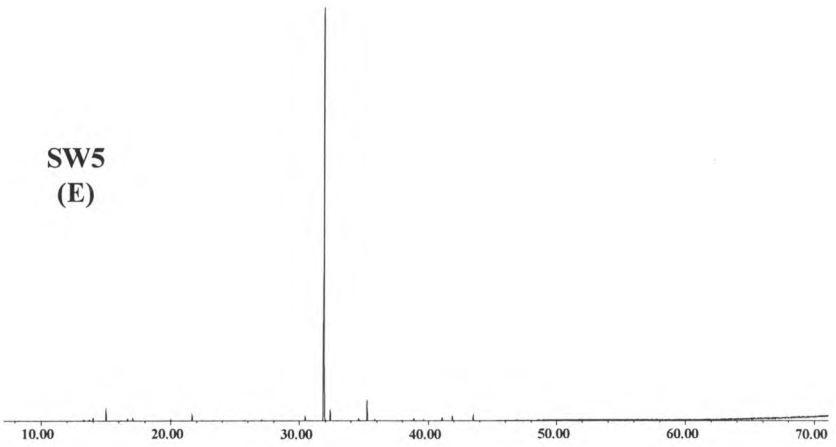


SW2
(B)




SW3
(C)
10.00
20.00
30.00
40.00
50.00
60.00
70.00


SW4
(D)
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00


SW5
(E)
10.00
20.00
30.00
40.00
50.00
60.00
70.00

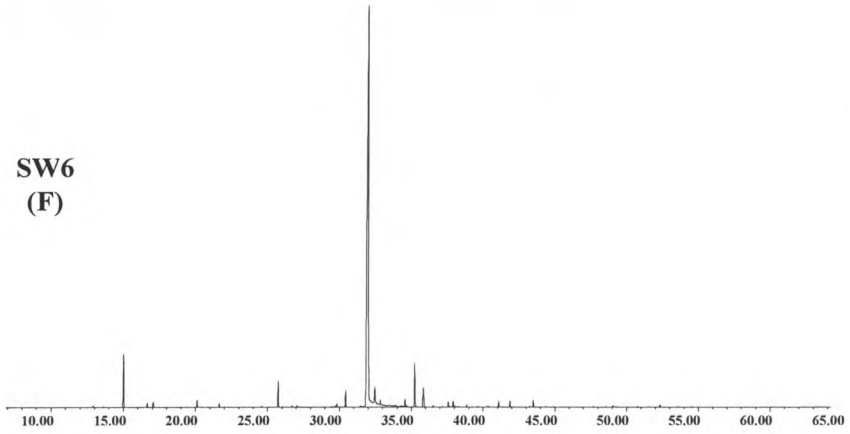


Figure 3.4. (A)-(F) GC/MS profiles for essential oils from six plants in the Swaziland population.

Table 3.2. Summarized GC/MS data for the Swaziland population with relative percentages of significant compounds.

RRI	Compound	SW1	SW2	SW3	SW4	SW5	SW6
1118	β-pinene	-	-	-	0.11	-	-
1132	sabinene	-	-	0.11	-	-	0.28
1174	**myrcene and α-phellandrene**	**1.11**	**28.8**	-	-	-	-
1174	myrcene	-	-	0.44	-	5.15	5.62
1203	limonene	**28.21**	0.35	**13.73**	**43.35**	0.35	0.49
1213	1,8-cineole and β-phellandrene	-	0.55	-	-	-	-
1213	1,8-cineole	-	-	0.84	0.7	2.14	0.61
1218	β-phellandrene	0.15	-	-	-	-	-
1246	(*Z*)-β-ocimene	0.53	-	-	-	-	-
1266	(*E*)-β-ocimene	0.39	-	-	-	-	-
1274	isomyrecenol (K.I) *tentative	-	1.9	-	-	-	0.75
1280	p-cymene	0.39	0.49	0.1	-	0.81	-
1290	terpinolene	-	-	-	-	0.46	-
1319	dihydrotagetone	-	1.21	-	-	1.92	0.3
1391	(*Z*)-3-hexen-1-ol	-	-	-	-	-	0.16
1398	6,7-epoxymyrcene BP 1979	-	1.79	-	-	0.87	1.78
1452	1-octen-3-ol (D.I) and *cis*-1,2-limonene epoxide	0.43	-	-	-	-	-
1452	1-octen-3-ol	-	0.65	0.28	0.03	0.53	0.15
1458	*cis*-1,2-limonene epoxide	-	-	0.09	0.36	-	-
1468	*trans*-1,2-limonene epoxide	0.14	-	0.03	1.81	-	-
1497	α-copaene	0.09	-	-	-	-	-
1522	*trans*-tagetone	-	0.3	-	-	-	-
1532	camphor	-	-	-	-	-	0.15
1535	β-bourbonene	0.47	0.16	0.41	0.13	-	-

RRI	Compound	SW1	SW2	SW3	SW4	SW5	SW6
1553	linalool	0.51	0.25	0.4	0.19	1.33	0.96
1586	**myrcenone**	-	**36.31**	0.11	0.38	**59.4**	**55.93**
1612	β-caryophylline	0.81	3.54	1.45	2.16	5.46	5.23
1626	2-methyl-6-methylene-3,7-octadien-2-ol	-	-	-	-	-	1.52
1639	*trans*-p-mentha-2,8-dien-1-ol	0.27	-	0.19	0.91	-	-
1661	alloaromadendrene	-	0.12	0.12	0.12	-	-
1678	*cis*-p-mentha-2,8-dien-1-ol	-	-	0.12	0.16	-	-
1678	ipsdienol	-	0.6	-	-	-	1.26
1681	(*Z*)-3-hexenyl tiglate	-	-	-	-	-	0.7
1687	α-humulene	0.12	-	0.04	0.13	-	0.34
1704	*cis*-tagetenone and α-terpineol	-	-	-	-	-	2.79
1704	*cis*-tagetenone	-	3.08	-	-	4.93	-
1706	α-terpineol	-	-	-	0.08	-	-
1719	borneol	0.1	-	-	-	-	-
1725	verbenone	-	1.31	-	-	-	-
1726	germacrene-d	0.29	4.34	0.83	2.44	0.53	0.86
1726	*trans*-tagetenone and α-terpineol	-	-	-	-	-	0.6
1726	*trans*-tagetenone	-	3.64	-	-	-	-
1747	*trans*-carvyl acetate	0.87	-	0.58	1.16	-	-
1751	**carvone**	**61.07**	-	**72.65**	0.97	-	-
1755	bicyclogermacrene	-	0.15	-	-	-	-
1786	ar-curcumene	0.12	-	-	-	-	0.22
1808	2-methyl-2-butenoic acid	-	-	-	-	-	0.16
1811	*trans*-p-mentha-1(7),8-dien-2-ol	0.1	-	0.09	-	-	-
1845	*trans*-carveol	0.33	-	0.26	0.9	-	-
1856	carvone oxide	1.01	-	1.98	-	-	-
1865	isopiperitenone	0.86	0.12	1.27	0.96	-	0.08
1882	*cis*-carveol (D.I)	0.05	-	-	-	-	-
1896	*cis*-p-mentha-1(7),8-dien-2-ol	-	-	0.07	-	-	-
1949	**piperitenone**	0.14	-	0.17	**39.88**	-	0.35
2001	isocaryophylline oxide	0.13	-	0.09	0.08	0.29	-
2008	caryophylline oxide	0.24	0.11	0.53	0.82	1.2	0.31
2050	(*E*)-nerolidol	-	-	-	0.03	-	-
2144	spathulenol	-	-	0.08	0.07	0.25	-
2186	eugenol	-	-	0.12	0.06	-	-
2202	germacrene-d-4-ol	-	-	0.07	0.17	-	-
2316	caryophylla-2(12),6(13)-dien-5β-ol	-	-	0.06	-	-	-
2324	caryophylla-2(12),6(13)-dien-5α-ol	-	-	0.16	-	-	-
2389	caryophylla-2(12),6-dien-5α-ol	-	-	0.07	-	-	-
	Total	**98.93**	**89.77**	**97.54**	**98.16**	**85.60**	**81.60**

From Table 3.2. above, it is clear that there are four distinct chemotypes in this population. SW1 and SW3 belong to a type with major compounds carvone and limonene

at 61.1% and 28.2%, and at 72.7% and 13.7% respectively. SW2 belongs to a different type, with myrcenone (36.3%) and myrcene and α-phellandrene (28.8%) as major compounds. It is of interest to note that carvone, which was the major compound in both SW1 and SW3, was not detected in SW2. SW4 belongs to yet another type, with limonene (43.4%) and piperitenone (39.9%) as major compounds. The final type observed in this population, is represented by SW5, which has major compounds myrcenone (59.4%), myrcene (5.1%) and β-caryophylline (5.5%) and SW6 with myrcenone (55.9%), myrcene (5.6%), and β-caryophylline (5.2%). The high myrcenone content in SW5 and SW6 was also observed in SW2, however, the high myrcene and α-phellandrene content in SW2 was not observed in appreciable quantities in any of the other samples.

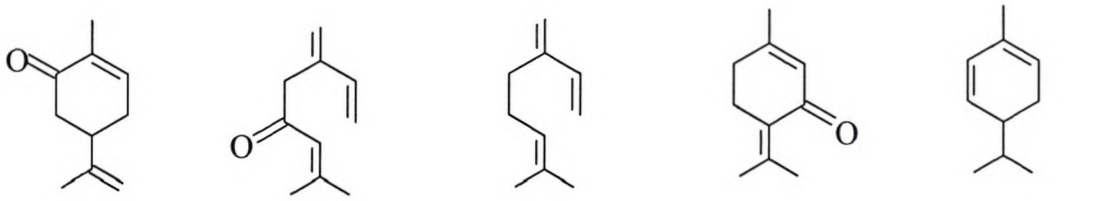


Figure 3.5. Chemical structures of some of the major terpenes identified in the essential oil from plants in the Swaziland population.

Nelspruit population:

Material was collected from three individual plants (N1, N2 and N3) from a natural population near 'Die Steiltes' in Nelspruit.

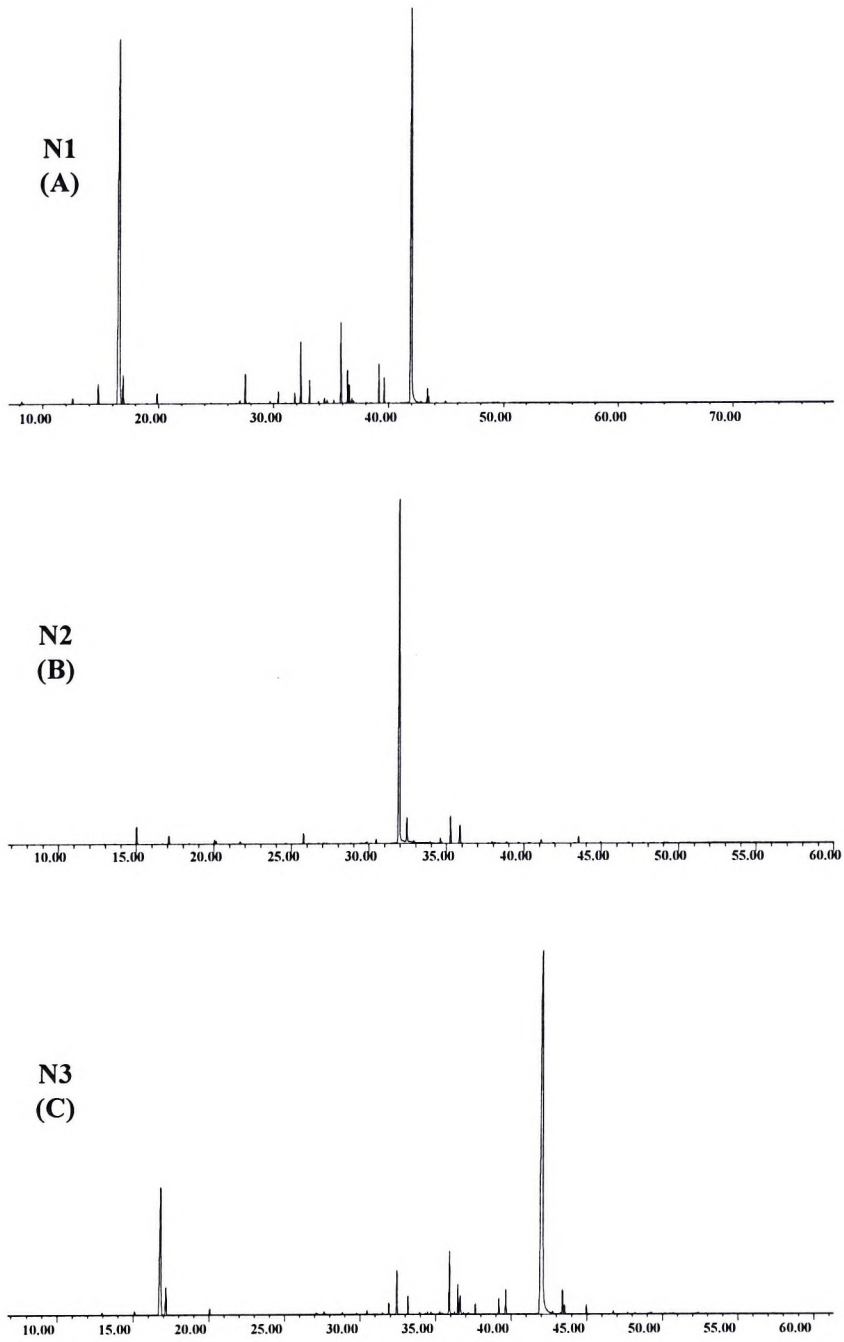
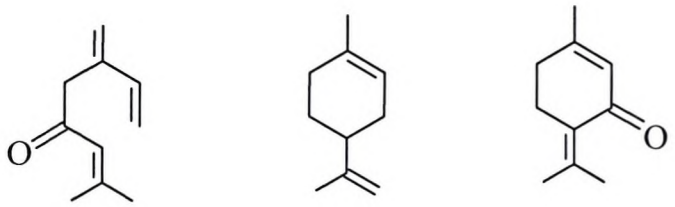


Figure 3.6. (A)-(C) GC/MS profiles for essential oils from three plants in the Nelspruit population.

Table 3.3. Summarized GC/MS data for the Nelspruit population with relative percentages of significant compounds.

RRI	Compound	N1	N2	N3
1032	α-pinene	0.14	-	-
1118	β-pinene	0.04	-	-
1132	sabinene	0.32	-	0.56
1174	myrcene	-	4.49	-
1176	α-phellandrene	0.94	-	0.77
1203	**Limonene**	**51.67**	-	**33.52**
1213	1,8-cineole	1.39	2.2	4.01
1280	p-cymene	0.04	0.93	0.76
1319	dihydrotagetone	-	0.42	-
1398	6,7-epoxymyrcene BP 1979	-	1.56	-
1452	1-octen-3-ol	-	-	0.35
1458	*cis*- and *trans*-1,2-limonene epoxide	-	-	0.3
1458	*cis*-1,2-limonene epoxide	0.19	-	-
1468	*trans*-1,2-limonene epoxide	1.11	-	-
1497	α-copaene	-	-	0.14
1535	β-bourbonene	0.1	-	0.08
1553	linalool	0.41	0.6	0.31
1586	**myrcenone**	0.3	**61.85**	0.35
1612	β-caryophylline	1.85	5.5	1.8
1639	*trans*-p-mentha-2,8-dien-1-ol	0.85	-	1.1
1661	alloaromadendrene	0.09	-	0.11
1678	*cis*-p-mentha-2,8-dien-1-ol	0.24	-	0.19
1678	ipsdienol	-	0.98	-
1687	α-humulene	0.11	-	0.13
1704	*cis*-tagetenone	-	4.03	-
1706	α-terpineol	0.17	2.35	0.14
1719	borneol	-	-	0.11
1726	germacrene-d	2.28	1.98	2.19
1726	*trans*-tagetenone	-	1.06	-
1747	*trans*-carvyl acetate	0.9	-	1.2
1751	carvone	0.61	-	0.62
1758	*cis*-piperitol	0.14	-	0.12
1771	γ-bisabolene	-	-	0.14
1786	ar-curcumene	-	-	0.25
1845	*trans*-carveol	1.22	-	0.65
1865	isopiperitenone	0.78	-	0.9
1949	**piperitenone**	**32.45**	-	**47.3**
2001	isocaryophylline oxide	-	-	0.21
2008	caryophylline oxide	0.44	1.1	0.72
2202	germacrene-d-4-ol	0.05	-	0.14
	Total	**98.83**	**89.05**	**99.36**

This population consists of two distinct chemotypes (Table 3.3.). The first type is represented by N1 with major compounds limonene (51.7%) and piperitenone (32.4%), and N3 with major compounds limonene (33.5%) and piperitenone (47.3%). This combination resembles SW4 from the Swaziland population. N2 belongs to a chemotype found in SW5 and SW6 (from the Swaziland population), with major compounds myrcenone (61.9%), myrcene (4.5%) and β-caryophylline (5.5%).



myrcenone limonene piperitenone

Figure 3.7. Chemical structures of some of the major terpenes identified in the essential oil from plants in the Nelspruit population.

Warmbaths population:

The leaves were harvested from three individual plants (W1, W2 and W3) in a natural population 10 km south of Warmbaths.

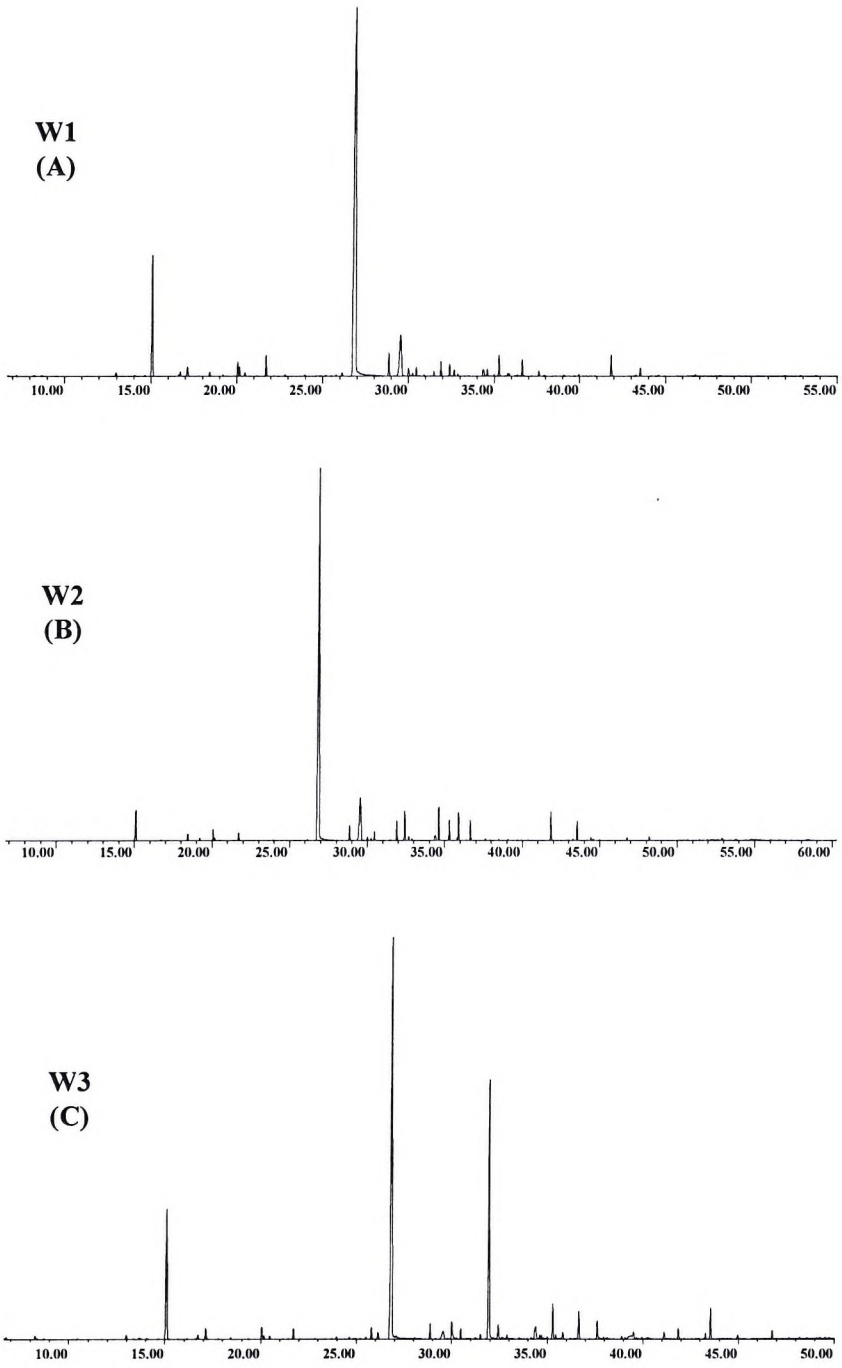


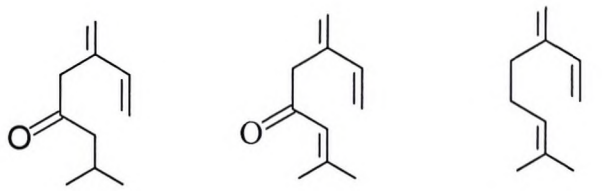
Figure 3.8. (A)-(C) GC/MS profiles for essential oils from three plants in the Warmbaths population.

Table 3.4. Summarized GC/MS data for the Warmbaths population with relative percentages of significant compounds.

RRI	Compound	W1	W2	W3
1017	4-methyl-2-pentanone	0.14	-	0.11
1132	sabinene	0.4	0.5	0.48
1174	**myrcene**	**11.33**	**13.62**	**13.8**
1203	2-methylbutyl isobutyrate	0.09	-	-
1203	limonene	0.39	-	0.43
1213	1,8-cineole and β-phellandrene	0.95	-	-
1213	1,8-cineole	-	-	0.98
1246	(*Z*)-β-ocimene	0.32	1.65	0.17
1266	(*E*)-β-ocimene	0.14	1.83	-
1274	isomyrecenol (K.I) *tentative	0.75	-	-
1280	p-cymene	0.97	-	0.97
1286	2-methylbutyl-2-methyl butyrate	0.18	-	0.16
1319	dihydrotagetone	1.11	1.11	0.62
1391	(*Z*)-3-hexen-1-ol	-	-	0.24
1398	6,7-epoxymyrcene BP 1979	-	-	0.73
1429	perillene	0.25	-	0.47
1444	**ipsenone** (tentative, Wiley)	**60.86**	**52.58**	**42.22**
1500	*cis*-tagetone	1.2	0.95	0.91
1522	*trans*-tagetone	4.9	4.8	1.07
1553	linalool	0.42	0.45	0.59
1577	α-cedrene	0.18	-	-
1586	**myrcenone**	0.47	0.64	**12.88**
1612	β-caryophylline	0.47	0.27	0.12
1626	2-methyl-6-methylene-3,7-octadien-2-ol	0.12	-	0.33
1678	ipsdienol	0.82	1.55	1.53
1685	isovaleric acid	0.21	0.13	0.35
1687	α-humulene	-	-	0.11
1706	α-terpineol	0.88	0.63	1.73
1719	borneol	-	-	0.22
1726	germacrene-d	0.09	0.89	-
1786	ar-curcumene	0.08	-	1.29
1853	calamenene	0.08	-	-
2001	isocaryophylline oxide	0.09	-	0.35
2008	caryophylline oxide	0.36	0.67	1.7
2071	humulene epoxide II	-	-	0.25
2144	spathulenol	0.1	-	0.56
	Total	**88.35**	**82.27**	**85.37**

From the Table above, (Table 3.4.), W1 with major compounds ipsenone (60.9%) and myrcene (11.3%) and W2 with major compounds ipsenone (52.6%) and myrcene (13.6%)

belong to a type different from those already discussed in the other populations. W3 presented with major compounds ipsenone (42.2%) and myrcene (13.8%) and it is very similar to W1 and W2 with however a notable difference in the myrcenone and *trans*-tagetone content. Myrcenone content in W3 is 12.9% compared to W1 (0.5%) and W2 (0.6%). Conversely the *trans*-tagetone content is higher in W1 with 4.9% and in W2 with 4.8% compared to W3 with a content of 1.1%. The level of *trans*-tagetone was found to be the highest in W1 in comparison to all other samples.



ipsenone myrcenone myrcene

Figure 3.9. Chemical structures of some of the major terpenes identified in the essential oil from plants in the Warmbaths population.

Long Tom Pass population:

Leaf material was collected from three individual plants (LTP1, LTP2 and LTP3) near the summit of the Long Tom Pass in Mpumalanga.

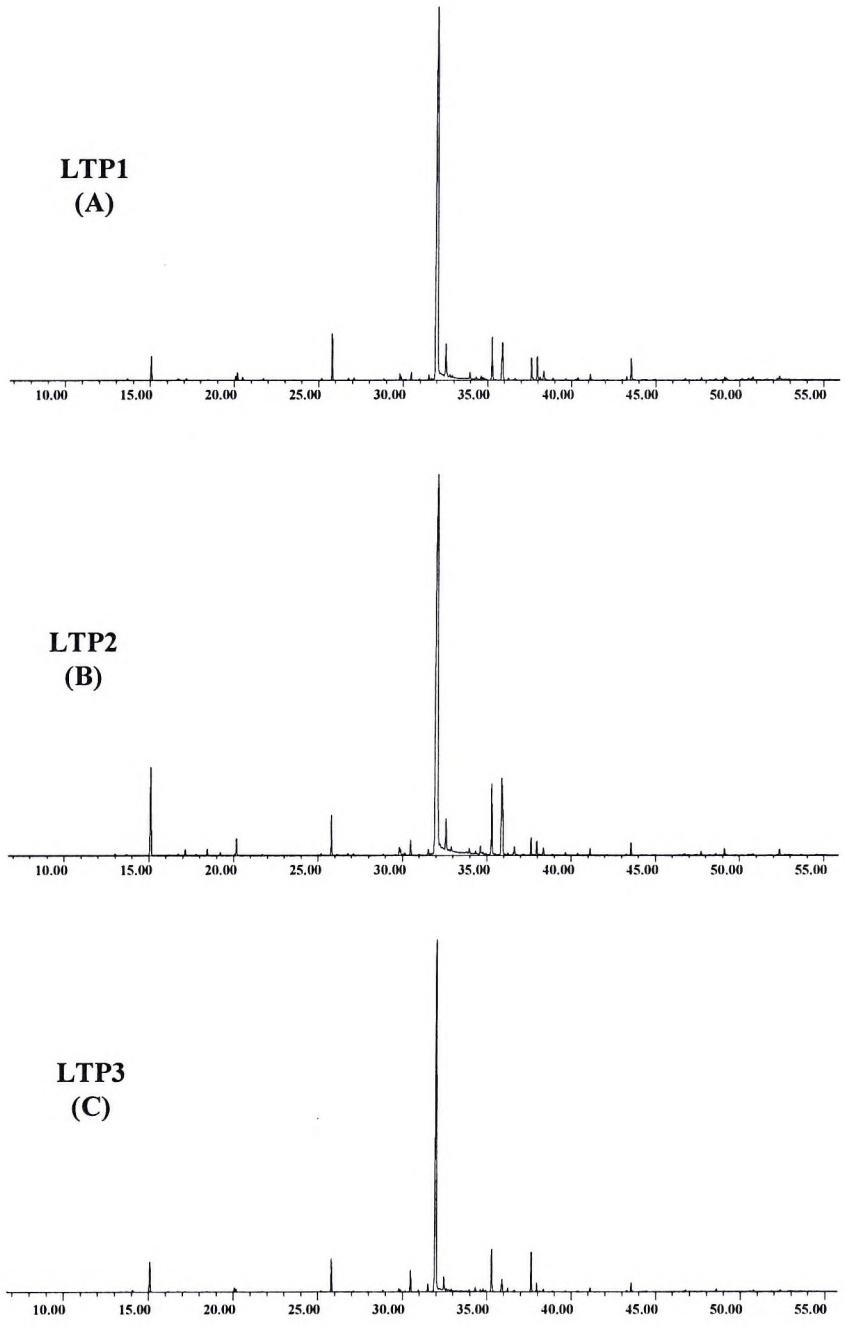


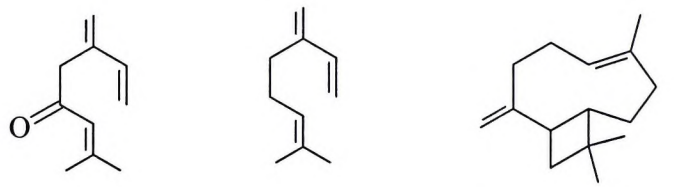
Figure 3.10. (A)-(C) GC/MS profiles for essential oils from three plants in the Long Tom Pass population.

Table 3.5. Summarized GC/MS data for the Long Tom Pass population with relative percentages of significant compounds.

RRI	Compound	LTP1	LTP2	LTP3
1032	α-pinene	-	0.03	-
1132	sabinene	-	0.12	-
1174	**myrcene**	**2.2**	**4.65**	**4.14**
1203	limonene	0.16	0.11	-
1213	1,8-cineole and β-phellandrene	-	0.4	-
1213	1,8-cineole	0.31	-	-
1225	(*Z*)-3-hexenal	-	0.13	-
1246	(*Z*)-β-ocimene	-	0.32	-
1266	(*E*)-β-ocimene	-	0.15	-
1274	isomyrecenol (K.I) *tentative	0.74	0.82	0.51
1280	p-cymene	0.35	0.09	0.64
1286	2-methylbutyl-2-methyl butyrate	0.19	-	-
1319	dihydrotagetone	0.16	0.06	-
1398	6,7-epoxymyrcene BP 1979	2.99	1.6	3.53
1400	nonanal	0.12	0.1	-
1429	perillene	-	0.07	-
1452	1-octen-3-ol	0.18	0.08	-
1497	α-copaene	0.11	0.02	0.29
1500	*cis*-tagetone	-	-	0.28
1522	*trans*-tagetone	-	2.63	0.82
1535	β-bourbonene	0.17	0.17	0.3
1541	benzaldehyde	-	0.28	-
1553	linalool	0.49	0.63	2.03
1568	*trans*-α-bergamotene	0.12	0.11	0.2
1577	α-cedrene	0.33	0.35	0.78
1586	**myrcenone**	**52.3**	**50.46**	**48.78**
1612	**β-caryophylline**	**4.69**	**4.2**	**4.84**
1626	2-methyl-6-methylene-3,7-octadien-2-ol	-	-	1.41
1661	alloaromadendrene	1.15	1.21	1
1668	(*Z*)-β-farnesene	-	-	1.36
1678	ipsdienol	1.16	0.65	1.24
1687	α-humulene	0.47	1.13	-
1690	α-acarodiene	-	-	0.58
1693	β-acarodiene	-	-	0.24
1695	(*E*)-β-farnesene	-	1.11	-
1704	*cis*-tagetenone and α-terpineol	-	3.3	-
1704	*cis*-tagetenone	2.56	-	-
1706	α-terpineol	0.09	-	4.58
1726	germacrene-d	1.53	3.2	0.61
1726	*trans*-tagetenone	1.41	-	-
1741	β-bisabolene	0.08	0.15	0.29
1755	β-curcumene	0.08	0.29	0.17

RRI	Compound	LTP1	LTP2	LTP3
1758	(*E*,*E*)-α-farnesene	-	0.17	-
1786	ar-curcumene	0.8	0.57	2.38
1808	2-methyl-2-butenoic acid	0.09	-	-
1865	isopiperitenone	0.1	0.14	-
2001	isocaryophylline oxide	0.15	0.08	0.12
2008	caryophylline oxide	0.8	0.46	0.52
2071	humulene epoxide II	0.13	-	-
2131	hexahydrofarnesyl acetone	-	0.05	-
2144	spathulenol	0.1	0.08	0.15
2186	eugenol	0.06	-	-
	Total	**76.40**	**80.17**	**81.79**

LTP1, LTP2 and LTP3 (in Table 3.5. above) belong to a type already identified in the Swaziland population (SW5 and SW6), with major compounds myrcenone, myrcene and β-caryophylline. LTP1 consists of myrcenone (52.3%), myrcene (2.2%), and β-caryophylline (4.7%). LTP2 consists of myrcenone (50.5%), myrcene (4.6%) and β-caryophylline (4.2%) and LTP3 with myrcenone (48.8%), myrcene (4.1%) and β-caryophylline (4.8%). LTP3 showed the highest content of α-terpineol across all populations with a composition of 4.6%.



myrcenone myrcene β-caryophyllene

Figure 3.11. Chemical structures of some of the major terpenes identified in the essential oil from plants in the Long Tom Pass population.

Fairland population:

Due to large volumes of essential oil required for continued antimicrobial studies, leaf material was collected from several plants growing on Fairland koppie.

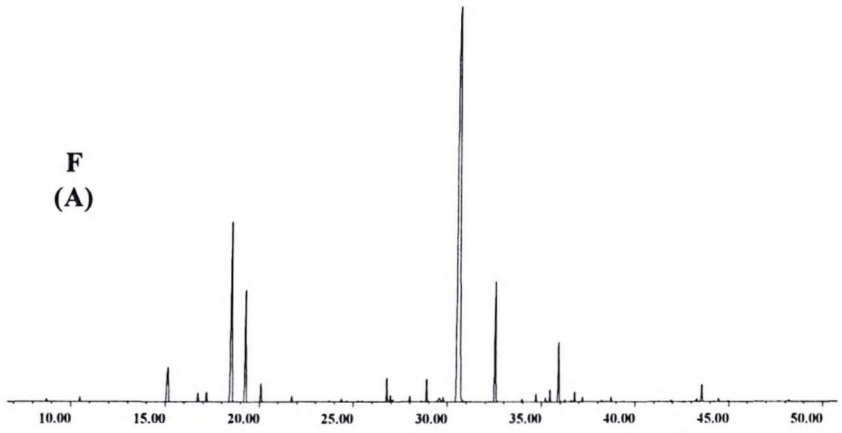


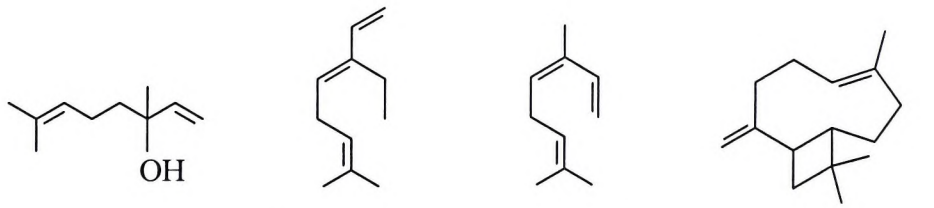
Figure 3.12. (A) GC/MS profile for an essential oil from one plant in the Fairland population.

Table 3.6. Summarized GC/MS data for the Fairland population with relative percentages of significant compounds.

RRI	Compound	F
1032	α-pinene	0.17
1076	camphene	0.3
1174	myrcene and α-phellandrene	2.6
1203	limonene	0.45
1218	β-phellandrene	0.46
1246	**(*Z*)-β-ocimene**	**12.97**
1266	**(*E*)-β-ocimene**	**6.21**
1280	p-cymene	0.87
1319	dihydrotagetone	0.21
1382	*cis*-alloocimene	0.18
1444	ipsenone (tentative, Wiley)	0.79
1450	*trans*-linalool oxide (*furanoid*)	0.2
1452	1-octen-3-ol	0.12
1478	*cis*-linalool oxide (*furanoid*)	0.16
1497	α-copaene	0.42
1500	*cis*-tagetone	0.18
1522	*trans*-tagetone	0.33
1532	camphor	0.18
1553	**linalool**	**65.19**

RRI	Compound	F
1612	**β-caryophylline**	**3.58**
1661	alloaromadendrene	0.07
1687	α-humulene	0.19
1704	γ-muurolene	0.1
1719	borneol	0.37
1726	germacrene-d	1.48
1740	α-muurolene	0.09
1755	bicyclogermacrene	0.09
1758	(*E*,*E*)-α-farnesene	0.21
1773	δ-cadinene	0.16
1830	2,6-dimethyl-3(*E*),5(*E*),7-octatriene-2-ol	0.1
2001	isocaryophylline oxide	0.07
2008	caryophylline oxide	0.42
2050	(*E*)-nerolidol	0.06
2071	humulene epoxide II	0.03
	Total	**99.01**

The essential oil belongs to a chemotype not identified in any of the other samples. Linalool (65.2%), (Z)-β-ocimene (13%) and β-caryophylline (3.6%) are accumulated as the major compounds in the essential oil.



linalool (E)-β-ocimene (Z)-β-ocimene β-caryophyllene

Figure 3.13. Chemical structures of some of the major terpenes identified in the essential oil from plants in the Fairland population.

Disk Diffusion Assays

All sixteen samples were tested in a disk diffusion assay against four bacteria; *Staphylococcus aureus* (Gram positive), *Enterococcus faecalis* (Gram negative), *Pseudomonas aeruginosa* (Gram negative) and *Escherichia coli* (Gram negative) and one yeast (*Candida tropicalis*). None of the samples (from Table 3.7. below) showed any apparent activity against *E. faecalis* or *P. aeruginosa*, except for sample F which showed an inhibition zone of 4 mm against *E. faecalis*. *S. aureus* and *E. coli* showed variable results, as did *C. tropicalis*.

Table 3.7. Disk diffusion assay results for sixteen samples (essential oils) tested on *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida tropicalis*.

Sample no.	Organism				
	S. aureus	*E. faecalis*	*P. aeruginosa*	*E. coli*	*C. tropicalis*
SW1	<1	-	-	1	2
SW2	-	-	-	1	<1
SW3	<1	-	-	1	<1
SW4	2	-	-	1.5	2
SW5	1	-	-	1	2
SW6	1	-	-	-	<1
N1	2	-	-	1	3
N2	1	-	-	-	2
N3	1	-	-	1	3
W1	<1	-	-	-	2
W2	<1	-	-	-	<1
W3	3	-	-	<1	2
LTP1	1	-	-	1	2
LTP2	1	-	-	<1	<1
LTP3	2.5	-	-	<1	3
F	2	4	-	1	3
Positive control	10	3	2	5	5
Negative control	No zone	No zone	No zone	No zone	No zone

(-) Indicates no apparent activity. Measurements are in millimeters, from the edge of the disk to the edge of the inhibition zone. Neomycin 50 µg/ml was used as a positive control for all micro-organisms except for *C. tropicalis*, where Nystatin 100 *iu.* was used.

In the Swaziland population, sample SW4 showed the highest activity against *S. aureus* and *E. coli*. Other samples in this population showed minimal to no activity against *S. aureus* (Figure 3.14). All samples also showed minimal activity against *E. coli*, except for SW6, which showed no apparent activity. Sample SW4 had the overall highest activity in this population, with larger inhibition zones against these organisms (Table 3.7.). In summary, SW1, SW4 and SW5 produced the highest activity against *C. tropicalis*. Samples SW2, SW3 and SW6 showed similar low activity against *C. tropicalis*.

From the Nelspruit population, N1 exhibited the highest activity. N1 and N3 showed similar activity against *S. aureus*, *E. coli* and *C. tropicalis*. It is interesting to note that N1 and N3 produced a similar inhibition in *C. tropicalis* together with LTP3 and F, with inhibition zones of 3 mm each. Sample N2 displayed the same activity against *S. aureus* as N3 (inhibition zone of 1 mm), but showed no activity against *E. coli.*

Within the Warmbaths population, W3 showed the highest activity. Samples W1 and W2 displayed the same activity against these organisms (Table 3.7.), except in *C. tropicalis* where W1 had higher activity. Both W1 and W2 had inhibition zones of less than 1 mm against *E. coli*. It is of note that W3 showed the largest inhibition zone against *S. aureus* (3 mm).

From the Long Tom Pass population, LTP1 showed the same level of inhibition against *S. aureus* and *E. coli* with an inhibition zone of 1 mm each, while LTP2 showed a lower activity against *E. coli* than *S. aureus*, with inhibition zones of less than 1 mm and 1 mm respectively. The highest activity against *S. aureus* was recorded by LTP3. It however showed very low activity against *E. coli.* Sample LTP3 had the highest and LTP2 the lowest activity (inhibition zone of less than 1 mm) against *C. tropicalis*.

Sample F showed variable inhibition for the four organisms. Inhibition of *S. aureus* was moderate and its activity against *E. coli* was low, but it had a high activity against *E. faecalis* (4 mm inhibition zone), and as mentioned earlier, was the only sample to show any activity here. It also had the highest inhibition zone (3 mm) against *C. tropicalis*.

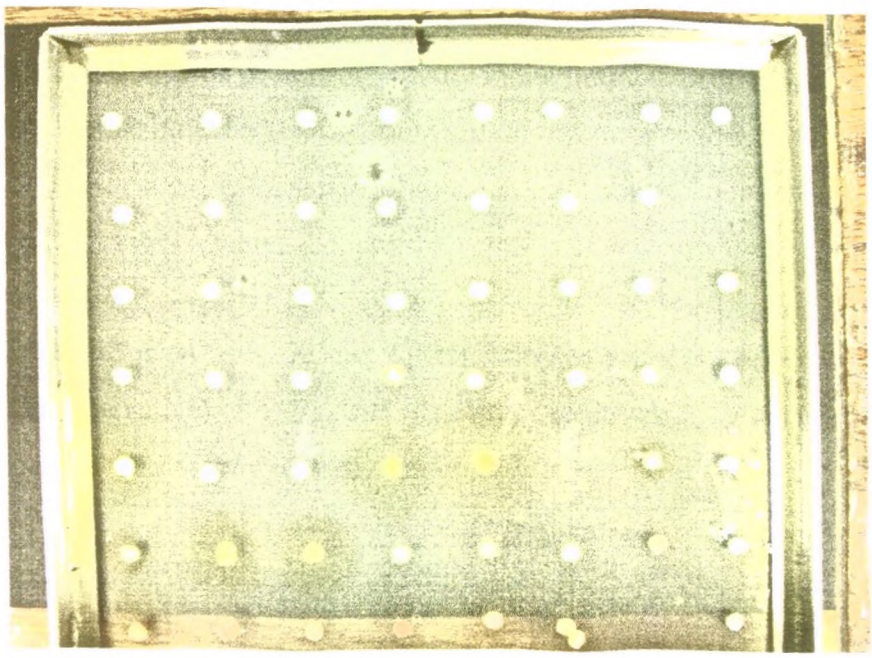


Figure 3.14. Disk diffusion plate of *Staphylococcus aureus*, showing zones of inhibition.

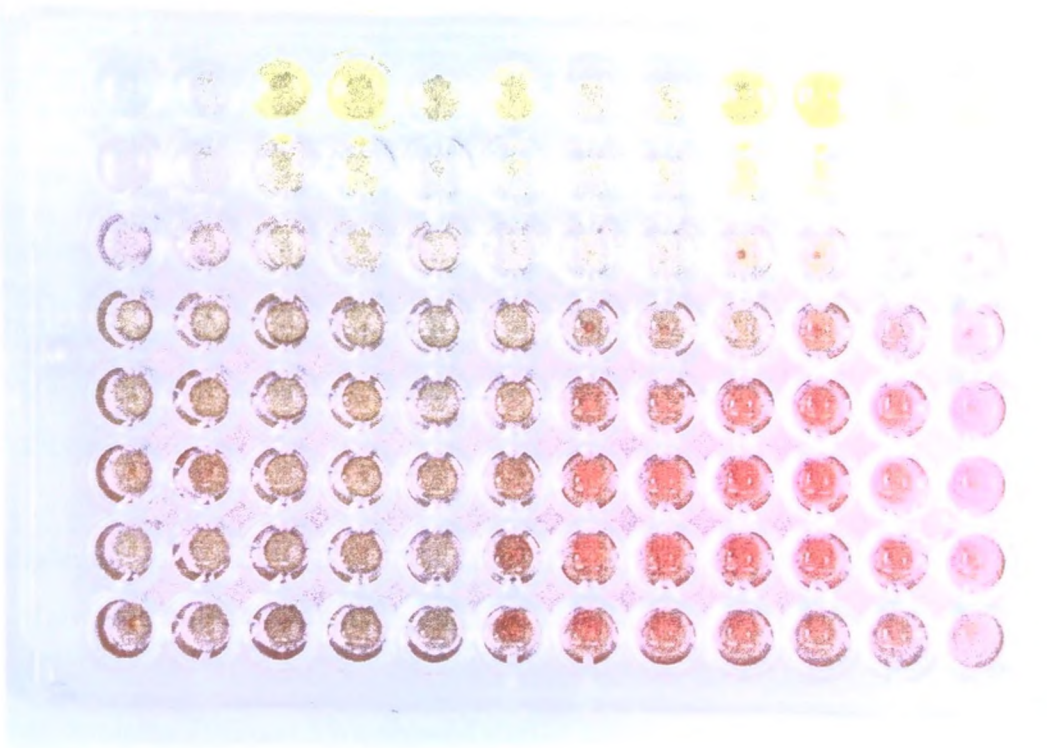


Figure 3.15. Micro-titre plate indicating the minimum inhibitory concentration of *Candida albicans*.

The oil samples that showed promising activity in the initial disk diffusion assay were tested against another four bacteria ie. *Serratia odorifera*, *Bacillus subtilis*, *Bacillus cereus* and *Staphylococcus epidermidis* (Table 3.8.). All showed no activity against *S. epidermidis* except for N2, which displayed low levels of activity.

Table 3.8. Disk diffusion assay results for ten samples tested on *Serratia odorifera* (Gram negative), *Bacillus subtilis* (Gram positive), *Bacillus cereus* (Gram positive) and *Staphylococcus epidermidis* (Gram positive).

Sample no.	Organism			
	S. odorifera	*B. subtilis*	*B. cereus*	*S. epidermidis*
SW1	1	3	2	-
SW2	-	2	1	-
SW3	1	2	<1	-
SW6	-	2	<1	-
N1	1	1	1	-
N2	-	-	1	1
W1	-	-	1	-
W2	-	-	1	-
LTP2	-	-	1	-
F	1	1	1	-
Positive control	6	10	10	12
Negative control	No zone	No zone	No zone	No zone

(-) Indicates no activity. Measurements are in millimeters, from the edge of the disk to the edge of the inhibition zone. Neomycin 50 µg/ml was used as a positive control for all micro-organisms.

Samples SW1, SW2, SW3 and SW6 were included in this second disk diffusion assay. All showed moderate activity against *B. subtilis* with SW1 showing the highest (inhibition zone of 3 mm). Sample SW1 also showed the highest activity against *B. cereus*. Samples SW2 and SW6 showed similar activity against *B. subtilis* and *B. cereus* and both showed no activity against *S. odorifera*. Extract SW3 showed low activity against *S. odorifera* and *B. cereus* but moderate activity against *B. subtilis*.

Samples N1 and N2 were selected for this second disk diffusion assay. Extract N1 showed low activity against *S. odorifera*, *B. subtilis* and *B. cereus*. Sample N2 showed low activity against *B. cereus* and *S. epidermidis*, with no activity observed against *S.*

odorifera or *B. subtilis*.

Samples W1 and W2 displayed low activity against *B. cereus* and showed no activity against *S. odorifera*, *B. subtilis* and *S. epidermidis*.

Sample LTP2 displayed the same activity as samples W1 and W2 against all four bacteria (Table 3.8.). Sample F showed low activity against *S. odorifera*, *B. subtilis*, and *B. cereus*.

Selected oil samples were tested against the fungi *Aspergillus niger* and *Cryptococcus neoformans* in disk diffusion assays (Table 3.9.). In general, the oils showed larger inhibition zones here than what has been observed previously (Table 3.7. and Table 3.8.).

Table 3.9. Disk diffusion assay results for fourteen samples tested on *Cryptococcus neoformans* and *Aspergillus niger*.

Sample number	Organism	
	C. neoformans	*A. niger*
SW1	2	-
SW2	1	-
SW3	3	-
SW4	3	10
SW5	3	-
N1	1	-
N2	3	-
N3	4	3
W2	1	-
W3	2	-
LTP1	2	12
LTP2	-	10
LTP3	3	-
F	4	-
Positive control	5	7
Negative control	No zone	No zone

(-) Indicates no apparent activity. Measurements are in millimeters, from the edge of the disk to the edge of the inhibition zone. Nystatin 100 *iu.* was used as a positive control for both micro-organisms.

Samples SW3, SW4 and SW5 showed moderate activity against *C. neoformans*. Sample

SW1 showed a slightly lower activity against *C. neoformans* compared to the others in its population, and sample SW2 showed the lowest activity against *C. neoformans*. Sample SW4 showed high activity against *A. niger* with an inhibition zone of 10 mm. None of the other samples in this population showed any activity against *A. niger*. Samples N1, N2 and N3 were all tested against the fungi, with sample N3 showing almost the same moderate activity against *C. neoformans* and *A. niger*. Sample N2 yielded a larger inhibition zone against *C. neoformans* compared to sample N1, but had no activity against *A. niger*. Sample W1 showed low activity against *C. neoformans* with sample W3 showing only marginally higher activity than sample W1. Both were inactive against *A. niger*. Sample W2 showed very low activity against *C. neoformans* and none against *A. niger*. Sample LTP2 was the only sample that showed no activity against *C. neoformans*. Samples LTP2 and LTP1 showed very high activity against *A. niger*, with sample LTP1 being the higher of the two and the highest of all the samples, with an inhibition zone of 12 mm. Sample LTP3 showed no activity against *A. niger* and minimal activity against *C. neoformans*. Sample F showed moderately high activity against *C. neoformans,* but no activity against *A. niger*.

Microdilution Assay

In the microdilution assay, six samples were evaluated for their activity against *C. albicans* (Figure 3.15.).

Table 3.10. Microdilution assay results for six samples tested on *Candida albicans.*

Rows of wells	Sample Number						Effective Concentration (mg/ml)
	SW3	SW4	W3	LTP1	N1	F	
A	-	-	-	-	-	-	32
B	-	-	-	-	-	-	16
C	+	+	+	+	+	+	8
D	+	+	+	+	+	+	4
E	+	+	+	+	+	+	2
F	+	+	+	+	+	+	1
G	+	+	+	+	+	+	0.5
H	+	+	+	+	+	+	0.25

(+) Indicates apparent growth in a well. (-) Indicates no apparent growth in a well.

The results remained constant after viewing at 30 minutes, 60 minutes, 120 minutes and at 24 hour intervals. The first two wells ie. A and B (represented by rows A and B in Table 3.10. above) for each sample showed no visible colour change. The effective concentration for well A was 32 mg/ml while well B was 16 mg/ml. From the definition mentioned earlier (Chapter 2), the minimum inhibitory concentration (MIC) was found to be 16 mg/ml. This shows that samples SW3, SW4, W3, LTP1, N1 and F, if diluted to a concentration of 8mg/ml or less, will be ineffective at inhibiting the growth of *C. albicans*.

Chapter 4 - Discussion

Figure 4.1. summarises the composition of major compounds in the sixteen samples of *Lippia javanica*. These bar graphs clearly depict the differences in content of the major compounds for all the populations. It is clearly evident that the plant exhibits qualitative and quantitative variations both within and between natural plant populations and that the plant exists in natural chemotypes.

Qualitative Cluster Analysis

For this cluster analysis (Figure 4.2.), data was scored for the presence or absence of compounds (binary data set). Therefore, if a sample presented with a set of compounds exactly like another in the dataset, the correlation co-efficient that links both would be 1; making them alike as far as presence or absence of compounds is concerned. If an arbitrary line A is drawn (Figure 4.2.), there is a split into three major clusters. A1, the first cluster, consists of 5 samples (SW1, SW3, SW4, N1 and N3), of which two samples bear the second highest resemblance to each other (SW4 and N1). This correlation is due to the limonene content in these five samples. Sample SW1 (A1,1), the outlying sample in this cluster least resembles the remainder of the cluster because of the absence of myrcenone. From the rest of the samples in this group, samples N1 and SW4 are closely clustered (A1,2) due to both having the same major compounds (limonene, piperitenone and β-caryophylline). Sample N3 falls into a branch of its own (A1,2,2) due to the absence of certain minor compounds e.g. α-pinene and β-pinene. Sample SW3 is also not included in the main cluster (A1,2,1,1) due to the presence of carvone oxide. Cluster A2 (the second cluster formed from line A) is a fairly large grouping and consists of the remainder of the samples (except for sample F) included in the dataset, that are clustered together due to the levels of myrcenone. Sample F falls into cluster A3 and bears the least resemblance to any other sample in the dataset and this is due to the presence of certain minor compounds e.g. camphene and cis- and trans-linalool oxide.

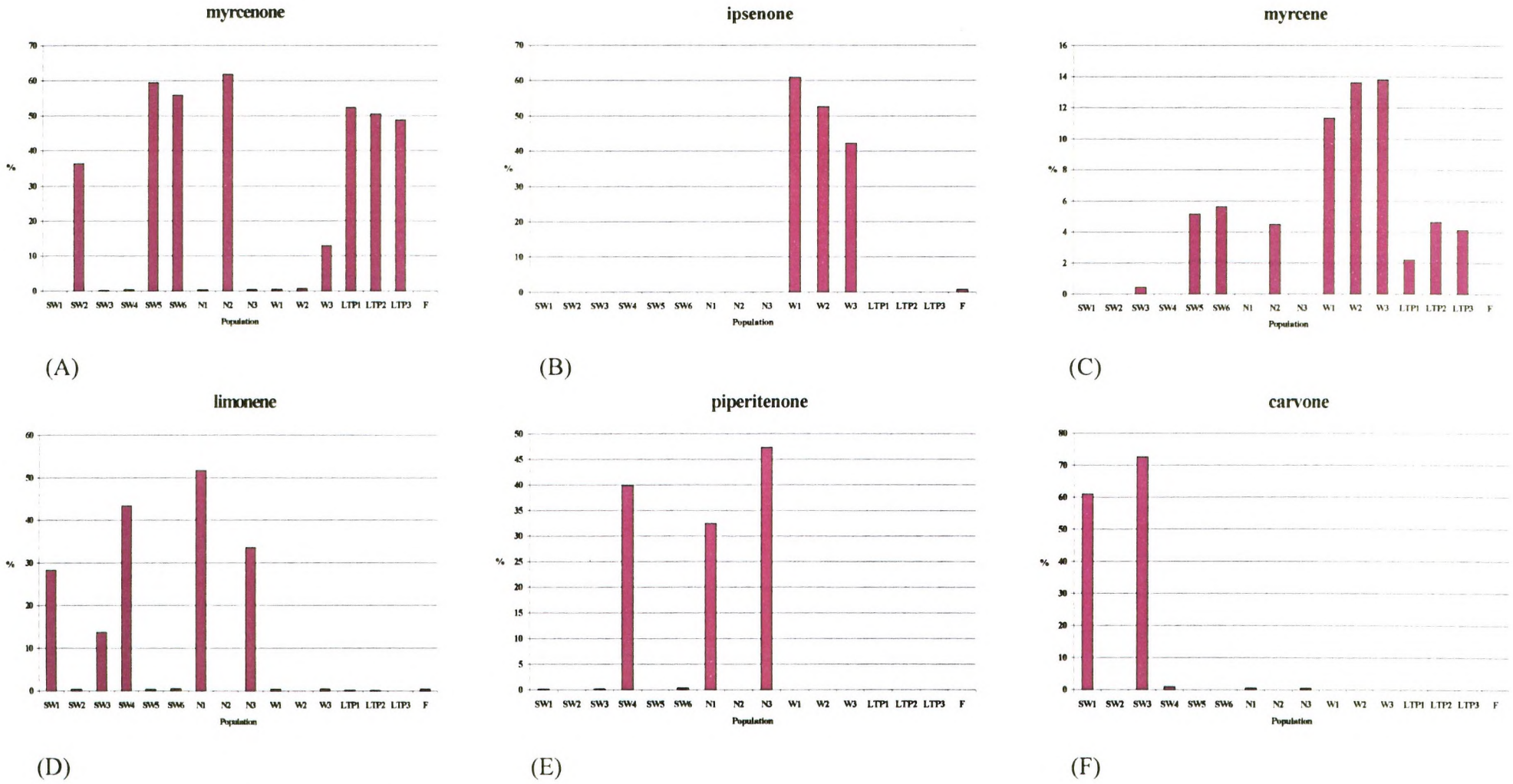


Figure 4.1. (A) – (F) Composition of myrcenone, ipsenone, myrcene, limonene, piperitenone and carvone in sixteen samples of *Lippia javanica*.

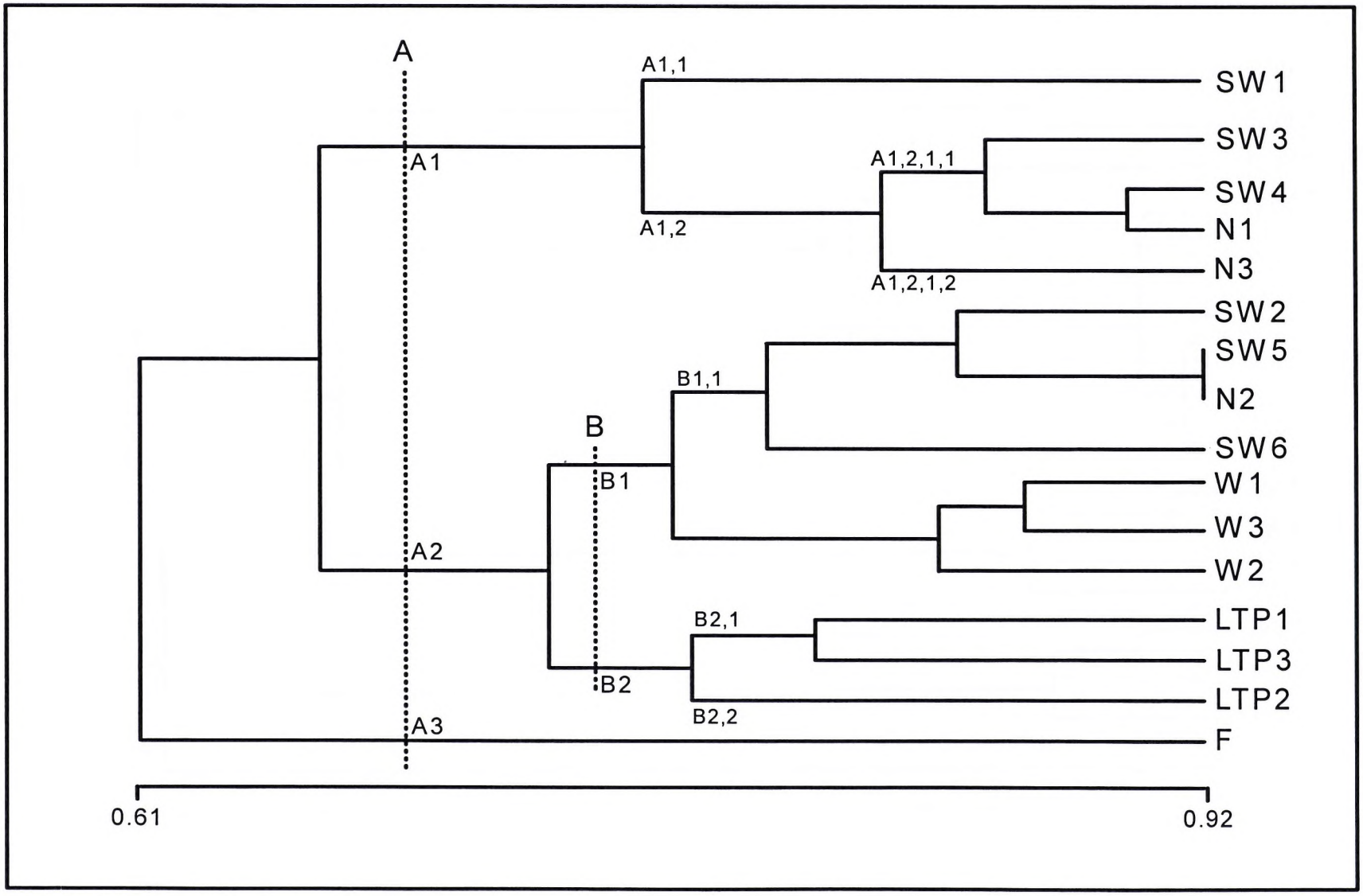


Figure 4.2. Qualitative cluster analysis performed on sixteen samples of *Lippia javanica*.

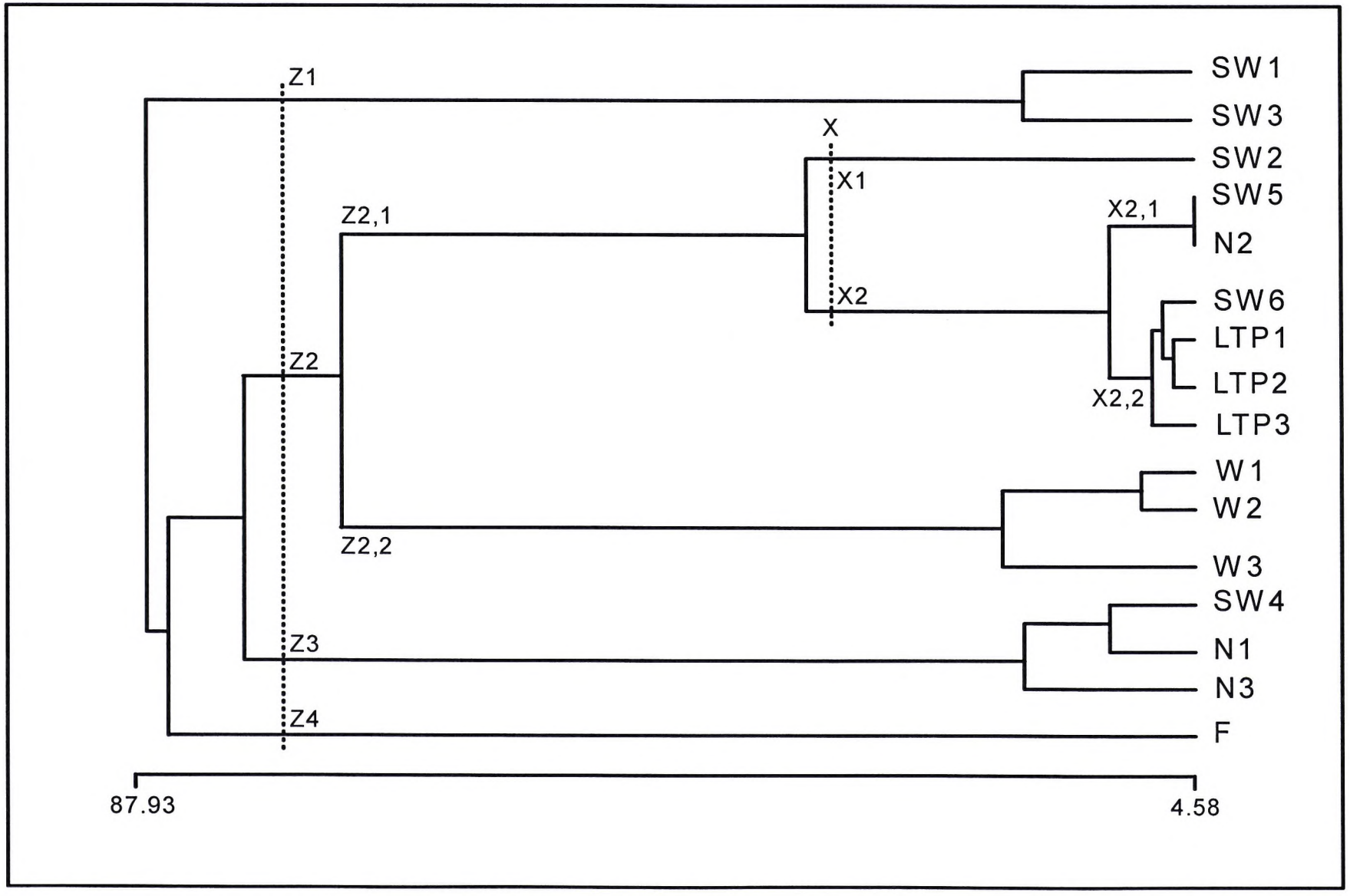


Figure 4.3. Quantitative cluster analysis performed on sixteen samples of *Lippia javanica*.

If another arbitrary line, B, is drawn, cluster A2 is further split into 2 groupings, B1 and B2. Cluster B2 (consisting of samples LTP1, LTP2 and LTP3) is correlated on the presence of α-copaene and β-bisabolene. Samples LTP1 and LTP3 are very alike, compared to sample LTP2, due to their presence of α-terpineol. (Z)- and (E)-β-ocimene is present in sample LTP2 (and not present in samples LTP1 and LTP3) and this separates it on its own branch (B2,2). Cluster B1,1 comprises SW2, SW5, N2 and SW6. The most outlying sample of these four is SW6, due to its content of piperitenone. From this group, samples SW5 and N2 have the highest correlation co-efficient in the complete dataset (0.92) and this is mainly due to them having the same major compounds (myrcenone, β-caryophylline, myrcene, cis-tagetenone and 1,8-cineole). Cluster B1,2 consists of samples W1, W2 and W3, with W1 and W3 being more alike than W2 (due to sample W2 not containing limonene).

Quantitative Cluster Analysis

In this analysis (Figure 4.3.) data was clustered on the basis of quantitative data or content. This means that if two samples have a very low co-efficient, they are very similar with respect to content. An arbitrary line Z results in four major groups, Z1, Z2, Z3 and Z4. Z1 is split off as the most outlying group due to SW1 and SW3 having limonene and the highest observed carvone content. Z4 consists only of sample F and is split off due to it having the highest observed linalool levels. Z3, the third group formed from line Z, consists of SW4, N1 and N3 and is formed due to the very high content of limonene and piperitenone. SW4 and N1 show a greater resemblance to each other than to N3 because they have a higher limonene than piperitenone content, while N3 has a higher piperitenone than limonene content. Z2, the last group formed from line Z, is further split into two groups i.e. Z2,1 and Z2,2. Z2,2 consists of W1, W2 and W3 and is split off due to the very high ipsenone content in these three samples. W1 and W2 show a closer resemblance to each other than to W3 and this is due to W3 having the lowest ipsenone content in this grouping. Z2,1 is split off Z2 because of higher myrcenone and β-caryophylline content and it consists of SW2, SW5, N2, SW6, LTP1, LTP2 and LTP3. If another arbitrary line, X, is drawn, it splits the original group Z2,1 into X1 and X2. X1 comprises sample SW2 only and this is due to it having a very low myrcenone content.

X2 is split into two further groups of X2,1 and X2,2. X2,1 consists of SW5 and N2 that have the lowest co-efficient in the whole dataset, which shows how much they resemble each other. This is mentioned previously in the qualitative cluster analysis. The grouping together of SW5 and N2 is due to the presence of the same major compounds (myrcenone, β-caryophylline, myrcene, *cis*-tagetenone and 1,8-cineole) in high proportions. This is similar to the previous observation where W1 and W2 are observed to resemble each other very closely. SW6, LTP1, LTP2 and LTP3 from group X2,2 resemble each other closely and this is seen from their co-efficients being very similar. LTP3 is the outlying sample and this is due to the low myrcenone content compared to samples SW6, LTP1 and LTP2.

For the *Lippia javanica* samples belonging to the Swaziland population (SW1, SW2, SW3, SW4, SW5 and SW6), it is clear that there is variation in the essential oil chemistry within the population. Samples SW1 and SW3 are of the same chemotype, which is not observed in the other samples in this population and consists of carvone and limonene as major compounds (Table 3.2.). Samples SW2, SW5 and SW6 constitute another chemotype which is rich in myrcenone (Table 3.2.). The outlying sample, SW4 shows a different profile that has no similarity to the other five samples and belongs to a type which is high in limonene and piperitenone as the major terpenoids. The qualitative and quantitative cluster analysis (Figure 4.2. and Figure 4.3.) both place samples SW2, SW5 and SW6 on the same branch, sample SW4 on a branch of its own and samples SW1 and SW3 in a separate cluster. This difference between individuals in a population is quite striking, but is common amongst plants that exhibit chemotypic activity for example in *Thymus vulgaris* as noted by Janssen *et al.* (1987). The chemical variability was also expressed in the antimicrobial activity for the samples from the Swaziland population. Samples SW1 and SW3 (both of the carvone and limonene chemotype) produced similar results in the disk diffusion assays, with respect to size of the inhibition zones and organisms inhibited (Table 3.7., Table 3.8. and Table 3.9.). They showed the highest zones of inhibition against *C. neoformans* and *B. subtilis* (2 mm or more). The myrcenone type, represented by samples SW2, SW5 and SW6, proved to be less active than the carvone and limonene type. The highest activity was reported against *B. subtilis* (SW2 and SW6 had inhibition zones of 2 mm each) and *C. neoformans* (SW5 had an

inhibition zone of 3 mm). Sample SW4 (producing limonene and piperitenone) had the highest inhibition zones against *S. aureus* (2 mm), *E. coli* (1.5 mm) and *A. niger* (10 mm). It was the only sample in the Swaziland population to show any activity against *A. niger*. In general, from the Swaziland population, three chemotypes were identified and the type that displayed the highest overall activity was the limonene and piperitenone type (SW4). Samples SW3 and SW4 showed an inhibition in the micro dilution assay equivalent to 16 mg/ml in *C. albicans* (Table 3.10.). The samples from this population showed higher activity against Gram positive than Gram negative organisms. These results are in agreement with previous studies, which concluded that essential oils in general are more active against Gram positive than Gram negative bacteria (Zohri *et al.* 1995). It was suspected that the essential oils were not able to enter the cell contents in order to have an effect, which pointed to a permeability issue. The physical nature of the Gram negative cell wall is a possible reason for the low permeability to hydrophobic molecules, because the porin channels in the cell wall only allow small quantities of hydrophobic molecules into the cell at a time. This gave the organism enough time to inactivate these small amounts rather than inactivate the saturated medium surrounding it (Nikaido and Vaara 1985).

The Nelspruit population is clearly divided into two chemotypes, which is also evident from the TLC plate (Figure 3.3.). The GC/MS data places samples N1 and N3 together as one type, with major compounds limonene and piperitenone, and this is the same as sample SW4 from the Swaziland population. Although sample N3 has a higher content of piperitenone than limonene (Table 3.3.), which is the opposite to samples N1 and SW4, it is still grouped together with these samples. This point is reinforced by the quantitative cluster analysis that places samples SW4, N1 and N3 on the same branch (Z3) (Figure 4.3.). Sample N2 belongs to the myrcenone chemotype identified in the Swaziland population (Table 3.2.), and it is this that makes it appear different on the TLC plate compared to samples N1 and N3 (Figure 3.3.). It is grouped with samples SW2, SW5 and SW6, in the qualitative cluster analysis (Figure 4.2.). The antimicrobial disk diffusion assay performed on these three samples from the Nelspruit population indicates a similar activity for samples N1 and N3 in the first assay (Table 3.7.). Sample N2 showed an overall lower activity here than in the disk diffusion assay that followed (Table 3.8.). In

the disk diffusion assay against *C. neoformans* and *A. niger* (Table 3.9.), the samples showed varying activity. Samples N1 and N2 only showed activity against *C. neoformans*, while N3 showed moderate activity against both *C. neoformans* and *A. niger*. N1 displayed the same activity as SW3 in the microdilution assay.

Contrary to what was observed in the preceding two populations, the Warmbaths population yielded very similar GC chromatograms and TLC profiles (Figure 3.3.). All three samples accumulate ipsenone and myrcene. The qualitative cluster analysis places all three samples together on branch B1,2 (Figure 4.2.) and quantitative cluster analysis also places them together on branch Z2,2 (Figure 4.3.). This coherence is due to high ipsenone content. The antimicrobial disk diffusion assays however, do not show all three samples behaving in a similar manner (Table 3.7, Table 3.8. and Table 3.9.). Samples W1 and W2 seem to be more alike due to the similar activity that they display in the first and second disk diffusion assays (Table 3.7. and Table 3.8.). Sample W3, as mentioned earlier, showed the same activity as the others that it was compared to in the microdilution assay (Table 3.10). From this it is clear that one has to be cautious in assuming that biological activity is restricted to the major chemical compounds only.

The Long Tom Pass population also produced samples that are alike, with all three producing the same profile on the TLC plate, very similar to that of samples SW2, SW5, SW6 and N2 (Figure 3.3.). The myrecenone content places these samples (Table 3.5.) into the chemotypes already identified in the Swaziland population (SW2, SW5 and SW6) and in the Nelspruit population (N2). The qualitative cluster analysis places LTP1, LTP2 and LTP3 together (on branch B2, Figure 4.2.). The quantitative cluster analysis places these three samples together with SW2, SW5, SW6 and N2 (on branch Z2,1 Figure 4.3.). In the initial disk diffusion assay (Table 3.7.), LTP1, LTP2 and LTP3 showed varying activity in similar organisms. Sample LTP2 showed minimal activity in the second disk diffusion assay (Table 3.8.). In the disk diffusion assay in Table 3.9, samples LTP1 and LTP2 showed high activity against *A. niger*, with sample LTP1 producing the largest zone of inhibition from all the populations. Sample LTP2 also produced a large inhibition zone here while sample LTP3 produced no activity. Sample LTP1 produced the same activity as the other samples tested in the microdilution assay (Table 3.10).

Sample F from the Fairland population proved to be the most divergent of the samples, due to its high linalool content. Qualitative and quantitative cluster analysis both place sample F on its own branch, separate from the other samples (branch A3 in Figure 4.2. and branch Z4 in Figure 4.3.). In the disk diffusion assays, it showed activity against *E. faecalis* where all other samples showed no activity (Table 3.7.). In the microdilution assay, it showed a minimum inhibitory concentration of 16mg/ml, the same as the other samples analysed here (Table 3.10.).

Published literature on *Lippia javanica* does make mention of variations in chemical composition. Most of the literature however has not focused solely on *Lippia javanica* and its occurrence as chemotypes. For example, certain studies have mentioned that *Lippia javanica* displays chemical variation but most of these studies have mentioned myrcenone as a major component (Mwangi *et al.* 1991, Velasco-Negueruela *et al.* 1993, Fujita 1965). Myrcenone as a major compound occurred in seven of the sixteen samples i.e. SW2, SW5, SW6, N2, LTP1, LTP2 and LTP3, which gives a 43.75% occurrence in the dataset which is quite a substantial number of samples that have a high myrcenone content. This high myrcenone content is represented by branch Z2,1 in the quantitative cluster analysis (Figure 4.3.). This is the biggest cluster indicating that this chemotype is the most abundant. Limonene is another compound that delineates the samples into another chemotype. This is evident in the qualitative cluster analysis (Figure 4.2.), where samples SW1, SW3, SW4, N1 and N3, are incorporated in branch A1. This high content of limonene was reported by Chagonda *et al.* (2000) in nine sites where wild *Lippia javanica* was harvested. However, in the quantitative cluster analysis, it is observed that this grouping of five samples, which incorporates three populations, is split. Samples SW1 and SW3 occur on a branch of their own, separate from the other samples because of the carvone content. Carvone was also detected in SW4, N1 and N3, but these quantities were very low, occurring in amounts of less than 1% (Table 3.2. and Table 3.3.). In contrast, these three above mentioned samples have high quantities of piperitenone occurring in a range of 32% to 48%. Piperitenone levels in samples SW1 and SW3 are less than 0.2%.

Thirteen samples were scattered across three distinct chemotypes. The first chemotype, a myrcenone rich chemotype, encompasses individuals from four populations with myrcenone composition ranging between 36% and 62%. The carvone rich type was only

observed in two samples from the same population (SW1 and SW3). The carvone type also had high amounts of limonene. Quantitative cluster analysis reinforces their grouping together and apart from the rest of the samples. The limonene rich chemotype occurred in two populations, once in SW4 and again in N1 and N3. This limonene chemotype also showed high amounts of piperitenone ranging from 32% to 48%. Chagonda *et al.* (2000) also found high amounts of limonene in *Lippia javanica* samples collected from three locations in Zimbabwe. The carvone chemotype, however was not mentioned, as this compound was apparently not present. As far as the literature is concerned, it does not seem as though a carvone chemotype has been previously identified in *Lippia javanica.* The same is true for the ipsenone chemotype. The ipsenone chemotype samples were W1, W2 and W3. These samples had varying ipsenone content ranging from 42% to 61%. This whole population group from Warmbaths fell into the ipsenone chemotype. There is no literature to substantiate the presence of *Lippia javanica* with high amounts of ipsenone. Sample F, however, can be classified as a chemotype because in the literature there is mention of high amounts of linalool in *Lippia javanica* samples analysed (Neidlein and Staehle 1974, Chagonda *et al.* 2000). Chagonda *et al.* (2000) found varying quantities of linalool in plants collected at approximately the same time of year. In three locations in that study, there was a difference of at least 30% between plants collected at the same time from the same location. Both cluster analyses place sample F on the 'outskirts' of the dataset, and in both cases it is the sample with the lowest correlation to any of the other samples.

The plant *Lippia javanica*, is used mainly in African traditional medicine to treat respiratory disorders, from coughs and colds to bronchitis. With such widespread use, it is not surprising that there could be therapeutic variability in efficacy. At present there are many essential oils being extracted from plants and sold commercially. For many of these oils, chemotypic variation has not been studied fully. *Lippia javanica* is not at a commercialisation stage yet, but should this occur, caution should be exercised in making sure that only the most favourable chemotypes are harvested.

Conclusion

Lippia javanica displays quantitative and qualitative variations both within and between natural plant populations. This variation seems to be random and is not correlated to the geographical distribution of the plant. Furthermore, from the natural plant populations assessed, five distinct chemotypes were noted, as summarised in Table 4.1. Chemotypic variability is an important factor in selecting favourable chemotypes for commercial development, especially in terms of chemical fingerprinting often required in quality control.

Table 4.1. Five different chemotypes showing their major compounds and the samples containing these major compounds.

	Compounds	Samples
Chemotype 1	carvone, limonene	SW1, SW3
Chemotype 2	myrcenone	SW2, SW5, SW6, N2, LTP1, LTP2, LTP3
Chemotype 3	limonene, piperitenone	SW4, N1, N3
Chemotype 4	ipsenone, myrcene	W1, W2, W3
Chemotype 5	linalool	F

The study also documented an ipsenone chemotype in *Lippia javanica* for the first time. The plant also displays moderate antimicrobial activity against respiratory pathogens, which justifies its wide use in African traditional medicine to treat coughs and symptoms associated with colds and flu. The highest activity was observed against the pathogens of fungal origin, which are commonly associated with opportunistic infections in immuno-compromised patients. For some samples the chemical content was correlated to the antimicrobial activity e.g. samples SW4, N1 and N3 displayed similar activity against *S. aureus, E. coli and C. tropicalis*. However, some plants having identical chemical profiles (especially the major compounds) displayed varying results in the antimicrobial assays.

Further antimicrobial studies performed on *Lippia javanica* (Burm.f.) Spreng should be cognisant of the occurrence of chemotypes in natural plant populations and the impact that this would have on the results of such studies.

Bibliography

Chagonda, L. S., Makanda, C. D., Chalchat, J. 2000. Essential oils of wild and cultivated *Lippia javanica* (Spreng) and *L. oatessi* (Rolfe) from Zimbabawe. J. Essent. Oil Res., 12, 1-6.

Compadre, C. M., Pezzuto, J. M., Kinghorn, A. D., Kamath, S. K. 1985. Hernandulcin: an intensely sweet compound discovered by review of ancient literature. Science 227, 417-419.

Costa, M., Di Stasi, L. C., Kirizawa, M., Mendacolli, S. L., Gomes, C., Trolin, G. 1989. Screening in mice of some medicinal plants used for analgesic purposes in the State of Sao Paolo. Part II. J. Ethnopharmacol. 27, 25-33.

Cowan, M. M. 1999. Plant products as antimicrobial agents. Clinical Microbiology Reviews, 12 (4), 564-582.

de Barros Viana, G. S., do Vale, T. G., Silva, C. M., de Abreu Matos, F. J. 2000. Anticonvulsant activity of essential oils and active principles from chemotypes of *Lippia alba* (Mill.) N.E. Brown. Biol. Pharm. Bull., 23 (11), 1314-1317.

Eloff, J. N. 1998. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Medica, 64, 711-713.

Essawi, T., Srour, M. 2000. Screening of some Palestinian medicinal plants for antibacterial activity. J. Ethnopharmacol., 70, 343-349.

Farnsworth, N. R. 1994. Ethnopharmacology and drug development. In Ciba Foundation Symposium 185, Wiley Chichester, 42-59.

Fujita, Y. 1965. Cubic system of classification of the genus *Lippia* (Verbenaceae) by the constituents of essential oils. Reports, Government Industrial Research Institute (Osaka), 306, 21-25.

Geissman, T. A. 1963. Flavonoid compounds, tannins, lignins and related compounds, 165. In Florkin, M., Stotz, E. H., (ed), Pyrrole pigments, isoprenoid compounds and phenolic plant constituents, vol. 9., Elsevier, New York.

Gelfand, M., Mavi, S., Drummond, R. B., Ndemera, S. B. 1985. The Traditional Medical Practitioner in Zimbabwe. Mambo Press, Gweru, Zimbabwe.

Govere, J., Durrheim, D. N., Du Toit, N., Hunt, R. H., Coetzee, M. 2000. Local plants as repellents against Anopheles arabiensis, in Mpumalanga Province, South Africa. Cent. Afr. J. Med., 46 (8), 213-216.

Hutchings, A. 1996. Zulu Medicinal Plants. Natal University Press, Pietermaritzburg.

Janssen, A. M., Scheffer, J. J. C., Baerheim Svendsen, A. 1987. Antimicrobial Activity of essential oils: 1976-1986 Literature Review. Aspects of the tests methods. Planta Medica, 5, 395-398.

Klayman, D. L. 1985. Qinghaosu (Artemisinin): An antimalarial drug from China. Science, 228, 1049.

Kleuger, P. A., Daros, M. R., Silva, R. M., Farias, M. R., De Lima, T. C. M. 1997. Neuropharmacological evaluation of crude and semipurified extracts from *Lippia alba* Will. N. E. Br. (Verbeneceae). Abstracts. International Joint Symposium. Chemistry, Biological and Pharmacological Properties of Medicinal Plants from the Americas. Poster Session, 2: B23.

Lawrence, B. M. 1984. The botanical and chemical aspects of oregano. Perf. & Flav., 9 (5),41-51.

Lopez, A. A. M., Rojas, H. N. M., Jiménez, M. C. A. 1979. Plant extracts with cytostatic properties growing in Cuba. Revista Cubana de Medicina Tropical, 31, 105-111.

Lukwa, N. 1994. Do traditional mosquito repellent plants work as mosquito larvicides? Cent. Afr. J. Med., 40 (11), 306-309.

Mabogo, D. E. N. 1990. The Ethnobotany of the Vhavenda. MSc Thesis. University of Pretoria.

Marsten, A., Hostettman, K. 1999. Biological and chemical evaluation of plant extracts and subsequent isolation strategy. Bioassay methods in Nat. Prod. Res. And Drug Development, Edited by Bohlin, L., Bruhn, J., Kluver Academic Publishing, Netherlands.

Mwangi, J. W., Addae-Mensah, I., Munavu, R. M., Lwande, W. 1991. Essential oils of Kenyan *Lippia* species. Part III. Flav. Fragr. J., 6, 221-224.

Mwangi, J. W., Addae-Mensah, I., Muriuki, G., Munavu, R. H., Lwande, W., Hassawnali, A. 1992. Essential oil of *Lippia* species in Kenya. IV. Maize weevil (*Sitophilus zeamais*) repellancy and larvicidal activity. Int. J. Pharmacogn., 30, 9-16.

Naranjo, P. 1995. The urgent need for the study of medicinal plants. In Ethnobotany: Evolution of a Discipline, edited by Schultes, R. E. and von Reis, S., Disocorides Press.

Neidlein, R., Staehle, R. 1974. Constituents of *Lippia javanica*. III. Dtsch. Apoth. Ztg., 40, 1588-1592 and 49, 1941-1943.

Neuwinger, H. D. 2000. African traditional medicine. A dictionary of plant use and applications. Medpharm GmbH Scientific Publishers, Stuttgart, Germany.

Nikaido, H., Vaara, M. 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev., 49, 1-32.

Pascual, M. E., Slowing, K., Carretero, M. E., Villar, A. 2001[a]. Antiulcerogenic activity of *Lippia alba* (Mill.) N. E. Brown (Verbenaceae). Farmaco., 56 (5-7) 501-504.

Pascual, M. E., Slowing, K., Carretero, E., Sánchez Mata, D., Villar, A. 2001[b]. *Lippia*: traditional uses, chemistry and pharmacology: a review. J. Ethnopharmacol., 76, 201-214.

Rates, S. M. K. 2001. Plants as source of drugs. Toxicon, 39, 603-613.

Rohlff, F. J. 1997. NTSYSpc-2.00. Department of Ecology and Evolution, State University of New York.

Smith, C. A. 1966. Common Names of South African Plants. Memoirs of the Botanical Survey of South Africa 35.

Terblanché, F. C., Kornelius, G. 1996. Essential oil constituents of the genus *Lippia* (Verbenaceae) – A literature review. J. Essent. Oil Res., 8, 471-485.

Uribe-Hernández, C. J., Hurtado-Ramos, J. B., Olmedo-Arcega, E. R., Martínez-Sosa, M. A. 1992. The essential oil of *Lippia graveolens* H. B. K. from Jalisco, Mexico. J. Essent. Oil Res., 4, 647-649.

Vale, T. G., Matos, F. J., de Lima, T. C., Viana, G. S. 1999. Behavioral effects of essential oils from *Lippia alba* (Mill.) N.E. Brown chemotypes. J. Ethnopharmacol., 67 (2),127-133.

Van Wyk, B.-E., van Oudshoorn, B., Gericke, N. 1997. Medicinal Plants of South Africa. Briza Publications, Pretoria, First Edition, 168-169.

Velasco-Negeureula, A., Pérez-Alonso, M. J., Guzmán, C. A., Zygadlo, J. A., Ariza-Espinar, L., Sanz, J., García-Vallejo, M. C. 1993. Volatile constituents of four *Lippia* species from Córdoba (Argentina). J. Essent. Oil Res., 5, 513-524.

Watt, J. M., Breyer-Brandwijk, M. G. 1962. The Medicinal and Poisonous Plants of Southern and Eastern Africa. Second Edition, Livingstone, London.

Zohri, A. N., Abdel-Gawad, K., Sabers, S. 1995. Antibacterial, antidermatophytic and antitoxigenic activities of onion. Microbiol. Res., 150, 167-172.

Appendix I

Poster presented at the 33rd International Symposium on Essential Oils, Lisbon (Portugal), September 2002.

ANTIMICROBIAL PROPERTIES AND GEOGRAPHICAL VARIATION IN ESSENTIAL OIL COMPOSITION OF FEVER TEA - *LIPPIA JAVANICA* (VERBENACEAE)

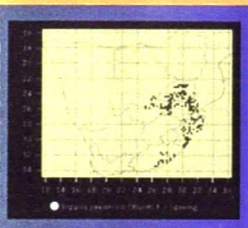
S Subramoney[1], SF van Vuuren[1], AM Viljoen[1], B Demirci[2], KHC Başer[2], A De Castro[3]

[1] Department of Pharmacy and Pharmacology, Faculty of Health Sciences, University of the Witwatersrand, 7 York road, Parktown 2193, South Africa

[2] Medicinal and Aromatic Plant and Drug Research Center (TBAM), Anadolu University, 26470-Eskişehir, Turkey

[3] De Castro and Brits Ecology Consultants, PO Box [illegible] Cresta 2118, JHohannesburg, South Africa

INTRODUCTION



Lippia javanica is distributed in Southern Africa and northwards into tropical Africa where it occurs as an erect woody shrub approximately two metres in height. Its hairy leaves have compound veins and are highly aromatic with a strong lemon smell. Dense rounded heads of small yellowish-white flowers appear during the flowering season (Van Wyk *et al.*, 1997).

The plant is used extensively in traditional medicine by both the layman and traditional healers to treat minor ailments. The leaves and stems are often used and in some cases the roots as well (Van Wyk *et al.*, 1997). Strong leaf infusions are made, which are commonly used as inhalants and also topically for scabies and lice (Van Wyk *et al.*, 1997; Gelfand *et al.*, 1985). More commonly, leaf and stem infusions are used as a tea to treat coughs, colds, fever and bronchitis (Watt and Breyer-Brandwijk, 1962; Hutchings, 1996). It has also been used for bronchial ailments and influenza (Hutchings A, 1996). Roots are used as antidotes for suspected food poisoning, bronchitis and sore eyes. *Lippia javanica* is commonly referred to as "fever tea" in English. This name is probably derived from its use as a weak infusion for fever symptoms. In Afrikaans it is referred to as "koorsbossie", which literally translated means "fever bush". In Zulu it is commonly referred to as "umsuzwane".

OBJECTIVES

- Record the essential oils profiles and identify the major compounds in the essential oil.
- Determine the chemo-geographical variation in essential oil composition.
- Document the scientific rationale for the traditional use of *Lippia javanica* by assessing the antimicrobial activity.

MATERIALS AND METHODS

Plant material was collected from three individual plants in four distinct natural plant populations:

- Balagane (Swaziland), hereafter abbreviated as **S1, S2 and S3**
- North of Nelspruit (Mpumalanga, South Africa), hereafter abbreviated as **N1, N2 and N3**
- South of Warmbaths (Limpopo Province, South Africa), hereafter abbreviated as **W1, W2 and W3**
- Long Tom Pass (Mpumalanga Province, South Africa), abbreviated as **L1, L2 and L3**

Plant material was distilled for three hours by hydro-distillation using a Clevenger apparatus

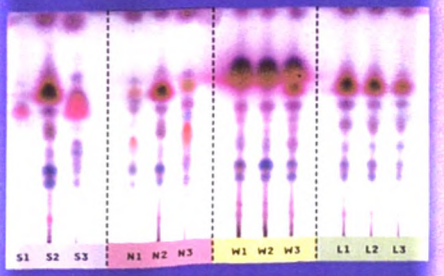
The essential oil was analysed by GC-MS operating under the following conditions: Column: HP-Innowax (60 m x 0.25 mm id., 0.25 µm film thickness), Temperatures: injection port 250°C, column 60 °C for 10 min., 4°C / min. to 220°C, 220°C for 10 min., 1°C / min. to 240°C (total = 80 min.).

Antimicrobial activity: Time kill studies were used to demonstrate the rate at which the pathogens are killed over a 24 hr period. Three respiratory pathogens were selected; *Klepsiella pneumonia* (NCTC 9633), *Cryptococcus neoformans* (ATCC 90112) and *Bacillus cereus* (ATCC 11778). The inactivation broth death kinetic method as described by Christoph & Stahl-Biskup (2000) was used. Cultures were grown in Tryptone Soya (*Oxoid*) broth and centrifuged for 10 min at 5000 rpm. The supernatant was discarded and the pellets resuspended in 10 mL of a 0.9 % NaCl solution. Oil concentrations of 0.25, 0.5, 0.75 and 1% were incorporated into 50 ml Tryptone Soya (Oxoid) broth with 0.5 % tween and a final inoculum of approximately 1 X 10^5 cfu / mL. The different concentrations were incubated at 37°C in a shaking water bath. At time intervals ranging from 0 min - 24 hrs, aliquots of 1 mL were transferred to 9 mL inactivation broth consisting of 0.1 % peptone (Oxoid), 5% lecithin (Merck) and 5% yeast extract (Oxoid). Five serial dilutions were performed in 0.9 % NaCl solution. From the inactivation broth, 100 µl was plated onto Tryptone Soya (Oxoid) agar for each oil concentration. The plates were incubated at 37 °C for 48 hr and colony forming units (CFU's) counted and death kinetics expressed in log10 reduction time-kill plots. Controls were included in the study having the same broth formulation but without the oil. The assay was performed in duplicate.

A - SEM photograph of the abaxial leaf surface of *Lippia javanica* showing cross-sections of glands containing the essential oil.
B - *Lippia javanica* (Verbenaceae).
C & D - Examples of commercial products made from *Lippia javanica*

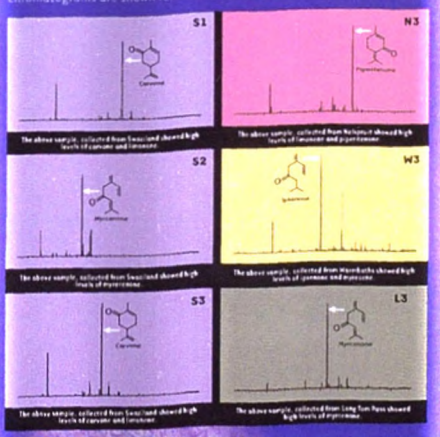
RESULTS

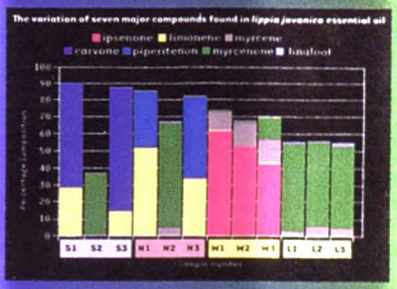
From the four populations analyzed by thin layer chromatography, it is apparent that *Lippia javanica* displays within and between population variations.



GC-MS data

GC-MS data indicates quantitative and qualitative variation within and between plant populations. The first three chromatograms are from three individual plants collected in Swaziland (S1 – S3). Representative chromatograms are shown for each of the other three populations.

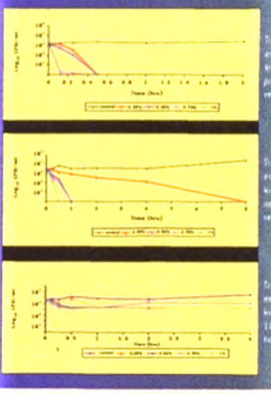




The chemical variation in the individual plants taken from four natural plant populations is shown for 7 essential oil compounds above. It is remarkable that carvone contributes 60% of the total essential oil composition in S1 and S3 but is virtually absent in all other plants analyzed. Ipsenone is characteristic for the Warmbaths population while piperitenone was only detected in two plants from the Nelspruit population.

ANTIMICROBIAL ACTIVITY

Antimicrobial assays were performed on the sixteen oil samples of *Lippia javanica*, with oil samples being tested against five gram positive bacteria, three gram negative bacteria and four fungi. Moderate inhibition was found with most species at varying localities. As *Lippia javanica* has been used traditionally to treat various respiratory ailments, which may be bacterial or fungal, assays were performed using respiratory pathogens. Comparison of the time-kill plots for the three organisms studied showed that the killing rate was the greatest for *Klebsiella pneumonia*, then *Cryptococcus neoformans* and very little reduction of microbial populations for *Bacillus cereus*.



Time kill plot on *Lippia javanica* essential oil showing death kinetics of *Klebsiella pneumonia* (NCTC 9633) represented over two hours.

Time kill plot on *Lippia javanica* essential oil showing death kinetics of *Cryptococcus neoformans* (ATCC 90112) represented over eight hours.

Time kill plot on *Lippia javanica* essential oil showing death kinetics of *Bacillus cereus* (ATCC 11778) represented over four hours.

CONCLUSIONS

- *Lippia javanica* displays quantitative and qualitative variation both within and between natural plant populations. The variation seems to be random and is not correlated geographical distribution.
- Various chemotypes of *Lippia javanica* can be identified which is an important factor in selecting favourable chemotypes for commercial development.
- *Lippia javanica* is used in African traditional medicine to treat respiratory disorders. The essential oil exhibits rapid cidal activity on *Klebsiella pneumonia* and *Cryptococcus neoformans*.

BIBLIOGRAPHY

- Christopher F., Kaulfers PM & Stahl-Biskup E. 2000. A comparitive study of the invitro Antimicrobial activity of Tea Tree oils s.l. with special reference to the activity of Trixetones; Planta Med; 66; 556-560.
- Gelfand, M., Mavi, S., Drummond, R.B., Ndemera, S.B. 1985. The Traditional Medicinal Practitioner in Zimbabwe. Mamba Press, Gweru, Zimbabwe.
- Hutchings A., and van Staden J. 1994. Plants used for stress-related ailments in traditional Zulu, Xhosa and Sotho medicine. Part 1: Plants used for headaches; Journal of Ethnopharmacology; 43; 89-124.
- Van Wyk B., van Oudtshoorn B. & Gericke N. 1997. Medicinal plants of South Africa; Briza publications, South Africa.
- Watt, J.M. & Breyer-Brandwijk, M.G. 1962. The Medicinal and Poisonous Plants of Southern Africa. 2nd Edition, Livingstone, London.