THE ANTIMICROBIAL ACTIVITY AND PHYTOCHEMISTRY OF AFRICAN WORMWOOD (*ARTEMISIA AFRA*)

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

Johannesburg, 2003

Declaration

I, Lehlohonolo Tebogo Gwebu declare that this research report is my own work. It is being submitted for the partial fulfillment of the requirements for the degree of Masters of Science in Medicine (Pharmaceutical Affairs) at the University of the Witwatersrand, Johannesburg. It has not being submitted before for any degree or examination at this or any other University.

13 day of JUNE, 2003

Dedication

This research report is dedicated to my husband Andrew Bhekumuzi Gwebu who has always had an interest in herbal products.

Abstract

Artemisia afra Jacq. Wild also known as African wormwood, "umhlonyane" (Xhosa and Zulu), "lengana" (Sotho and Tswana) and "wildeals" (Afrikaans) is an aromatic shrub belonging to the Asteraceace. It is widespread in South Africa extending from the mountainous regions of the South Western Cape, along the eastern coast to the Northern Province (van Wyk *et al.*, 1997). Due to the popular use of *A. afra,* herbal tinctures have been prepared for commercial distribution. As the chemotypic variation remains unrecorded it has been impossible to standardize extracts containing *A. afra*. The aerial parts of sixteen samples from four natural populations were hydrodistilled and the essential oil analysed by GC-MS and tested for antimicrobial activity on a number of bacteria and fungi. The essential oil composition varied quantitatively and qualitatively within and between natural populations. With the aid of cluster analysis, several chemotypes have been identified based on the presence and quantity of the following compounds; α-thujone (5.55-77.65%), β-thujone (1.37-57.73%), camphor (1.00-48.99%), 1.8-cineole (2.31-50.09%), artemisia ketone (14.47-27.97%), artemisia alcohol (9.31-27.76%) and santolinyl acetate (4.14-24.32%). The variation did not correlate to geographical distribution. The essential oil of all the plants was active against all organisms studied except *Pseudomonas aeruginosa* and *Enterococcus facaelis*. The oil exhibited higher antifungal properties than antibacterial activity. The pure terpene standards of major compounds detected in *A. afra* (thujone, cineole, camphor etc.) showed no antimicrobial activity and it can be assumed that the oil could exert antimicrobial properties by working in a synergistic way. *Artemisia afra* varies within and between natural populations and standardizing of commercial products will be problematic without cloning of a favourable chemotype. Selection of favourable chemotypes should be based on efficacy (i.e. wide spectrum anti-microbial activity) and safety index (e.g. low thujone content).

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I would like to give a big thank you to my supervisor, Dr Alvaro Viljoen, for the quality of supervision, dedication, commitment and support during this research. It was real a pleasure to work with a person of his caliber.

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A heart-felt thank you to the TBAM staff in Turkey, especially Prof. Başer for collaborating on this project. To Betül and Fatih, thank you for their warm welcome and for being helpful during my stay in Turkey. Thank you to all staff members of the Department of Pharmacy and Pharmacology (Witwatersrand) for your help and support. Thank you to my colleague Noku for her moral support and prayers.

Lastly, I would like to thank my parents, my brother and sister for their continuous support and to my husband for being understanding and supportive even when I had to leave him for two weeks as part of research.

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Abbreviations

DD	Disc Diffusion assay
1A	Setibeng plant A
1B	Setibeng plant B
1C	Setibeng plant C
2A	Giant's Castle plant A
2B	Giant's Castle plant B
2C	Giant's Castle plant C
3A	Klipriversberg plant A
3B	Klipriversberg plant B
3C	Klipriversberg plant C
3D	Klipriversberg plant D
3E	Klipriversberg plant E
3F	Klipriversberg plant F
3G	Klipriversberg plant G
4A	Qwa-qwa plant A
4B	Qwa-qwa plant B
4C	Qwa-qwa plant C
TLC	Thin Layer Chromatography
GC	Gas Chromatography
GC\MS	Gas Chromatography Mass Spectrometry
Kp	*Klebsiella pneumoniae*
Ec	*Escherichia coli*
St	*Salmonella typhi*
Sa	*Staphylococcus aureus*

Preface

It is estimated that there are 250 000 to 500 000 species of plants on earth with most of them are used for medicinal purposes (Borris, 1996). The use of plants as antimicrobials dates back to ancient times, but the interest to use plant derivatives as antimicrobials was lost during the advent of antibiotics in 1950 and people moved from over prescription of toxic drugs to "unconventional" therapy.

However, recently plants have provided a source of inspiration for novel drug compounds, as plant derived medicine has made a large contribution to human well-being and health. Their role in developing new drugs is; they could become the base for the development, a natural blueprint for development of new drugs or a phytomedicine to be used for the treatment of a disease (e.g. artemisinin from *Artemisia annua* for malaria).

African wormwood (*Artemisia afra*) is one of the most popular medicinal plants in South Africa and it has been extensively used in traditional herbal preparations. Extracts of *A. afra* are used to treat coughs, colds, fever, flu, stomach discomforts, pneumonia, malaria etc. (Watt & Breyer-Brandwijk, 1962). The main constituents of African wormwood essential oil are α-thujone, β-thujone, camphor, 1.8-cineole and borneol. According to Graven *et al.* (1992) the oil has shown variation in chemical composition, but specific GC/MS data remains undocumented.

African wormwood essential oil is said to be similar to European wormwood (*A. absinthium*) because of the presence of thujone. Toxic and hallucinogenic effects has been associated with overdose or continued use over long periods of thujone. Therefore, a high content of thujone needs to be avoided for the sake of the consumer safety. Thus commercial preparations need to be standardized.

Therefore we set out to investigate the chemical composition of *A. afra* essential oil samples within and between natural populations to establish if there is any chemotypic variation and also to evaluate antimicrobial activity as it is used as a phytomedicine to treat bacterial and fungal infections.

CHAPTER 1: INTRODUCTION

The genus *Artemisia*:

Artemisia is a fairly large genus within the Asteraceace family housing approximately 350 species (Tan *et al.*, 1998). *Artemisia* species are widespread in nature and are predominately distributed in the northern temperate hemisphere. There is one indigenous (*Artemisia afra*) and one localized (*Artemisia vulgaris*) species in Southern Africa. Most of the *Artemisia* species yield essential oils (Pappas & Sheppard-Hanger, 1996). Members of the genus have been frequently used to treat diseases such as malaria, diabetes, hepatitis and infections caused by bacteria, fungi and viruses.

Botanical description of *Artemisia afra:*

Artemisia afra is also known as African wormwood, "umhlonyane" (Xhosa, Zulu), "lengana" (Sotho, Tswana) and "wildeals" (Afrikaans). It is a multi-stemmed perennial shrub, growing up to two meters in height (Figure 1). It is characterized by glaucous feather like leaves with a pungent aromatic smell. The leaves are six centimetres long, alternately arranged and oval in shape. The inconspicuous flowers are pale yellow and borne on branch ends. In colder area, the branches die back in winter and regenerate from the base. *Artemisia afra* is easily disseminated from the seeds, cuttings and rooted pieces and it grows well in the wild and in cultivation (Graven *et al.*, 1992).

Habitat and distribution:

Artemisia afra is widespread in the southern tropical eastern area of Africa (e.g. Kenya, Zimbabwe, Tanzania, and Angola etc.). The northern limit of the distribution range is in Ethiopia. In South Africa, the plant is abundantly distributed in mountainous regions of the South Western Cape, common along the eastern coast and extends through to the Northern Province (Figure 2). *Artemisia afra* is clump-forming common in highland areas at the altitude ranging between 1500 and 3000 m. The soils range from volcanic ash, loamy sands, to sandy or calcareous clay loams of volcanic or granite origin.

Figure 1: *Artemisia afra* in habitat.

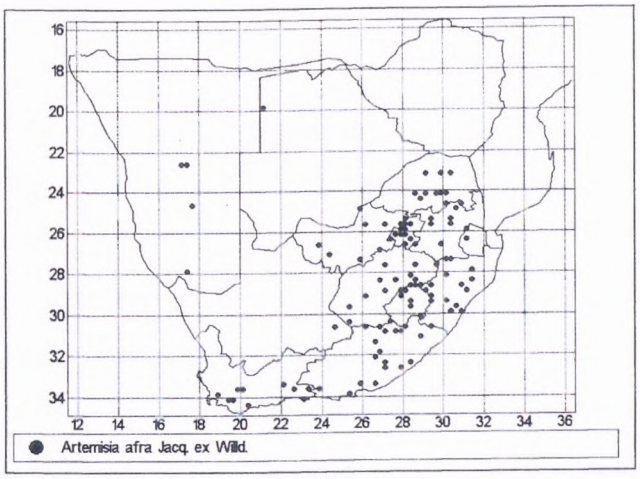


Figure 2: Distribution map of *A. afra* in southern Africa.

Figure 3: An example of a commercial product of 'lengana' incense used in inhalation therapy.

Figure 4: *Artemisia afra* tincture used for mild fever and stomach discomfort.

Medicinal use of *Artemisia afra*:

African wormwood is one of the most commonly used medicinal plants in South Africa and the major ethnomedicinal uses of *A. afra* are summarized in Table 1. Although the leaves are mostly used, other plant parts such as the roots are also used as ingredients in herbal preparations. The dry and fresh leaves together with the young stems are used to prepare infusions, decoctions, molasses and tinctures. The infusions are often made syrupy by the addition of sugar or honey to mask the bitter taste. *Artemisia afra* preparations are primarily used for colds, coughs, influenza, as it is said to clear the respiratory and bronchial passages (Watt & Breyer-Brandwijk, 1962). Graven *et al.* (1992) reported that *Klebsiella pneumoniae* and *Staphylococcus aureus*, which may cause the above conditions, were inhibited by *A. afra.* (No MIC data or an attempt at correlating observed activity to essential oil composition was made in that study).

Watt and Breyer-Brandwijk (1962) reports the use *A. afra* as a tea for fever and influenza or as an enema for febrile complaints. The leaves may be warmed and used as a dressing (poultice) for an inflamed throat and fever in children (Bally, 1937). The leaves may also be heated and the vapour inhaled for colds and flu (Jacobs & Bath, 1995). The vapour is also used to steam the throat of scarlet fever. The genitalia are steamed with vapour for menstrual chills and also after childbirth, while leaf decoctions have been administered for extended labour (Gelfand *et al.*, 1985).

Leaf infusions may also be held at the mouth to ease the pain of gumboils and to hasten their bursting, and it is also used as a gargle and mouthwash (Von Koenen, 1996). In the South Western Cape the leaf infusion is used as eye lotion and also drunk for acne, pimples, skin ulcers and it is believed to act as a blood purifier (Watt & Breyer-Brandwijk, 1962). It is interesting to note that one of the ethnopharmaceutical properties claimed for *A. afra* is in the treatment of skin conditions (acne, pimples) related to bacterial infections. The leaf poultice (usually moistened with brandy) may be applied locally to relieve neuralgia and swelling in mumps, or to treat pimples, ulcers and colic in children (Watt & Breyer-Brandwijk, 1962).

Plant material is often pulverized together with milk and used in enemas to treat constipation and intestinal worms in children (Hutchings *et al.*, 1996). A brandy tincture is taken orally for colic while plant ash has been taken as an expectorant (Lindsay, 1978).

Most of the stomach upsets are caused by *Escherichia coli* and food spoiling microorganisms such as *Aspergillus niger*, *Salmonella typhi* etc. According to Gundidza (1993), *A. afra* showed significant anti-fungal activity against *Alternaria alternata*, *Aspergillus niger* and *Penicillium citrum*.

Watt and Breyer-Brandwijk (1962) reports the inhalation of leaf decoctions for blocked nose, headache, or alternatively the tip of a fresh plant is inserted into the nose to treat the same conditions. Figure 3 displays an example of commercial product of 'lengana' incense used in inhalation therapy. Fresh twigs may also be inserted into a hollow tooth to relieve toothache.

Previous work on *A. afra*:

The aerial part of *A. afra* is rich in essential oils. Essential oils are aromatic plant products used in food industry, perfumery, cosmetics and pharmaceutics (Jain, 1985). They are secreted from oil cells in secretion ducts or stored in glandular hairs. Essential oils are complex mixtures of terpenes. Terpenes are chemical compounds with an isoprene unit structure and they can occur as monoterpenes, sesquiterpenes and diterpenes.

Previous studies showed that the main chemical constituents found in the essential oil of *A. afra* are α-thujone, β-thujone, camphor, borneol and 1.8-cineole (van Wyk *et al.*, 1997). Chagonda *et al.*, (1999) reported that major components of *A. afra* oils from different countries exhibited chemical composition variation. For instance the major component reported from Ethiopian oils was artemisyl acetate (24.4-32.1%), 1.8-cineole (63.37%) in the Kenyan oil and α- and β-thujone (52%) in the Zimbabwean oil and α-thujone (52.5-54.2%) in South African oil.

Changonda *et al.*, (1999) reported 1.8-cineole as the major component in cultivated South African oil. This gave rise to a study where wild and two organically cultivated *A. afra* oils from Zimbabwe were compared on the basis of their chemical components.

Table 1: A summary of ethnomedicinal uses of *Artemisia afra*.

PLANT PART USED	TREATMENT	PREPARATION	REFERENCES
Leaf	Blocked nose	Inhale decoction, Infusion	van Wyk *et al.*, (1997)
		Tea	van Wyk *et al.*, (1997)
Leaf	Colds	Decoction	Jacobs *et al*,. (1995)
Roots		Infusion	Watt & Breyer-Brandwijk, (1962)
Leaf	Coughs, bronchitis, whooping coughs	Decoction / bath	Watt & Breyer-Brandwijk, (1962) Hutchings *et al*,.(1996)
Leaf	Flu	Infusion	Jacobs *et al.*, (1995)
		Decoction	Watt & Breyer-Brandwijk, (1962)
		Ash Decoction	Watt & Breyer-Brandwijk, (1962)
Leaf	Fever		Neuwinger, (2000)
Root			Hutchings *et al.*, (1996)
Plant			Gelfand *et al.*, (1985)
Leaf	Colic, digestive disorder		van Wyk *et al.*, (1997)
Leaf	Acne, pimples, skin ulcers		Von Koenen, (1996)
Plant	Malaria		Schlange *et al.*, (2000)
	Diabetes		Lindsay, (1978)
	Expectorant		Watt & Breyer-Brandwijk, (1962)
Leaf	Toothache, mouthwash, gumboils		Von Koenen, (1996)

The results showed that the major components of plants in the wild were artemisia ketone (6.3-41.9%), 1.8-cineole (0.1-27.9%), α-copaene/camphor (8.5-27.1%) and santolina alcohol (3.1-10.1 %). The analysis of the oils of cultivated *A. afra* reflected the presence of two chemotypes: one dominated by artemisia ketone (32.1-34.8%), α-copaene/camphor (21.8-24.4%), 1.8-cineole (10.9-16.9%) and one cultivated in Harare, by 1.8-cineole (23.5-28.7%), α-copaene /camphor (20.2-21.3%) and borneol (14.2-17.0%).

According to Janssen *et al.* (1987), the same species can differ in essential oil composition and this impact on selecting favourable chemotypes for commercial development and to identify superior clones from a chemical perspective. To support the above report, *A. afra* collected from Fort Hare (in the Eastern Cape) had higher α-thujone (78.68%), β-thujone (13.13%) and lower 1.8-cineole (8.19%) content than that reported by Graven *et al.* (1992) and the oil did not contain any camphor (Mangena *et al.,* 1999).

Artemisia afra is pungently aromatic due to the accumulation of essential oils. The constituents of essential oils (mono- and sesquiterpenes) are known to be effective antimicrobials (Tan *et al.*, 1998). Traditionally, *A. afra* is administered by inhalation (usually after heating of the plant material). Hence, we have been prompted to investigate the antimicrobial activity of the essential oil (volatile) compounds.

A preliminary study indicated that the essential oil of *A. afra* has some antimicrobial and antioxidative properties (Graven *et al.*, 1986). The oil has been reported to be active against a broad spectrum of microorganisms. Mangena *et al.* (1999) reported the activity of African wormwood oil towards *B. subtilis*, *E. coli*, *K. pneumoniae*, and *S. aureus*. The oil showed significant activity against *C. albicans*, *A. alternata* and *A. niger* (Gundidza, 1993). According to Graven's report (1986) the oil has a value as a biological agent with greater antimycotic than antibacterial activity.

Pharmacological studies

Natural products are good but not everything that is green is safe and good. Although the *A. afra* oil had antimicrobial and antioxidative properties it exhibits some toxicity. The volatile oil has been shown to be an abortifacient when administered *per os* to rabbits. The extracts are known to produce haemorrhage nephritis and pulmonary oedema (Watt

& Breyer-Brandwijk, 1962). A worker who swallowed a tablespoon of essential oil suffered severe poisoning with confusion and other symptoms reported to be caused by thujone.

Study objectives:

- To produce a chemical profile (GC/MS data) for various clones of *A. afra*.
- To determine the antimicrobial activity of the essential oils and thus providing scientific basis for the traditional uses.
- To determine the correlation between chemical composition and antimicrobial activity.
- To emphasize the need to standardize favourable clones for phytomedicinal purposes (efficacy and safety).

CHAPTER 2: MATERIALS AND METHODS

Phytochemistry

Fieldwork:

A total of sixteen samples of *A. afra* were collected from four natural populations. The sample populations were Setibeng in Lesotho, Giant's Castle in Kwa-Zulu Natal Province, Phuthaditshaba (Qwa-qwa) in Free State Province and Klipriversberg Nature Reserve in Gauteng Province (Table 2). Three individual plants were selected in each population except from Klipriversberg, where seven plants were collected. The leaves of the plant were harvested near the base (Figure 5).

Table 2: A selection of plants from four natural populations and corresponding abbreviations.

Plant population							
Setibeng (Lesotho)	1A	1B	1C				
Giant's Castle (KwaZulu-Natal)	2A	2B	2C				
Klipriversberg (Gauteng)	3A	3B	3C	3D	3E	3F	3G
Qwa-qwa (Free State)	4A	4B	4C				

Isolation of essential oils:

The essential oils were obtained from the aerial parts through hydro-distillation in a Clevenger apparatus (Figure 6) for $3^1/_2$ hours. The temperature of the apparatus was kept constant (104 °C). The oils were dried with anhydrous sodium sulphate (Saarchem). The pure oils were collected in amber vials to protect them from the negative effect of light. The tightly closed amber vials were stored at 4 °C to prevent the oils from resinifying.

Thin layer chromatography:

Once the essential oils were extracted from the plant, they were analysed by thin layer chromatography (TLC). This technique is usually used for separation and it is the simplest and cheapest method for screening a large number of samples. Essential oil was diluted (1:7) with hexane (Rochelle Chemicals) and *ca.* 2 µl was spotted on a silica gel (Merck) TLC plate using a calibrated micro-capillary tube and developed in mobile phase system of toluene:ethyl acetate (93:7) for 10- 20 minutes. The plates were visualized

under UV light (365 and 254 nm) and sprayed with vanillin sulphuric acid (1 % ethanolic vanillin, 10 % ethanolic sulphuric acid) spray reagent. The plates were heated in the oven at 110 °C for 10 minutes.

Gas chromatography (GC):

Gas chromatography is used to separate volatile liquid in the gaseous state. Samples were diluted 2:8 with hexane and 1 µl was injected into the GC system which operated as follows; Shimadzu 17A gas chromatograph; column: J&W-DB1 (60m x 0.25 mm id., 0.25 µm film thickness), temperatures: injection port 250 °C, column 60 °C and flame ionization detector (FID) 260 °C.

Gas chromatography \ mass spectrometry (GC/MS):

Gas Chromatography coupled with mass spectrometry was completed by myself at The Medicinal and Aromatic Plant and Drug Research Center (TBAM), Anadolu University, Turkey (Figure 7).

The oil was analysed by GC/MS using a Hewlett-Packard GCD system. Innowax FSC column (60 m x 0.25 mm) was used with helium as the carrier gas. GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min. Split flow was adjusted to 50mL/min. The injector and detector temperatures were at 250°C. MS were taken at 70 eV. Mass range was from m/z 35 to 425. Relative percentage amounts of the separated compounds were calculated automatically from peak areas of the total ion chromatogram. Library search was carried out using Wiley and Adams GC/MS Library and TBAM library of essential oil constituents.

Figure 5: Harvesting African wormwood from Klipriversberg.

Figure 6: Hydrodistillation of plant material using the Clevenger apparatus.

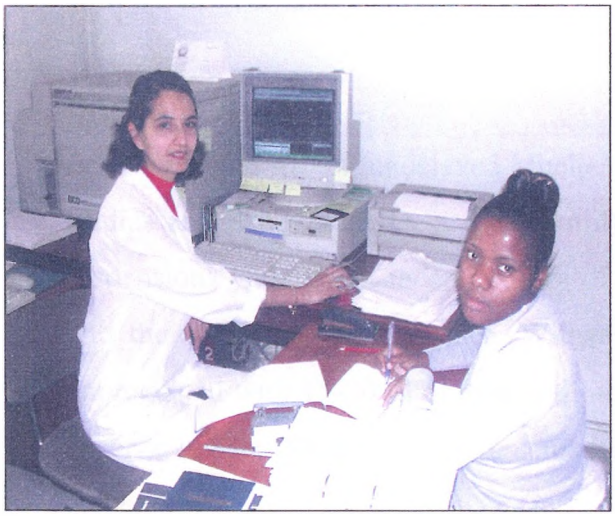


Figure 7: Analyzing the essential oil using GC/MS.

Figure 8: Paper discs saturated with essential oil for the antimicrobial assay by disc diffusion.

Antimicrobial assays

Test organisms:

Antimicrobial disc diffusion assays were performed using the hydro-distilled essential oils. Ten reference bacterial strains; *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATTC 15244), *Salmonella typhimurium* (ATCC 14028), *Staphylococcus epidermidis* (ATTC 2223), *Enterococcus faecalis* (ATCC 29212), *Bacillus cereus* (ATCC 11778), *Klebsiella pneumoniae* (NCTC 9633) and *Serratia odorifera* (ATCC 33132) were tested. Four fungal strains; *Candida albicans* (ATCC 10231), *Cryptococcus neoformans* (ATTC 90112), *Aspergillus niger* (clinical strain) and *Alternaria alternata* (clinical strain) were tested.

Preparation of cultures:

The bacterial and fungal cultures were harvested from stock agar plates. The bacterial cultures were inoculated in Mueller Hinton (Oxoid) broth, whilst the fungal cultures were harvested from Sabourauds Dextrose (Oxoid) agar. Compared the turbidity of cultures with 0.5 Mc Farlands standard.

Disc diffusion assay:

Base layers (15 ml) of autoclaved Mueller Hinton (Oxoid) agar was prepared for bacterial studies and Sabourauds Dextrose (Oxoid) agar was prepared for fungal cultures. Spore suspensions yielding an inoculum size of approximately 1 $X10^9$ were thoroughly mixed into the overlaying agar surface. With aseptic manipulation, sterile 6 mm discs were impregnated with 15-20 µl of essential oils. The discs were placed onto the set agar with a sterile needle (Figure 8). The essential oils were left to prediffuse in the fridge (4°C) before incubation.

Neomycin discs (30 µg, Oxoid) were used for positive bacterial controls. Nystatin discs (100 iµ, Oxoid) were used for fungal controls. Antimicrobial activity of the following individual chemical standards; artemisia ketone, cineole, (-)-camphor, (+)-camphor, borneol, (+)-α- and β-thujone were determined. All plates were incubated at 37 °C for 48 hr with the exception of the moulds, which were incubated at 25 °C for seven days. Tests were done in triplicate. The antimicrobial activity was determined by measuring the zone of inhibition from the discs.

Minimum inhibition concentration (MIC):

The minimum inhibition concentration (MIC) is the first concentration at which the growth of the bacteria is inhibited. This technique is more accurate and gives qualitative data compared to the disc diffusion assay. In this study, the MIC was determined by using the INT microplate bioassay technique (Eloff, 1998). The micro titre plate (96 wells) was aseptically prepared by adding 100µl of sterile water in all the wells and adding 100 µl of oil in the first row. Serial dilution of essential oils (100 µl) was done, and the inoculum in a suitable broth (100 µl) was added to all the wells. The starting concentration in the first row was 32 mg/ml. The plate was incubated at 37 °C for 24 hours. *p*-Iodonitrotetrazolium violet (INT) solution was added to the wells to determine colour change in relation to concentration of microbial growth. The INT acts by binding to the DNA of growing bacteria.

Essential oil yield:

Figure 9 below represents the yield of essential oil expressed as the percentage of essential oil in the fresh leaf material. The essential oil obtained from plants collected at Klipriversberg (3A-3G) and Qwa-qwa (4A-4C) produced relatively little essential oil in comparison with plants from Setibeng (1A-1C) and Giant's Castle (2A-2C). Plants 2B and 2C (Giant's Castle) produced the highest volume of essential oil at 1.72 and 1.91 % respectively. The Qwa-qwa population produced little essential oil (0.32, 0.68 and 0.32% respectively) despite the fact that the plants were bulky, very fresh and had a pungent smell.

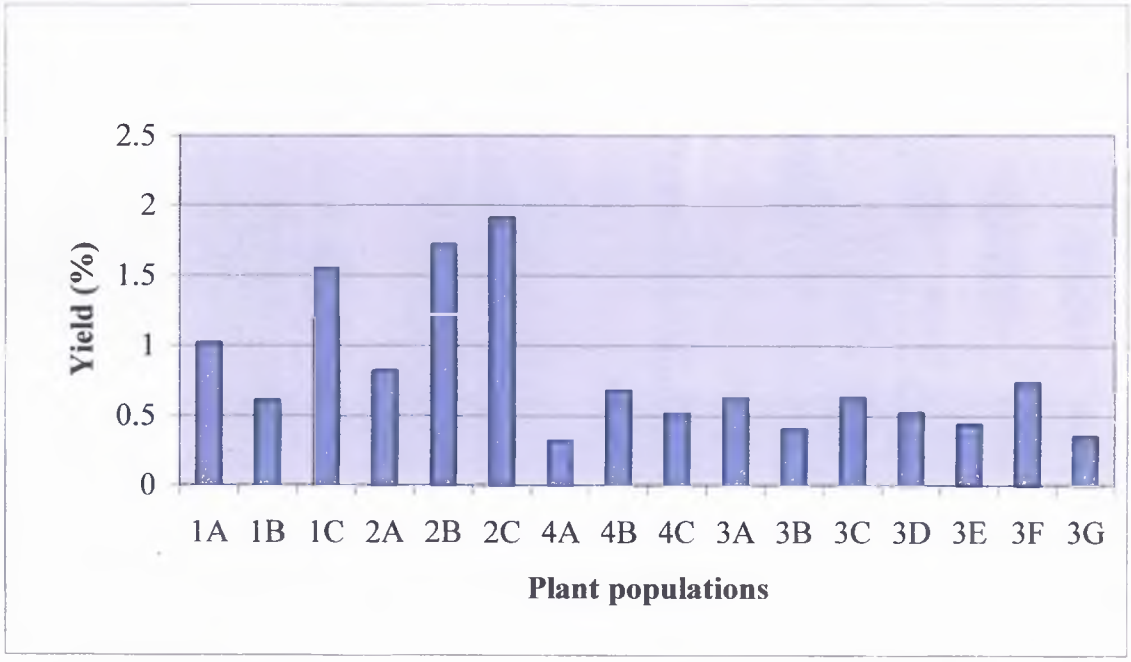


Figure 9: The essential oil yield of sixteen individual *A. afra* plants representing four natural populations.

The growth vigour and longetivity of *A. afra* may be severely reduced by frequent cutting of the plant. *Artemisia afra* has four reproductive stages: floral initiation or development, anthesis, seed set and seed development. The study done by Graven *et al.* (1992) gave evidence that significantly (p=0.01) higher oil yield can be obtained when the plant was harvested during anthesis and early seed set as opposed to the earlier or later harvesting in the reproductive period.

Analytical chemistry:

Thin layer chromatography (TLC):

All sixteen samples were screened for the presence of the chemical compounds using thin layer chromatography. It was observed that essential oil of plant 1A and 1B had almost identical TLC profiles whereas plant 1C, showed absence of most of the compounds present in the other two. The TLC screening (Figure 10) clearly shows the qualitative variation in essential oil composition both within and between natural populations. This variation prompted us to study the essential oil chemistry by gas chromatography coupled with mass spectrometry (GC/MS).

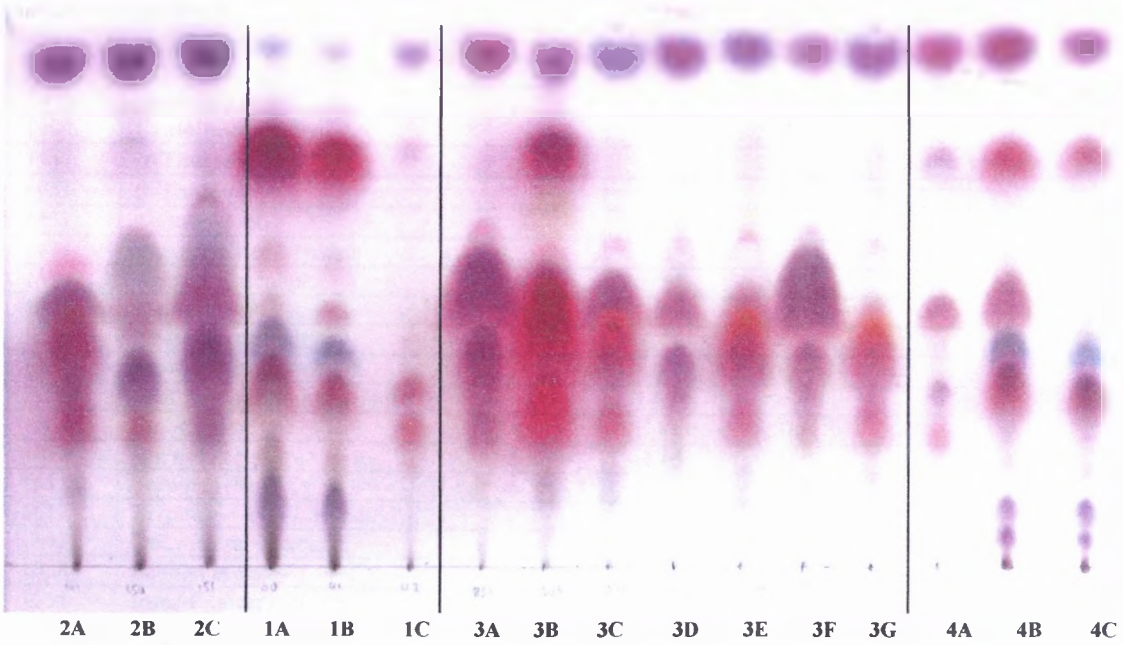


Figure 10: TLC plate showing the separation of essential oil compounds.

Essential oil composition:

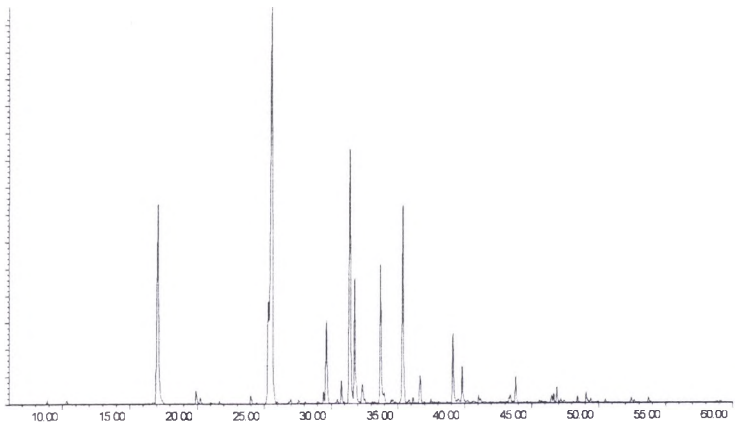
Setibeng population

Setibeng is a nature reserve in Lesotho. Three plants were collected, which were close to each other within the locality.

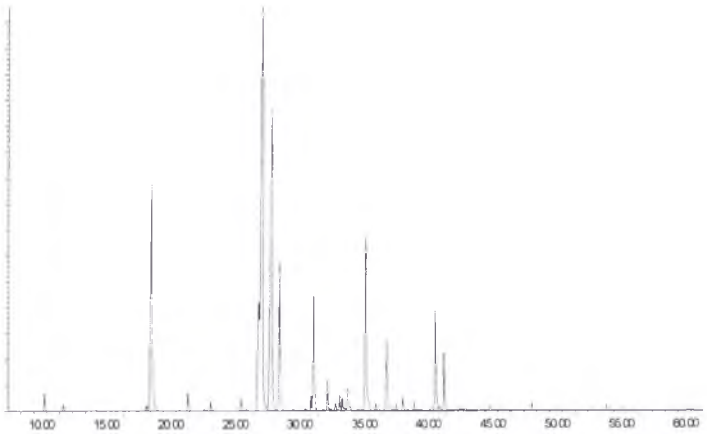
Table 3: Identified chemical compounds and area percentage of essential oil from the Setibeng population.

RRI	CHEMICAL COMPOUNDS	1A	1B	1C
1032	α-Pinene	0.60	1.30	-
1076	Camphene	0.57	0.55	-
1132	Sabinene	-	0.17	-
1213	**1.8-cineole**	**12.02**	**10.48**	**2.31**
1280	p-Cymene	0.61	0.64	0.25
1285	Isoamyl isovalerate	0.19	-	-
1299	(Z)-Methyl butryl isovalerate	0.32	-	-
1405	**Santolina alcohol**	**8.93**	**3.18**	-
1409	**Santolinyl acetate**	**24.32**	**20.19**	-
1437	**α-Thujone**	-	**23.81**	**77.65**
1451	**β-Thujone**	-	**5.64**	**16.81**
1452	1-Octen-3-ol	0.20	-	-
1474	*trans*-Sabinene hydrate	0.17	-	0.24
1510	Artemisia alcohol	0.57	0.57	-
1532	**Camphor**	**2.84**	**3.86**	-
1556	*cis*-Sabinene hydrate	0.16	-	0.17
1571	*trans*-p-menth-2-en-1ol	-	1.11	-
1582	***cis*-Chrysaenthenyl acetate**	**15.15**	-	-
1586	Pinocarvone	-	0.31	0.19
1597	**Bornyl acetate**	**4.45**	0.43	-
1611	Terpinen-4-ol	-	-	0.35
1617	Terpinen-4-ol + Lavanduyl	1.44	0.44	-
1643	Dehydro sabinaketone	-	0.23	0.18
1658	Sabinyl acetate	-	-	0.19
1664	*trans*-Pinocarveol	0.62	-	0.28
1684	Dehydro carvyl acetate	-	-	0.26
1686	Lavandulol	0.26	-	-
1706	α-Terpineol	-	-	0.18
1719	**Borneol**	**8.20**	1.97	-
1720	*trans*-Sabinol	-	-	0.12
1729	*cis*-1.2-epoxy-terpin-4-ol	0.23	0.16	-
1742	β-Selinene	0.29	0.44	-
1755	Bicyclogermcrene	-	-	0.16
1764	*cis*-Chrysaenthenol	1.65	-	-
1786	*ar*-Curcumene	0.18	0.39	-
1802	Cumin aldehyde	-	-	0.24
2008	Caryophyllene oxide	0.41	0.25	-
2033	4-α-Hydroxy achipendol	-	0.31	-
2144	Spathulenol	0.18	0.36	-
2606	β-Costol	-	0.61	-
2607	1-Octadecanol	-	0.27	-
	TOTAL	**84.56%**	**77.67%**	**99.58%**

RRI- Retention indices calculated against n-alkanes



A



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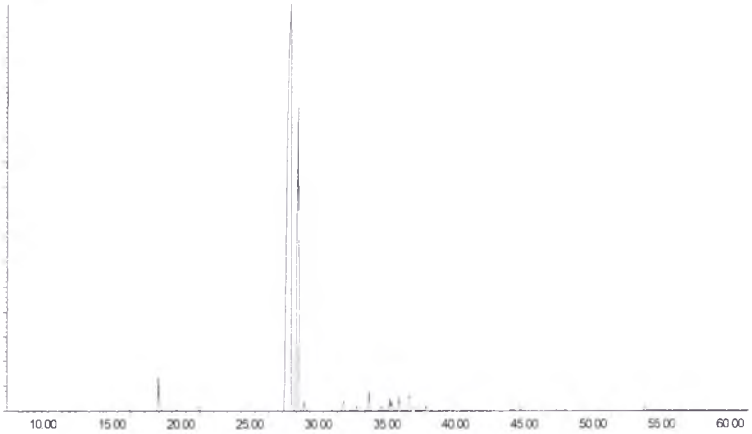


C

Figure 11: (A-C): The GC chromatograms showing the essential oil composition of three individual plants from the Setibeng population.

There were 25 chemical compounds identified in plant 1A (84.56%) and 1B (77.67%). Essential oil of plant 1C was composed of 16 chemical compounds and 99% of those compounds were identified. The GC/MS profile showed that plant 1A and 1B were similar as both had approximately the same levels of 1.8-cineole (11-12%), santolinyl acetate (20-24%) and camphor (3-4%). Plant 1A and 1B had two interesting chemical compounds, santolina alcohol (9% and 3%) and santolinyl acetate (24% and 20 %) which were not present in the other samples except the Qwa-qwa population. Santolina alcohol was also found present in the Zimbabwean *A. afra* oil (Chagonda *et al.*, 1999).

The thujone isomers (α-and β) were absent from plant 1A and were present in higher quantities in plant 1C (α-thujone 77% and β-thujone 16%). The bicyclic monoterpenoid *cis*-chrysanthenyl acetate (15%) was only present in plant 1A. Plant 1A accumulated the highest quantity of borneol (8%) and bornyl acetate (4%) compared to plant 1B.

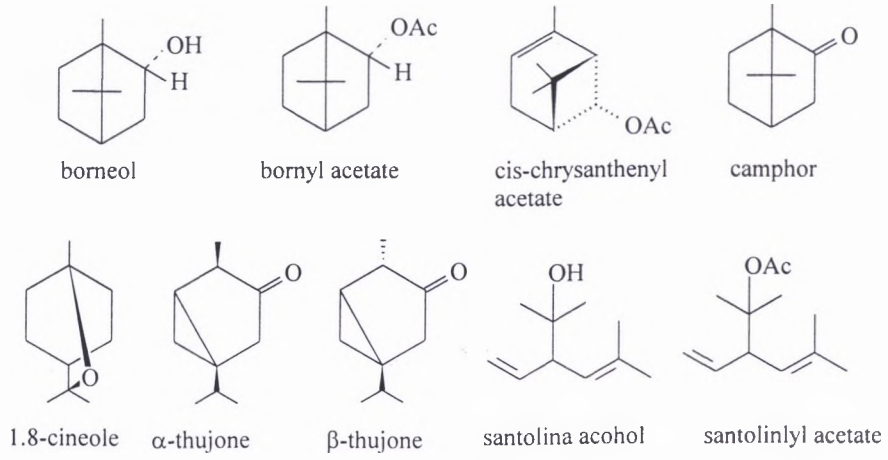


Figure 12: Chemical structures of major compounds detected in the three plants analysed from the Setibeng population.

Giants Castle population

Three plants were collected from Champagne Pools area near Giant's Castle, Kwa-Zulu Natal province just on the outskirts of Lesotho.

Table 4: Identified chemical compounds and area percentage of essential oil from the Giant's Castle population.

RRI	CHEMICAL COMPOUNDS	2A	2B	2C
1014	Tricyclene	-	-	0.22
1032	α -Pinene	-	0.22	0.27
1076	**Camphene**	-	1.59	**6.54**
1118	β-Pinene	-	-	0.42
1132	Sabinene	0.20	1.03	0.14
1174	Myrcene	0.25	0.40	0.71
1188	α-Terpinene	-	0.20	0.40
1195	Dehydro-1.8-cineole	-	-	0.14
1203	Limonene	-	0.11	0.72
1213	**1.8-cineole**	**12.69**	**4.84**	**14.77**
1246	(Z)-β-Ocimene	0.42	0.55	0.45
1255	γ-Terpinene	0.29	0.18	0.55
1280	p-Cymene	0.44	0.95	0.97
1290	Terpinolene	-	-	0.15
1358	**Artemisia ketone**	**14.47**	-	-
1403	**Yomogi alcohol**	**3.54**	-	-
1437	**α-Thujone**	**38.63**	0.94	-
1451	**β-Thujone**	**7.90**	**57.73**	-
1474	*trans*-Sabinene hydrate	0.42	0.34	1.08
1497	α-Copaene	0.23	-	0.18
1510	**Artemisia alcohol**	**9.31**	-	-
1532	**Camphor**	-	**19.38**	**48.99**
1556	*cis*-Sabinene hydrate	0.28	0.33	0.65
1571	*trans*-p-menthol-2-en-1ol	0.11	0.13	2.28
1582	*cis*-Chrysaenthenyl acetate	-	-	0.36
1586	Pinorcarvone	-	0.76	-
1597	Bornyl acetate	-	-	0.25
1611	Terpinen-4-ol	1.43	0.67	1.97
1612	β-Caryophyllene	-	0.59	0.32
1638	*cis*-p-menth-2-en-1-ol	-	-	1.75
1639	*trans*-p-menth-2.8-dien-1-ol	-	0.18	-
1643	Dehydro sabinaketone	-	1.59	-
1648	Myrtenal	-	-	0.12
1651	Sabinaketone	-	0.42	-
1663	*cis*-Verbenol	-	-	0.10
1664	*trans*-Pinocarveol	-	1.17	-
1668	(Z)-β-Faresene	0.31	-	0.24
1682	δ-Terpineol	0.24	-	0.27
1687	α-Humelene	-	0.14	-
1689	*trans*-Piperitol	-	-	1.12
1706	α-Terpineol	-	0.59	0.46
1719	Borneol	-	0.99	1.88
1720	*trans*-Sabinol	0.30	-	-
1726	Germcrene D	1.62	0.73	0.74
1742	β-Selinene	-	0.23	0.35
1748	Pipertone	-	-	1.02
1754	*trans*-Piperitone oxide	-	0.21	-
1755	Bicyclogermcrene	0.37	1.05	-
1758	***cis*-Piperitol**	-	-	**2.91**
1764	*cis*-Chrysaenthenol	0.63	-	1.82
1773	δ-Cadinene	0.28	-	0.24
1802	Cumin aldehyde	-	0.23	-

RRI	CHEMICAL COMPOUNDS	2A	2B	2C
1804	Myrtenol	-	-	0.30
1811	*trans*-p-menth-1(7),8-dien-2-ol	-	-	0.16
1864	p-Cymene-8-ol	-	-	0.11
1889	Ascaridol	-	0.19	-
1889	Ascaridol + Isopipertone	-	-	0.25
1957	Cubebol	-	-	0.17
2008	Caryophyllene oxide	0.27	-	0.16
2144	Spathulenol	0.23	0.12	0.41
2209	T-Muurolol	0.86	-	0.94
2241	p-Isopropyl phenol	-	0.11	-
2247	*trans*-alpha-Bergamotol	-	-	0.12
2264	Intermediol	-	-	0.45
	TOTAL	**95.72%**	**98.99%**	**98.62%**

RRI- Retention indices calculated against n-alkanes

The total numbers of chemical compounds identified in each plant were 2A (26), 2B (34) and 2C (47). The major compounds were 1.8-cineole, artemisia ketone, yomogi alcohol, artemisia alcohol, camphor, camphene, α- and β-thujone.

Plant 2C had the highest amount of camphene (6%). Artemisia ketone (14%), yomogi alcohol (4%) and artemisia alcohol (9%) were only present in plant 2A. Plant 2A had a higher quantity of α-thujone (39%) than plant 2B (0.9%). On the contrary, β-thujone was higher in plant 2B (58%) than plant 2A. Camphor was only observed in plants 2B (19%) and 2C (49%).

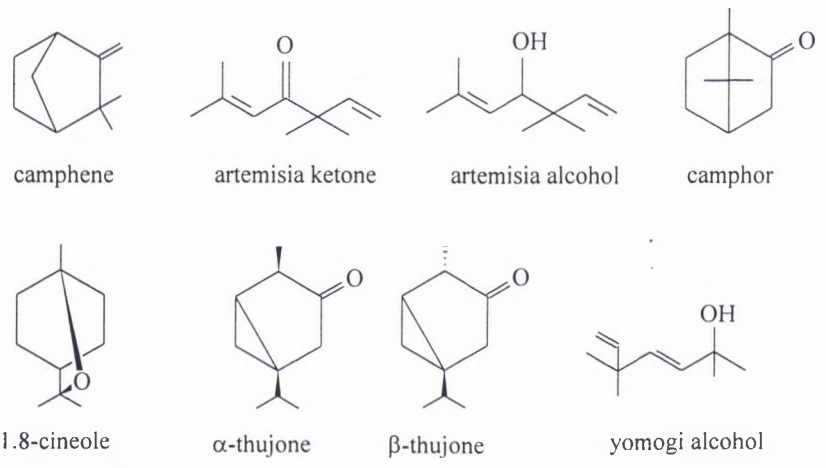
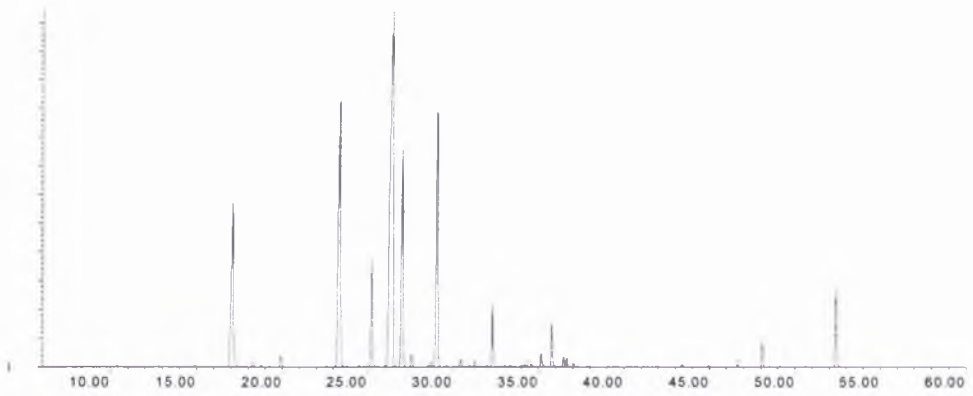


Figure 13: Chemical structures of major compounds detected in the three plants analysed from the Giant's Castle population.



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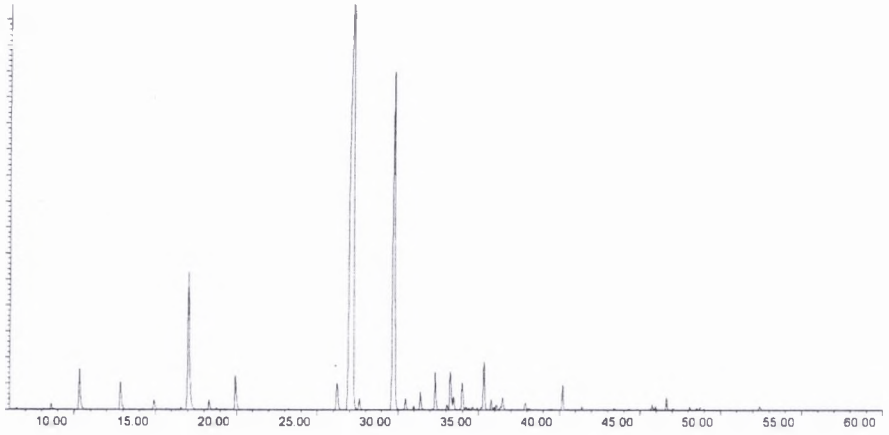

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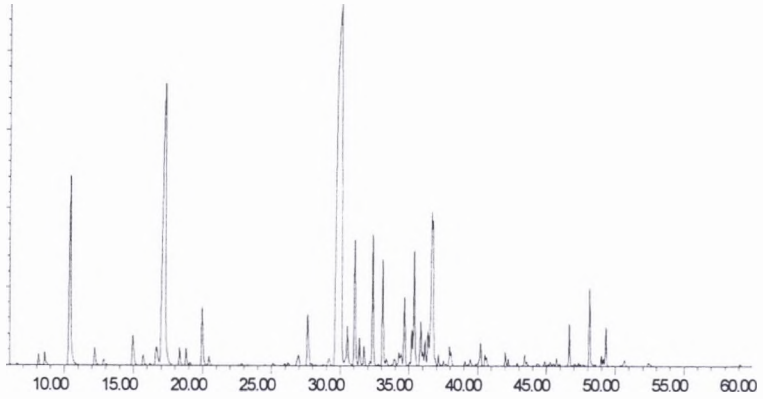

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Figure 14: The GC chromatograms showing the essential oil composition of three individual plants from the Giant's Castle population.

Klipriversberg population

Klipriversberg is a Nature Reserve in Gauteng province. Seven plants were collected from this population.

Table 5: Identified chemical compounds and area percentage of essential oil from Klipriversberg population.

RRI	CHEMICAL COMPOUNDS	3A	3B	3C	3D	3E	3F	3G
1014	Tricyclene	-	0.11	-	0.16	-	0.15	-
1032	α -Pinene	0.98	0.11	0.86	1.39	1.19	1.34	0.76
1035	α -Thujene	-	-	-	-	-	0.09	-
1076	**Camphene**	**2.21**	0.10	1.48	**4.71**	**2.10**	**3.43**	**2.18**
1118	β-Pinene	0.85	-	0.30	1.13	0.26	0.89	0.37
1132	Sabinene	0.28	0.08	0.09	0.19	0.18	0.27	0.06
1174	Myrcene	0.66	0.14	0.24	0.96	0.20	0.54	0.63
1188	α-Terpinene	0.80	0.13	0.36	0.75	0.43	0.86	0.22
1195	Dehydro-1.8-cineole	0.11	-	-	0.07	0.15	0.42	-
1203	Limonene	0.11	-	0.11	0.11	0.18	-	0.15
1213	**1.8-cineole**	**43.26**	**6.62**	**20.63**	**24.44**	**11.38**	**50.09**	**8.93**
1246	(Z)-β-Ocimene	1.53	0.41	0.38	2.36	0.67	0.80	0.90
1255	γ-Terpinene	1.77	0.18	0.75	1.18	0.37	1.68	0.43
1266	(E)-β-Ocimene	0.27	0.10	0.09	0.45	0.12	0.14	0.17
1280	p-Cymene	0.35	0.21	0.30	0.61	0.22	0.65	0.18
1285	Isoamyl isovalerate	0.30	-	-	-	-	0.13	-
1290	Terpinolene	0.45	-	0.17	0.25	0.10	0.35	0.11
1358	**Artemisia ketone**	-	**15.62**	**26.69**	-	**27.97**	-	**23.91**
1386	1-Octenyl acetate + (Z)-3-Hexen-1-ol	0.11	-	-	-	-	0.12	-
1386	1-Octenyl acetate	-	-	0.23	-	-	-	0.15
1391	*cis*-3-Hexanol	-	0.07	-	-	-	-	-
1403	**Yomogi alcohol**	-	-	**3.15**				
1405	**Yomogi alcohol+santolina Alcohol**	-	**10.27**	-	-	**2.04**	-	**2.36**
1437	**α-Thujone**	0.10	**5.55**	-	-	-	-	-
1450	*trans*-Linalool oxide (Furanoid)	0.07	-	-	-	-	-	0.14
1451	β-Thujone	-	1.37	-	-	-	-	-
1452	1-Octen-3-ol	0.50	-	0.76	0.87	0.51	0.72	0.58
1474	*trans*-Sabinene hydrate	3.39	0.41	1.14	1.39	0.86	2.79	0.65
1497	α-Copaene	0.19	0.21	-	0.37	-	0.13	0.36
1510	**Artemisia alcohol**	-	**27.76**	**12.07**	-	**10.13**	-	**9.57**
1532	**Camphor**	**15.64**	-	**17.63**	**31.46**	**27.17**	**16.83**	**29.54**
1540	**Chrysanthenone**	-	**4.65**	-	-	-	-	-
1553	Linalool	0.25	-	-	-	-	-	-
1556	*cis*-Sabinene hydrate	1.42	0.28	0.69	0.69	0.60	1.78	0.10
1571	*trans*-p-menth-2-en-1-ol	0.38	1.17	0.22	2.99	0.14	0.44	-
1586	Pinocarvone	0.56	0.31	0.47	0.55	0.34	0.57	0.64
1597	Bornyl acetate	1.75	-	0.08	0.33	0.50	0.31	0.16
1611	**Terpinen-4-ol**	**5.15**	0.75	**2.63**	**2.95**	1.52	**4.93**	1.53
1612	β-Caryophyllene	0.38	0.21	0.11	0.74	-	0.43	0.89
1617	Lavanduyl acetate	-	0.75	-	-	-	-	-
1638	*cis*-p-menth-2-en-1-ol	0.24	0.82	0.18	2.13	0.14	-	0.38
1648	Myrtenal	0.34	-	-	0.38	0.17	0.53	0.29
1664	*trans*-Pinocarveol	0.43	0.12	0.47	0.58	-	0.71	0.52
1682	δ-Terpineol	0.57	-	0.37	0.36	0.29	0.59	0.30
1686	lavundulol	-	0.36	-	-	-	-	-
1689	*trans*-Piperitol	0.10	0.57	-	0.67	0.21	0.11	0.30
1706	α-Terpineol	1.83	0.07	0.30	0.41	0.52	0.69	0.18
1719	**Borneol**	**5.64**	-	0.30	**2.88**	1.12	1.76	0.86
1726	Germcrene D	0.72	-	-	1.62	-	0.98	1.44

RRI	CHEMICAL COMPOUNDS	3A	3B	3C	3D	3E	3F	3G
1742	β-Selinene	-	-	0.45	0.36	0.15	-	0.53
1743	δ-Cadinene	0.13	0.17	-	0.31	0.19	-	0.29
1748	Pipertone	-	1.54	-	1.55	-	-	-
1755	Bicyclogermcrene	1.16	-	0.50	-	0.13	0.14	0.57
1758	***cis*-Piperitol**	-	1.39	-	**2.67**	-	0.13	0.46
1804	Myrtenol	0.23	-	0.11	-	-	0.37	0.14
1811	*trans*-p-menth-1(7).8-dien-ol + Myrtenol	-	-	-	0.32	-	-	-
1864	p-Cymene-8-ol	-	-	-	0.07	-	-	-
1864	Isopipertone	-	0.09	-	-	-	-	-
1889	Ascaridol	-	-	-	0.28	-	-	-
1896	*cis*-p-menth-1(7).8-dien-2-ol	-	-	-	-	0.23	-	0.15
1957	Epicubebol	0.18	0.18	-	-	-	0.13	0.32
1957	Cubebol	0.34	0.27	-	0.33	0.30	0.15	0.42
1969	*cis*-Jasmone	0.07	-	-	-	-	-	-
2008	Caryophyllene oxide		0.30	-	-	0.34	0.14	0.28
2008	p-menth-1,8-dien-10-ol + Caryophyllene oxide	0.33	-	0.29	0.18	-	-	-
2050	(E)-Nerolidiol	-	-	-	-	-	-	0.08
2074	Caryophylla-2(12).6(13)-dien-5-one	0.08	-	0.14	-	-	-	-
2130	1-Epi-cubenol	0.13	0.10	-	-	0.11	-	0.10
2144	Spathulenol	0.43	-	0.07	0.09	-	-	-
2202	Germcrene-D-4-ol	-	-	-	0.07	-	-	0.11
2209	T-Muurolol	1.27	1.21	0.08	1.24	1.40	0.58	1.66
2247	*trans*-α-Bergamotol	0.08	-	-	-	-	-	-
2255	α-Cadinol	0.08	-	-	0.08	-	-	0.09
2261	Intermediol	0.96	1.53	0.72	1.02	1.50	0.44	1.04
2324	Caryophylladienol II	0.15	0.10	0.14	0.07	0.10	-	0.12
2606	β-Costol	-	-	0.11	-	-	-	0.13
	TOTAL	**99.31**	**95.99**	**95.86**	**98.91**	**96.53**	**98.33**	**96.53**

RRI- Retention indices calculated against n-alkanes

The major compounds found in all plants were 1.8-cineole, camphene and terpinen-4-ol. The following major compounds were found in all but plant 3B: artemisia ketone and camphor. The main constituents of plant 3B were artemisyl acetate (9%) and a mixture of yomogi alcohol and santolina alcohol (10%) and chrysanthenone. Artemisia alcohol was a major compound, which was only found in plant 3B (28%), plant 3C (12%).

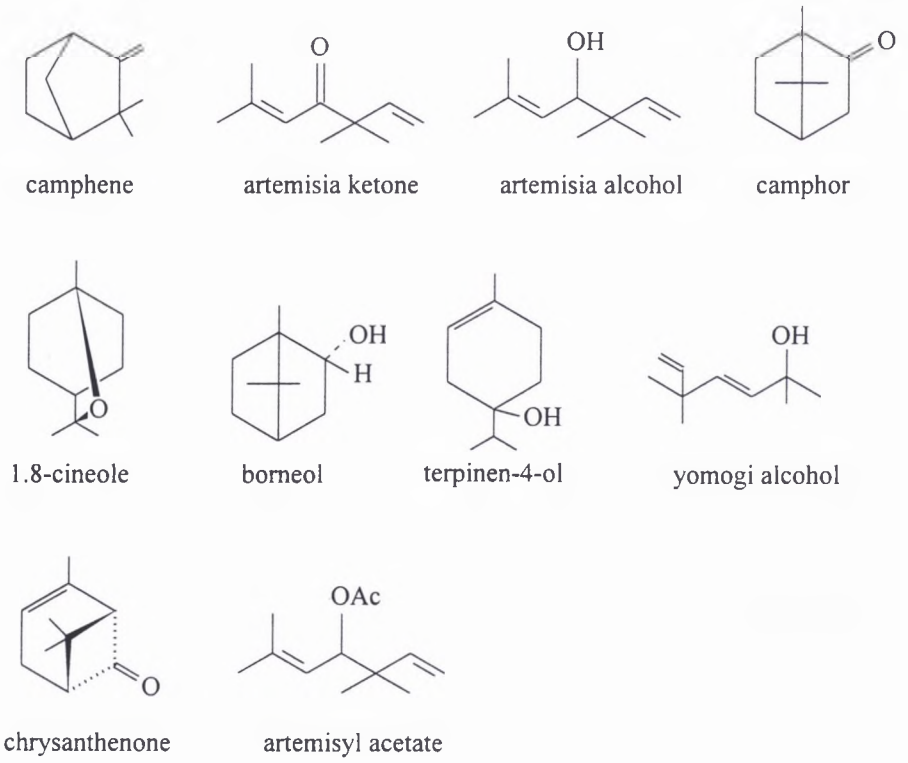
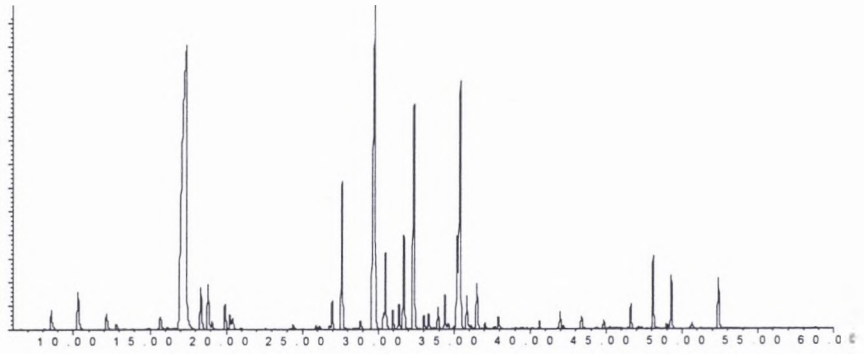


Figure 15: Chemical structures of major compounds detected in the seven plants analysed from the Klipriversberg population.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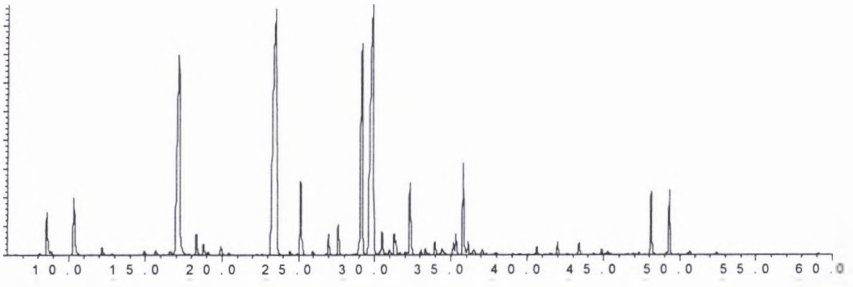
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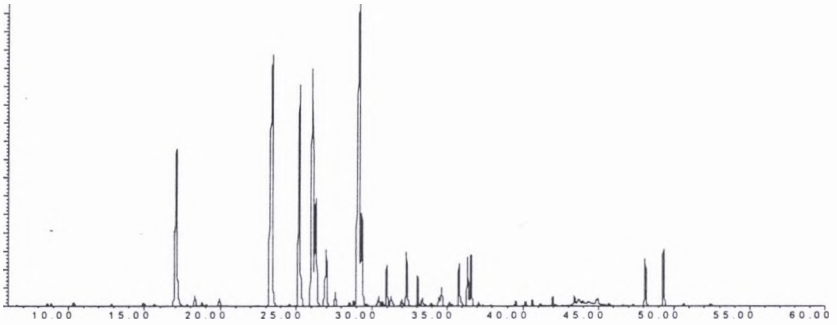
B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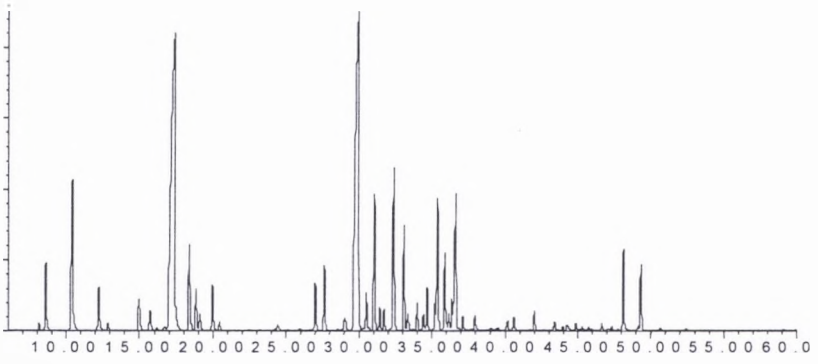
C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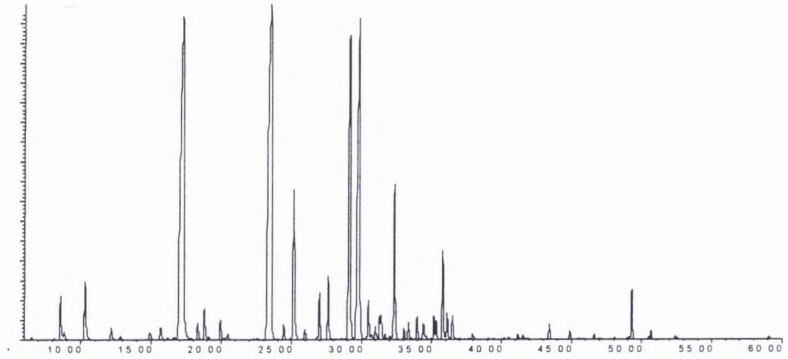
D



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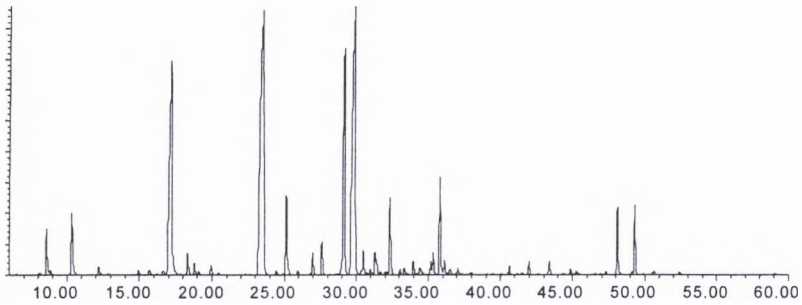

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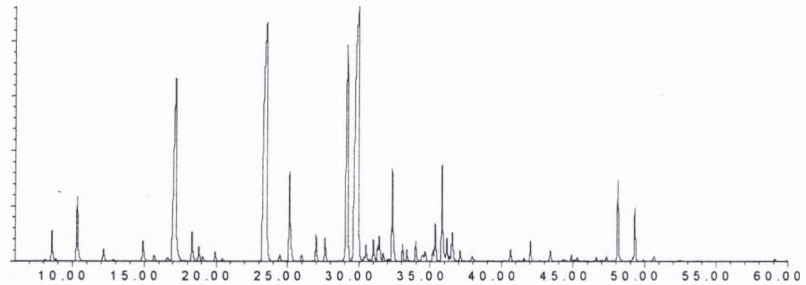


G

Figure 16: The GC chromatograms showing the essential oil composition of seven individual plants from the Klipriversberg population.

Qwa-qwa population

Three plants were collected just off the campus of the University of North in Qwa-qwa (Phuthadijahaba) in the Free State province.

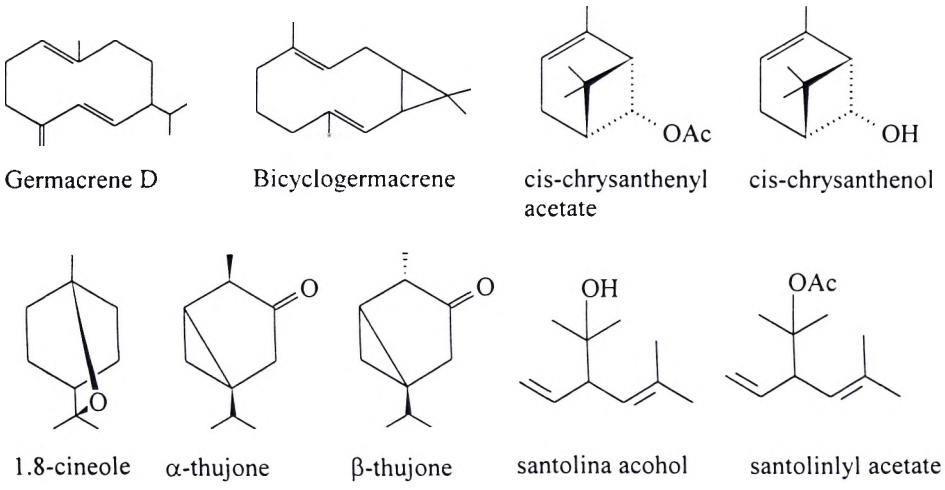
Table 6: Identified chemical compounds and area percentage of essential oil from Qwa-qwa population.

RRI	CHEMICAL COMPOUNDS	4A	4B	4C
1032	α-Pinene	0.12	0.89	0.38
1076	Camphene	0.22	-	-
1132	Sabinene	0.61	-	0.21
1151	Propyl-2-methyl butyrate	-	0.22	-
1174	Myrcene	0.67	0.21	0.10
1188	α-Terpinene	0.30	0.41	-
1195	Dehydro-1.8-cineole	0.15	0.14	-
1213	**1.8-cineole**	**12.60**	**22.52**	**2.58**
1246	(Z)-β-Ocimene	1.24	0.95	0.98
1255	γ-Terpinene	0.57	0.69	0.17
1266	(E)-β-Ocimene	0.21	0.16	0.12
1280	p-Cymene	0.49	1.05	-
1290	Terpinolene	0.12	-	-
1403	Yomogi alcohol	0.57	-	-
1405	**Santolina alcohol**	-	**9.29**	**4.14**
1409	**Santolinyl acetate**	0.75	**7.97**	-
1437	**α-Thujone**	**27.48**	**15.01**	**55.56**
1448	**Artemisyl acetate (Adams)**	**4.02**	-	-
1451	**β-Thujone**	**29.87**	**4.26**	**11.60**
1474	*trans*-Sabinene hydrate	0.10	0.18	-
1495	Bicylcoelemene	-	0.24	0.20
1510	Artemisia alcohol	0.16	-	-
1532	Camphor	1.00	0.59	-
1556	*cis*-Sabinene hydrate	-	0.17	-
1571	*trans*-p-menth-2-en-1-ol	0.18	0.29	-
1582	***cis*-Chrysanthenyl acetate**	**6.14**	-	-
1586	Pinocarvone	-	0.34	0.14
1611	**Terpinen-4-ol**	1.29	**2.15**	0.50
1612	β-Caryophyllene	0.49	1.91	0.33
1638	*cis*-p-menth-2-en-1-ol	0.13	0.22	-
1643	Deydro sabina ketone	0.10	-	0.14
1648	Myrtenal	0.37	0.27	-
1651	Sabina ketone	-	-	0.10
1658	Sabinyl acetate	0.17	-	-
1664	*trans*-Pinocarveol	0.62	0.67	-
1668	(Z)-β-Faresene	0.17	0.67	0.46
1686	lavundulol	-	0.48	0.18
1687	α-Humelene	0.12	-	-
1706	*trans*-Sabinol	0.74	-	-
1719	**Germcrene D**	1.38	**3.46**	1.15
1720	(E)-β-Farenese	-	-	0.12
1726	α-Terpineol	0.23	0.40	-
1726	α-Zingiberene	0.45	-	0.59
1740	α-Muurelene	-	-	0.27
1741	β-Bisanolene	-	-	0.11
1755	Borneol	-	0.16	-
1755	**Bicyclogermecrene**	1.65	**3.94**	1.31
1764	***cis*-Chrysaenthenol**	**2.20**	-	-
1773	δ-Cadinene	0.16	0.5	0.15

RRI	CHEMICAL COMPOUNDS	4A	4B	4C
1786	*ar*-Curcumene	-	-	0.30
1804	Myrtenol	0.11	0.16	-
1889	Ascaridol	-	0.18	-
1957	Epicubebol	-	0.19	0.28
1957	Cubebol	-	0.13	-
1977	4-β-Hydroxy achipendiol	-	0.14	-
2008	Caryophyllene oxide	-	0.23	0.11
2033	4-α-Hydroxy achipendol	-	0.90	0.46
2050	(E)-Nerolidiol	-	0.17	0.13
2057	1-Epi-cubenol	-	0.13	-
2073	p-menth-1.4-dien-7-ol	0.98	-	-
2098	Globulol	-	0.13	-
2144	Spathulenol	0.18	1.02	0.84
2209	T-Muurolol	0.11	0.74	0.28
2232	α-Bisobolol	-	-	0.14
2247	*trans*-α-Bergamotol	-	0.32	0.21
2255	α-Cadinol	-	0.14	0.13
2324	Caryophylladienol II	-	0.16	-
	TOTAL	**99.22**	**85.15**	**84.47**

RRI- Retention indices calculated against n-alkanes

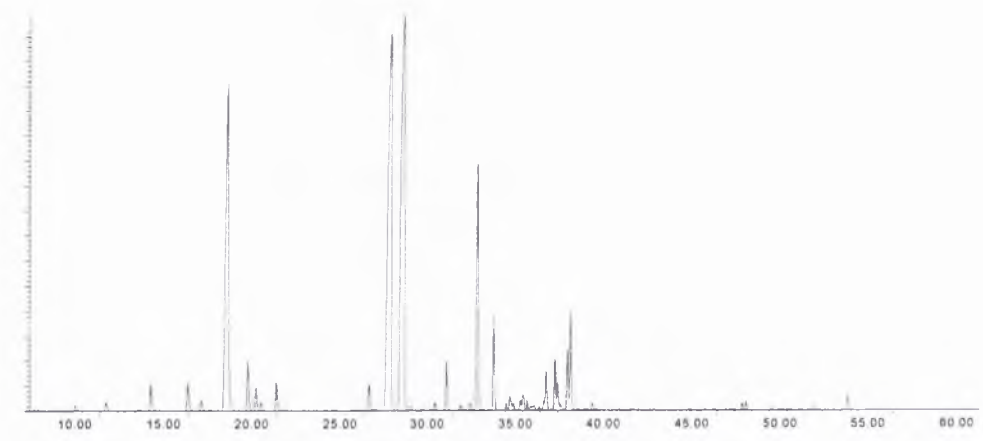
A total of 42 chemical compounds were identified in plant 4A (99%), 47 in plant 4B (85%) and 35 in plant 4C (84%). The major compounds were 1.8-cineole, santolina alcohol, artemisyl acetate, terpinen-4-ol, α- and β-thujone and two sesquiterpene hydrocarbons (germcrene D and bicylogermcrene). Santolina alcohol was found in plants 4B (9%) and 4C (4%). All the plants had relatively high levels of α- and β-thujone with the highest being reported from plant 4C (α-thujone 56%). Plant 4A was the only one sample producing *cis*-chrysaenthenyl acetate (6%).



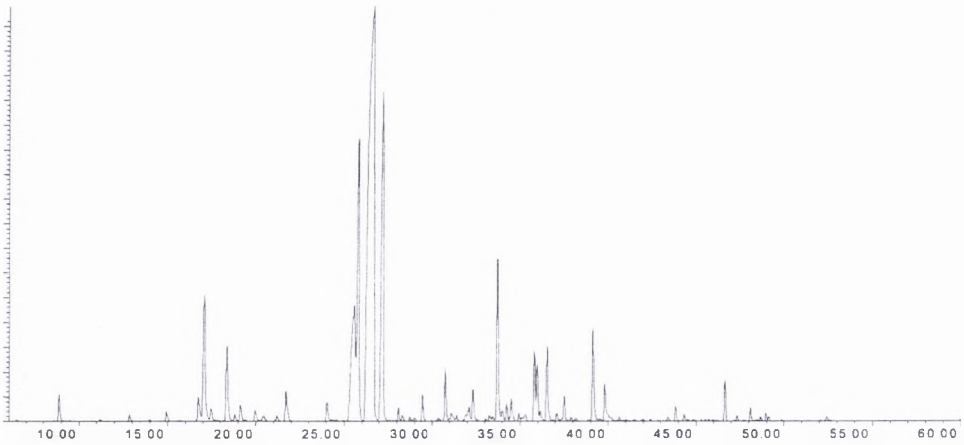
Germacrene D | Bicyclogermacrene | cis-chrysanthenyl acetate | cis-chrysanthenol

1.8-cineole | α-thujone | β-thujone | santolina acohol | santolinlyl acetate

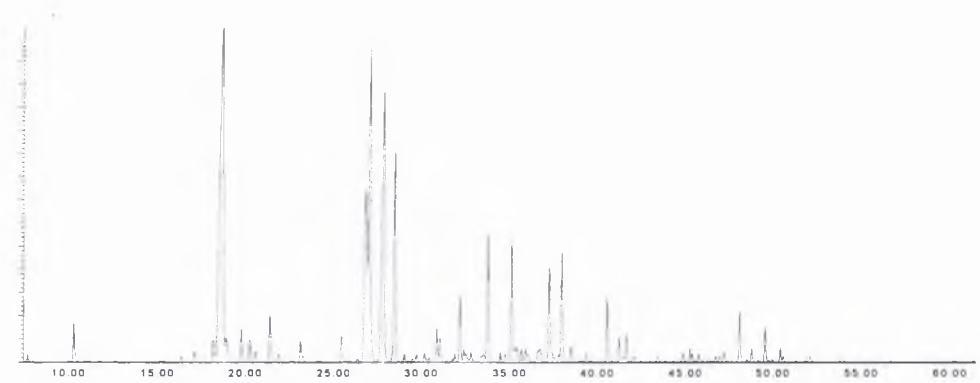
Figure 17: Chemical structures of major compounds detected in the three plants analysed from the Qwa-qwa population.



A



B



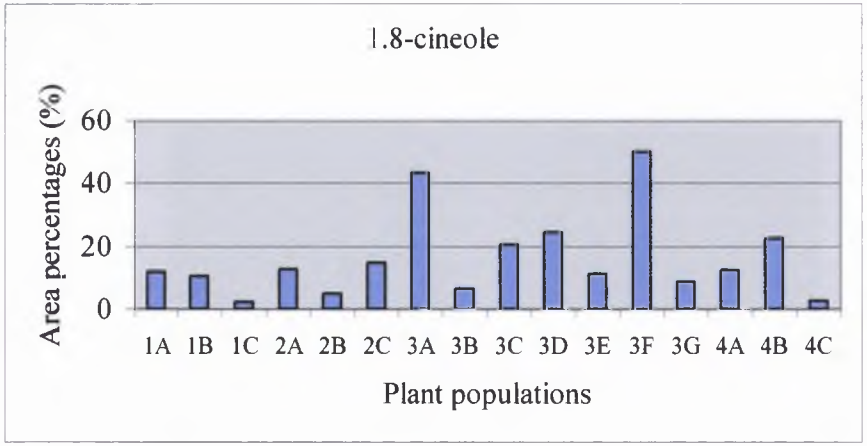
C

Figure 18: The GC chromatograms showing the essential oil composition of three individual plants from the Qwa-qwa population.

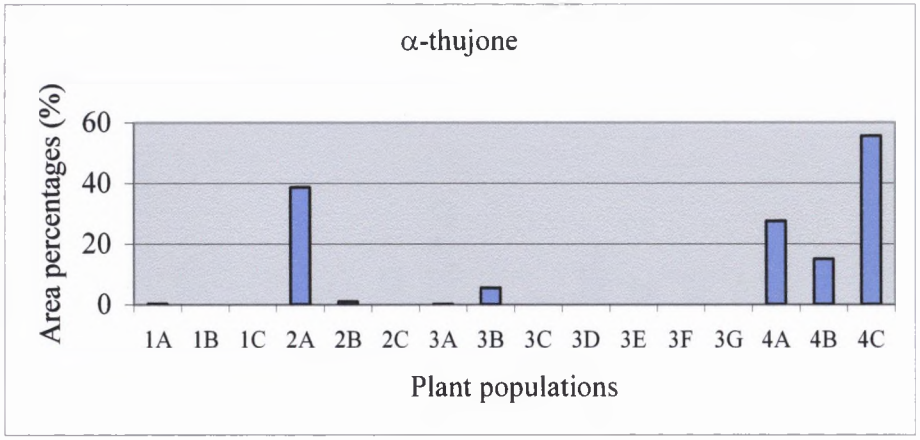
Variation in major constituents of *A. afra* oil

Individual chemical compounds, which are commonly found in *A. afra* oil as main constituents were selected to show the variation within and between the populations (Figure 19; A-H). Thujone is one of major constituents where immense variation was observed. This chemical compound is toxic with the α-form being more toxic than the β-form. The European people used absinth for making alcoholic beverages, but due to the harmful effects of thujone it was discontinued (Pappas & Sheppard-Hanger, 1996). Samples from Qwa-qwa population (Figure19 B & C) accumulate both α- and β-thujone. On the other hand the same samples lacked artemisia ketone and artemisia alcohol (Figure 19 D & G). It is interesting to note that samples 1B and 1C (Figure 19 B & C) have lower levels of β-thujone while α-thujone is totally absent. If thujone is administered in high doses it may cause confusion, convulsions and coma (Watt & Breyer-Brandwijk, 1962). Therefore caution has to be taken with respect to plants 2A, 4C and 2B, if they are to be used in preparation of commercial tincture as these plants have high content of α- and β-thujone. It is advisable that the oil should not be taken internally and should be used with caution during pregnancy and for epileptics.

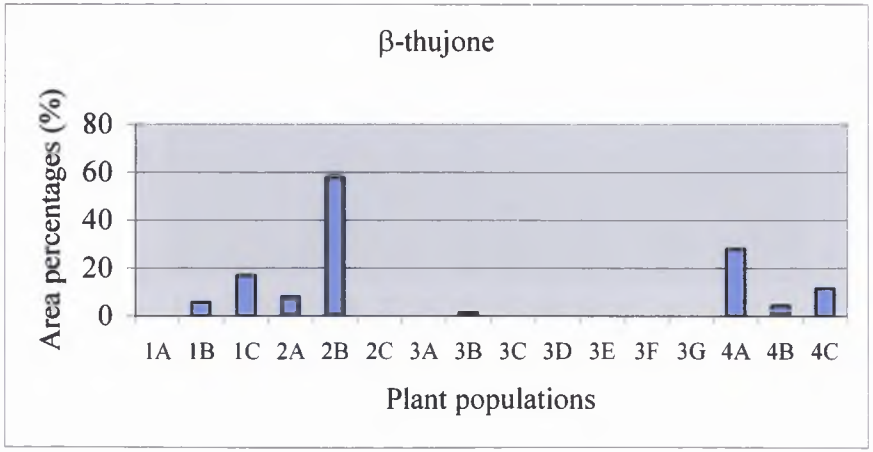
According to Silvestre *et al.* (1999), several species have shown abundance of 1.8-cineole in their essential oil composition but the *A. afra* oil varied in quantities within and between populations (Figure 19 A). Samples 3A and 3F had the highest quantities of 1.8-cineole at 43 % and 50 % respectively. Artemisa ketone (Figure 19 D) was present in plants from Klipriversberg population except in samples 3A, 3D and 3F. In figure 19 (E and H) sample 3B had the highest quantities of artemisia alcohol and santolina alcohol, while they were devoid of camphor and borneol.


1.8-cineole
Area percentages (%)
60
40
20
0
1A 1B 1C 2A 2B 2C 3A 3B 3C 3D 3E 3F 3G 4A 4B 4C
Plant populations

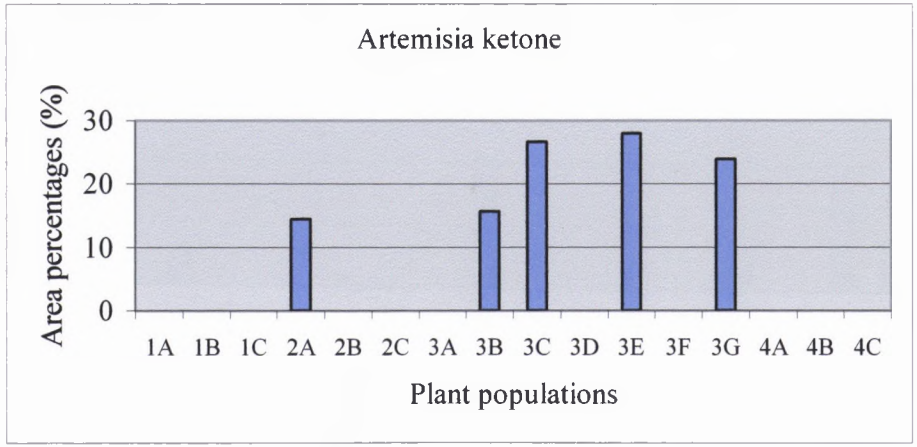
A


α-thujone
Area percentages (%)
60
40
20
0
1A 1B 1C 2A 2B 2C 3A 3B 3C 3D 3E 3F 3G 4A 4B 4C
Plant populations

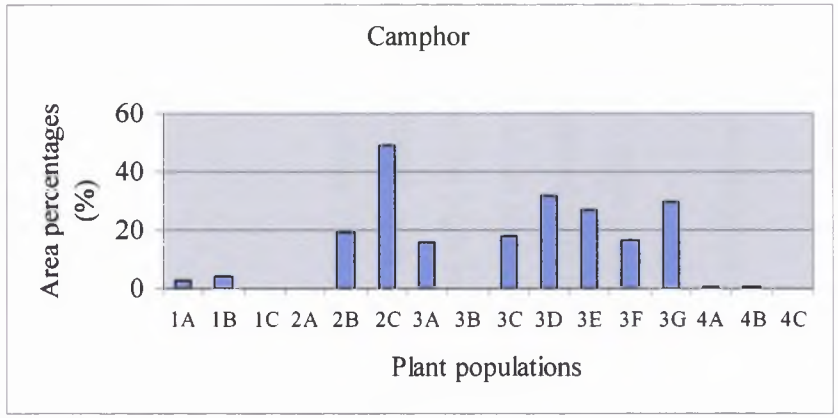
B


β-thujone
Area percentages (%)
80
60
40
20
0
1A 1B 1C 2A 2B 2C 3A 3B 3C 3D 3E 3F 3G 4A 4B 4C
Plant populations

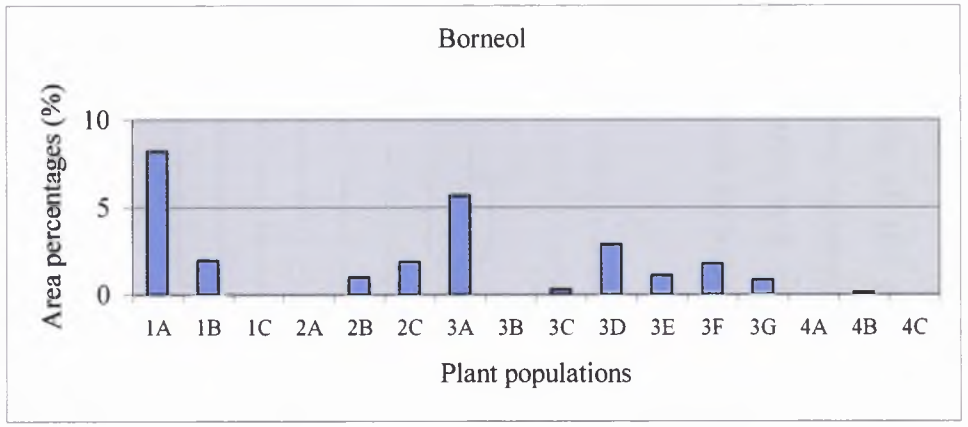
C


Artemisia ketone
Area percentages (%)
30
20
10
0
1A 1B 1C 2A 2B 2C 3A 3B 3C 3D 3E 3F 3G 4A 4B 4C
Plant populations

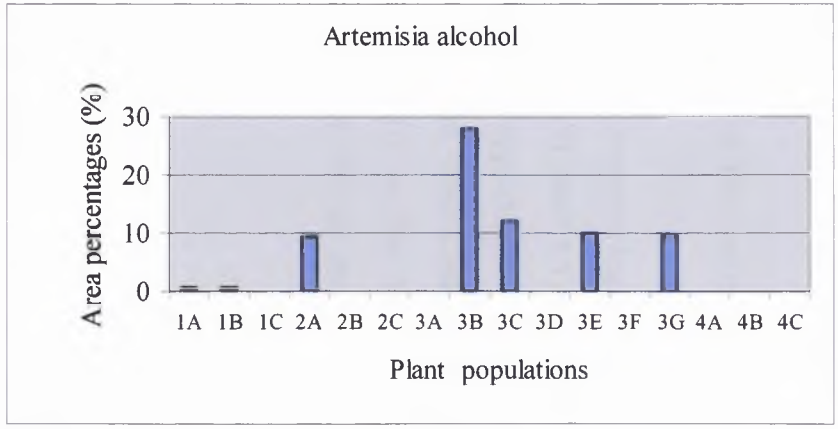
D



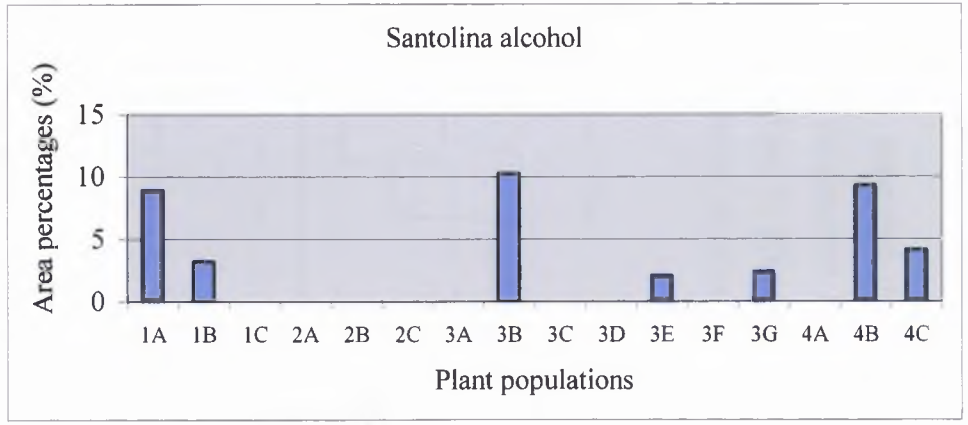
E



F



G



H

Figure 19: Bar graphs showing variation within and between the plant populations of major chemical compounds commonly found in *A. afra* oil.

Cluster analysis

Cluster analysis of 107 chemical compounds was generated using NTYSpc-2 and a dendrogram was constructed (Rholf, 1997). The dendrogram below shows that the chemical variation in *A. afra* is erratic and not correlated to geographical distribution. In essence the plants from the same locality were expected to be clustered together.

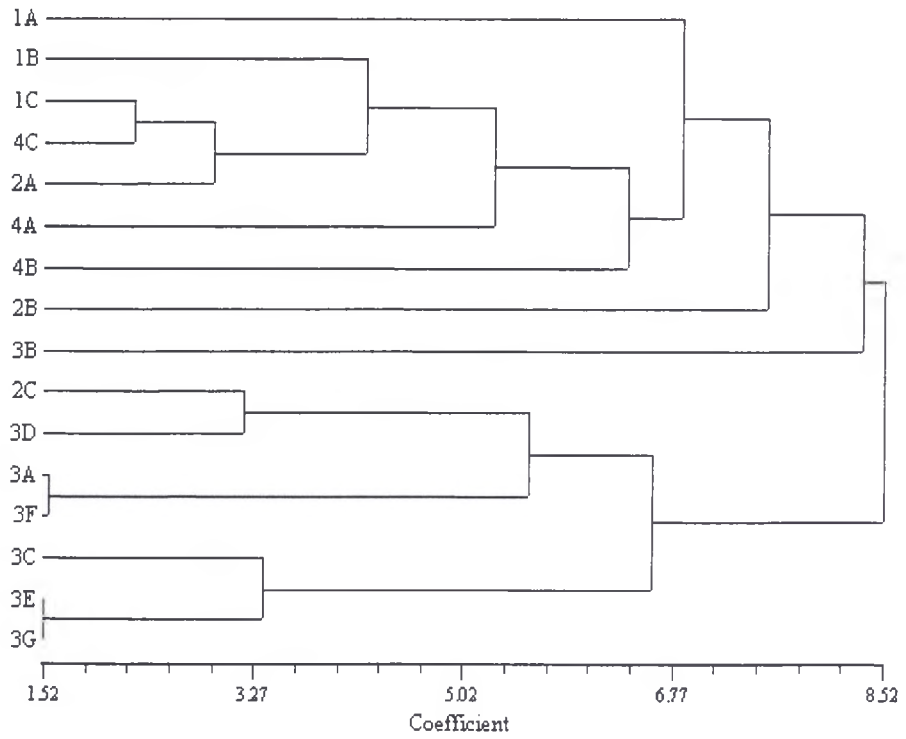


Figure 20: The dendrogram of chemical compounds from four natural populations.

For instance, the three plants from Setibeng were not clustered together instead plant 1C was closely clustered with plant 4C from Qwa-qwa and plant 1A and 1C were outliers. This cluster could be linked to either the presence of approximately same levels of 1.8-cineole (2%) and the high levels of α-thujone (56% and 78%). Chemical compounds that could be associated with plant 1A being an outlier is the major compound *cis*-chrysaenthnyl acetate (15%) and *cis*-chrysaenthanol (1.7%) as they only appear in this sample.

Plant 3A and 3F were also tightly clustered together due to almost the same quantities of sabinene (0.3%), high 1.8-cineole (43-50%), γ-terpinene (1.7%), *trans*-sabinene hydrate (3%) and terpinen-4-ol (5%). Plants 3E and 3G were tightly clustered together and had the coefficient of 1.52. Most of the chemical compounds were generally equal in both the plants, especially the terpene alcohols, (e.g. yomogi alcohol and santolina alcohol 2%, artemisia alcohol 10% and terpinen-4-ol 1.5%). Plant 3C formed a cluster with the two above-mentioned plants.

Antimicrobial activity

Qualitative disc diffusion assay

The selection of the microbes for disc diffusion assays and minimum inhibitory concentration assays was done on the basis of the traditional use of *A. afra*. Table 7 represents the zones of inhibition measured in millimetres including the disc of 6 mm. The essential oils of the all plants showed no activity towards *P. aeruginosa* and *E. faecalis*, while inhibiting *B. cereus and C. neoformans*. The highest inhibition towards the latter organism was shown for plants 4A and 4C (10 –10.5 mm). In contrary to the high inhibition the essential oil of all three plants from Qwa-qwa population were not active towards *S. aureus*, which was sensitive to all other essential oil. The same population had no activity towards *K. pneumoniae*.

Table 7: The zones of inhibition were measured for sixteen samples by including the 6 mm of the disc.

	1A	1B	1C	2A	2B	2C	3A	3B	3C	3D	3E	3F	3G	4A	4B	4C	Co
B. cereus	*	*	*	7	8	8	8	8	7	7.5	7	7	7.5	7	7	7	15
B. subtilis	*	*	*	6	7	7.5	7	7	6.5	7.5	6	6	7.5	6.5	6.5	6.5	15
S. epidermidis	*	*	*	6	6	6	6	6	6	6	7	7	6	6	6	6	15
S. aureus	7	7	7.5	8	7	7	7	8	8	8	8	8	8	6	6	6	15
E. coli	6.5	6.5	6.5	6.5	6.5	6.5	6.5	7	7	7	7	7	7	7	7	7	15
E. faecalis	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	15
K. pneumoniae	6	8	6	6	7	7	6	6	7	6	6	7	8	6	6	6	15
P. aeruginosa	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	15
S. typhi	6	7	6	7	9	6	6	7	6	7	6	6	7	6	6	6	15
S. odorifera	*	*	*	7	8	8	7	7	7	7	6.5	7	7	6.5	6.5	6.5	15
C. albicans	6	9	9	6	11	10	7	8	8	8	9	7	7	8	9	7	16
C. neoformans	9	9	9	7	9	7	7.5	8	7	8	8	7.5	7	10.5	10	9	16
A. alternaria	*	*	*	10	8	8	6	6	6	6	6	6	6	6	6	6	16
A. niger	*	*	*	6.5	6	6	6	6	6	6	6	6	6	6	6.5	6	16

* not determined due to insufficient oil

(Setibeng 1: A, B, C; Giants Castle 2: A, B, C; Klipriversberg 3: A, B, C, D, E, F, G; Qwa qwa 4: A, B, C; Co = neomycin for bacteria and nystatin for yeasts and fungi)

The plants from Giant's Castle were the only plants that had activity towards *A. alternaria*. Essential oil of plants 3E and 3F were the only ones that inhibited *S. epidermidis*. Antibacterial activity for *K. pneumoniae* was only observed for plants 1B, 2B, 2C, 3C, 3F and 3G. In general the gram-positive bacteria were fairly sensitive to the *A. afra* oil as compared to gram-negative bacteria. The fungi were more sensitive to the essential oil compared to bacteria. The disc diffusion assay also affirmed the variation within and between populations.

Minimum inhibitory concentration

It was observed from figure 21 that plants 2B and 3B had a MIC of 16 mg/ml against *K. pneumoniae*, *S. aureus* and *S. typhi*. While plants 3C and 3E had a MIC of 16 mg/ml against *E. coli* and *S. aureus*. The MIC results showed slight variation between plant 2B and 2C from the same population. The minimum inhibitory concentration for plant 2B was 16 mg\ml and 2C was 8 mg\ml against *S. aureus*.

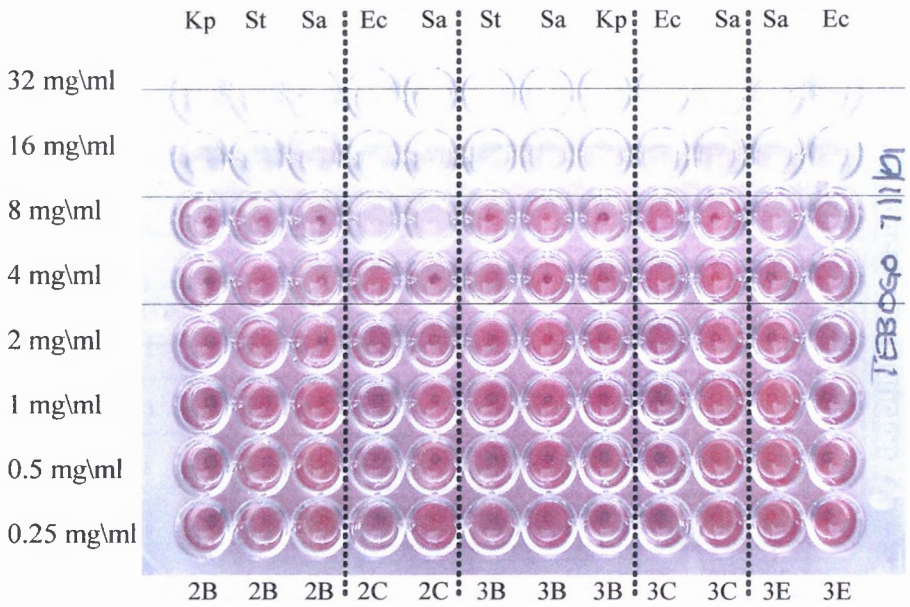


Figure 21: A 96 well microtitre plate used to measure minimum inhibitory concentration.

Correlation between the chemical composition and antimicrobial activity

It is difficult to correlate the observed antimicrobial activity to chemical composition, as no pattern is obvious. The antimicrobial results (Table 7) were subjected to a qualitative cluster analysis. Data extracted from GC/MS analysis as presented in Figure 20 were superimposed onto the antimicrobial activity dendrogram.

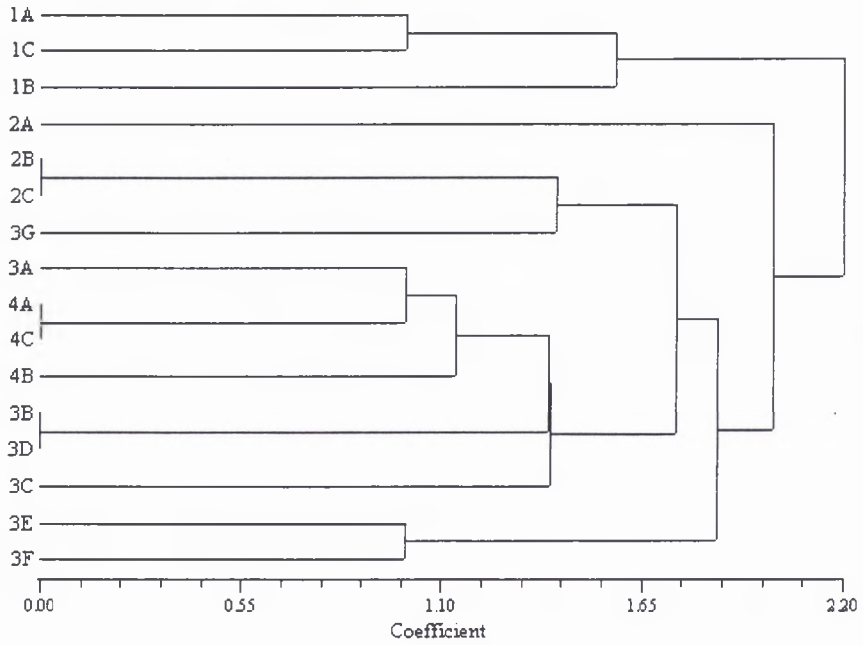


Figure 22: The qualitative cluster analysis of antimicrobial activity of sixteen essential oil samples.

Samples 2B and 2C displayed the same spectrum of activity (Table 7) and are hence tightly clustered together in Figure 22. Both essential oil samples showed almost identical levels of activity towards *B. cereus*, *B. subtilis*, *S. aureus S. odorifera*, and *A. alternaria*. The yeast, *Candida albicans* was the most sensitive to essential oils from 2B and 2C exhibiting zones of inhibition of 11 and 10 mm respectively. Based on the similar antimicrobial properties, one is inclined to expect some chemical congruence in terms of essential oil composition for these two samples. Figure 20 clearly shows that these two samples are nested in two distinct sub-clusters indicating differences in chemical composition. These quantitative and qualitative differences are also obvious in Table 4 with reference to compounds such as camphene, 1.8-cineole, β-thujone and *cis*-piperitol.

Similarly two plants from the Qwa-qwa populations (4A and 4C) were closely clustered together on the basis of their general inactivity although good activity was recorded for *C. neoformans*. These two samples occupied distant positions in the chemical cluster

analysis in Figure 20. The two samples show immense quantitative differences for santolina alcohol and santolinyl acetate, 1.8-cineole and α- and β-thujone. The inhibition of *C. neoformans* could possibly be associated with the presence of inhibitory sesquiterpenes (β-caryophyllene 2%, δ-cadinene 0.5% and (E)-nerolidiol), alcohols (santolina alcohol and yomogi alcohol) and monoterpene ascaridol, which are known for inhibition of yeasts and moulds (Pauli, 2001).

One is inclined to expect plants with similar chemistries to also display comparable biological activities, in this case, antimicrobial activity. From Figure 20 it is observed that samples 3E and 3G have a very high correlation co-efficient and from Table 3 quantitative and qualitative similarities in essential oil composition is apparent (e.g. 1.8-cineole, artemisia ketone, artemisia alcohol, camphor). These two samples however show different antimicrobial activity profiles (Figure 22). Compared to sample 3G, sample 3E exerted more superior activity against the yeasts (*C. neoformans* and *C. albicans*). Sample 3G however displayed better activity against *K. pneumoniae* and *B. subtilis* when compared to 3E.

Biological activity is merely a manifestation of the chemical composition. Pure terpene standards of major compounds (artemisia ketone, 1.8-cineole, borneol, (-)-camphor, (+)-camphor, α- and β-thujone) identified in *A. afra* oil were evaluated on the same microrganisms listed in Table 7. The compounds were tested singularly and in various combinations. All the individual chemical standards as well as the combinations showed no activity. This indicates that one should be cautious in assuming that the activity is caused by major compounds only as it clear that minor compounds are exerting strong antimicrobial activity. In previous studies it was suggested that not only major compounds contribute to the total activity, but the minor compounds should also be considered (Mangena *et al.*, 1999).

It could be possible that the essential oil compounds in *A. afra* are operating in a synergistic manner. Onawunmi *et al.* (1985) illustrated synergism of the essential oil compounds by using the main constituents of lemon grass, where mycrene showed no activity on its own but it did enhance the activity of α- and β-citral, the latter which was shown to be the antimicrobial factor in lemon grass oil. It is also speculated that the essential oil constituents may have different modes of action. Certain chemical

compounds may desensitize the cell wall and the others enter to inhibit the growth of the microorganism. It was established from research on sage and rosemary oils that 1.8-cineole exerted antimicrobial action by the destruction of the fungi cell wall and impairment of metabolism (Steinmetz *et al.*, 1988).
To establish which compounds are active and their mode of action remains a daunting task considering that over 102 steam volatile compounds are present in *A. afra* essential oil.

The mechanism of action or terpenes is not fully understood but it may involve membrane disruption by the lipophilic compounds. Investigations have shown that the site of action of cyclic hydrocarbons including terpene hydrocarbons and tea tree oil is at the cell membrane (Cox *et al.,* 1998; Sikkema *et al.,* 1995). Tea tree oil caused K^+ leakage in *E. coli*, whilst β-pinene has been shown to have an effect on K^+, H^+ leakage and respiration in yeast (Uribe *et al.,* 1985). In a study conducted by Griffin *et al.* (1999), α-, β-pinene and sabinene caused the permeability of an artificial membrane.

Besides the quantitative presence of the compounds, chemical structures also plays a major role in the determination of the biological activity. Essential oil chemistry as presented in the chromatograms (Figure 11, 14, 16, and 18) is simply a 'snap shot ' of the chemical constituents as specificity of the enantiomers are not recorded.

As mentioned above, the specific chemical structure of a compound plays a major role in the determination of antimicrobial activity. Generally the compounds with a phenolic structure showed activity towards most of the microorganisms. According to Pelczer *et al.* (1988) some phenolics are known for their bactericidal and bacteriostatic properties depending on the concentration used. Dorman *et al.* (1998) has reported that the alcohols are known to possess bactericidal rather than bacteriostatic effect against vegetative cells. In a study done by Pelczer *et al.* (1988), the alcohol terpenoids exhibited activity against test microorganisms, potentially acting as either protein denaturing agents, solvents or dehydrating agents.

The role of structure and molecular properties of terpenoids are important in determining their antimicrobial activity. Molecular properties, such as hydrogen bonding parameters and molecular volume of monoterpenes or sesquiterpenes, molecular surface area and

hydrophilic/lipophilic balance have been associated with activity patterns against microorganisms (Griffin *et al.*, 1999).

High levels of monoterpene hydrocarbons and sesquiterpenes were found to lower the anitmicrobial activity of essential oils (Chalchat *et al.*, 1997). According to Griffin *et al.* (1999), aldehydes (e.g. cumin-aldehyde) are known to possess powerful antimicrobial activity. Griffin *et al.* (1999) however reported that alcohols had more activity against *S. aureus* than aldehydes. A number of essential oil samples in this study contained ketones as major compounds. The presence of an oxygen function in the framework increases the antimicrobial properties of terpenoids. Ketones are interesting for the treatment of mucupurulent infectious states (usually a strictly indirect action): verbenone, thujone, camphor, pinocamphone, cryptone, fenchone, menthone, piperitone and carvone.

Plant 1C did not inhibit *C. albicans* and the possible contributors could be *cis*-chrysaenthenyl acetate and bornyl acetate. It has been proven that terpene acetates and hydrocarbons tend to show little activity against bacteria and yeast regardless of their structural type. The little activity has been associated with low water solubility; log k_{ow} and hydrogen bonding capacity (Griffin *et al.*, 1999).

Specificity and level of activity were not always defined by the functional group present but were associated with hydrogen-bonding parameters in all cases, and for gram-negative organisms a combination of hydrogen-bonding parameters and molecular size parameters. The importance of subtle structural differences of the *p*-menthane terpenoids on molecular properties and activity, especially *Pseudomonas aeruginosa*, has also been demonstrated (Griffin *et al.*, 1999). The carbonylation of terpenoids was shown to increase the bacteriostatic and fungistatic activities specifically by contact method (Naigre *et al.*, 1996).

The gram-negative bacteria showed less sensitivity to the essential oil compared to the gram-positive organisms. This phenomenon may be related to the structure of the outer membrane of gram–negative bacteria. For example, *E. faecalis* and *P. aeruginosa* were not inhibited by any of the oils. But these bacteria are known for having an outer membrane, which is an effective permeability barrier (Nikaido & Vaara, 1985). Beside the impermeable outer membrane, many of the essential oils accumulate monoterpenes

and in general, monoterpenes perform poorly against *P. aeruginosa* (Naigre *et al.*, 1996). In enteric bacteria the outer membrane has been shown to provide protection from the detergent action of bile salts and degradation of digestive enzymes (Nikaido& Nakae, 1979). The reason why *E. coli*, which is an enteric bacterium was more sensitive to the essential oil compared to *P. aeruginosa* is that the porins of *P. aeruginosa* are larger than *E. coli* porins (Brown, 1975). According to Griffin *et al.,* (1999) it was established that the pre-treatment with polymixin B nonapeptide (PMBN), an outer membrane permeabilising agent significantly increased the initial rates and overall numbers of cells killed for all compounds.

According to Janssen (1987) the antiseptic efficacy of essential oils is generally proportional to their liposolubility. Besides the specific organism under study the following factors may hamper the evaluation of antimicrobial activity; variation of neat oil on the disc, disc size, the volatility of the oils at room temperature, the water insolubility of the oils, their complexity, technique of testing, quantity of the oil used for testing, agar composition and type of medium used. Medium constituents may react with essential oil components such as aldehydes, which can react with the sulfhydryl groups in proteins (Muenzing *et al.*, 1972). The incubation period for moulds is longer than for bacteria and the volatility of the essential oil may be responsible to the negative inhibition results obtained. Recently, Inouye *et al.* (2001) illustrated that the MIC value of essential oil were lowered 2-8 fold when evaporation was prevented.

Respiratory diseases are common ailments of the population in developing countries where there is high incidence of infection of the respiratory tract and bacterial complications of the common cold (Hall & McBride, 1988). There is also a problem of microorganisms being resistant, thus, it was important to establish if there is any scientific basis for the use of *A. afra* to treat colds, flu, cough, blocked nose and bronchitis, which are either of viral or bacterial origin. Some of the *A. afra* oil had an abundance of 1.8-cineole, which according to Silvestre *et al.* (1999), is economically important for the products to have, because of its pharmacological uses in the treatment of respiratory problems and antibacterial properties. The activity of 1.8-cineole was tested against a large number of bacteria and fungi. According to Pattnaik (1997), it was found that 1.8-cineole inhibited large number of bacteria including *S. aureus* at a concentration of 6.7 mg/ml^{-1} with the exception of two strains of *P. aeruginosa,* which

were resistant. In contrast Raman *et al.* (1995) did a study on tea tree oil and found 1.8-cineole to be inactive against *S. aureus* using TLC-bioautographic assay.

It was interesting to note that the oil from Qwa-qwa showed significant activity towards *C. neoformans* and plant 2B and 2C towards *C. albicans* because *C. neoformans* causes respiratory ailments. Phytomedicine have shown great promise in the treatment of respiratory ailment infections diseases including opportunistic AIDS infections (Iwu *et al.,* 1999). It has been proven that *Crytococcus neoformans* and *Candida* appear in the last stage of the illness (Verden & van Dremmer, 1992). Therefore it was interesting to note antimicrobial activity for *A. afra* oil. Due to the volatility of the oil aerosols can be made to treat pulmonary diseases, in complement to classic therapy. Plant volatile oils with antifungal activity can be incorporated into lotions that can be used topically to treat fungal skin infections.

Commercial preparations need to be standardized as *A. afra* oil showed immense chemical variation. The problems associated with standardization is a very complex matter with herbal products as the activity may not be ascribed to a single chemical entry, but ascribed to a mixture of constituents, some of which have not yet been identified. At the present time, most herbal products are standardized on the basis of the concentration of a single active or marker compound in a concentrated extract. If the active or marker compound is present in appropriate quantity, it is assumed that all the other necessary components are also represented and uniform activity is assumed. In this study plant 2C from Giant's Castle population proved to be the favourable plant to be cloned for cultivation. This plant accumulated camphene (6%), 1.8-cineole (15%), high content of camphor (49%) and no thujone. The essential oil showed activity towards nine microorganisms out of fourteen. The plant proved to be effective and safe to be used in the preparation of commercial products.

CHAPTER 4: CONCLUSIONS

- *Artemisia afra* displayed chemical variation both within and between natural populations. This observation was clearly illustrated by TLC and GC/MS analysis of the essential oils. With reference to essential composition it can be concluded that *A. afra* is a highly variable species. It was noted that the essential oils displayed variation in major and minor essential oil compounds.

- The chemical variation was erratic and did not correlate to geographical distribution. This was demonstrated by the cluster analysis. For example, plants from Klipriversberg in Gauteng Province may have the same chemical composition as plants from Giant's Castle (in KwaZulu Natal Province).

- The antimicrobial activity of *A. afra* also showed variation. This was not surprising as biological activity is a mere manifestation of the chemical composition. The *A. afra* essential oil had higher antifungal properties than antibacterial. The gram-positive bacteria were more sensitive to the essential oil compared to the gram-negative bacteria.

- The major compounds of *A. afra* when accessed on their own had no activity. Thus suggesting that the chemical compounds may be acting in a synergistic manner (i.e. combinations). Further studies need to be conducted to identify the active compounds. The activity could also be ascribed to minor compounds. Our investigation was narrowly focused on the major essential constituents.

- The use of essential oil of *A. afra* to treat respiratory ailments, colic and blocked nose has some scientific rationale, where the oil showed a moderate antimicrobial activity towards *S. aureus* and *K. pnuemoniae*. The essential oil can also aid people suffering from infections caused by *C. albicans* and *C. neoformans*.

- Due to chemical variation mentioned above, the commercial preparations need to be standardised. Standardisation may be achieved by the selection of a favourable chemotype based on efficacy (e.g. broad spectrum of antimicrobial activity) and safety (e.g. low thujone content) for cloning and cultivation.

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APPENDIX

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

African wormwood – essential oil composition, geographical variation and antimicrobial properties of a coveted traditional herbal remedy

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AFRICAN WORMWOOD – ESSENTIAL OIL COMPOSITION, GEOGRAPHICAL VARIATION AND ANTIMICROBIAL PROPERTIES OF A COVETED TRADITIONAL HERBAL REMEDY

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INTRODUCTION

Artemisia is a large and widespread genus in the family Asteraceae housing approximately 350 species (Tan *et al.* 1998). Members of the genus have been frequently used to treat diseases such as malaria, diabetes, hepatitis and infections caused by bacteria, fungi and viruses. Some of the traditional medicinal uses of *Artemisia afra* are summarised in Table 1.

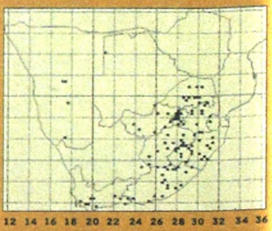

Figure 1: Geographical distribution of *Artemisia afra* in southern Africa.

Figure 2: An example of a commercial product derived from *Artemisia afra*.

Artemisia afra is also known as African wormwood, "umhlonyane" (Xhosa, Zulu), "lengana" (Sotho, Tswana) and Wilde als (Afrikaans) (Van Wyk *et al.* 1997). It is a multi-stemmed perennial shrub, growing up to two meters in height. It is characterized by glaucous feather-like leaves with a pungent aromatic smell. In South Africa, the plant is abundantly distributed in mountainous regions from South Western Cape, generally along the eastern coast extending through to the Northern Province.

Figure 3: *Artemisia afra* in habitat.

Table 1: Summary of the primary medicinal uses of *Artemisia afra*.

PLANT PART USED	TREATMENT	PREPARATION	REFERENCES
Leaf	Blocked nose	• Fresh leaf put into nose • Decoction / inhalant	Van Wyk *et al.* 1997 Van Wyk *et al.* 1997
Leaf	Colds	• Decoction / inhalant	Jacobs *et al.* 1995
Leaf	Coughs, Bronchitis, whooping coughs	• Infusion • Decoction	Watt & Breyer-Brandwijk, 1962 Hutchings *et al.* 1996
Leaf	Flu	• Decoction / inhalant • Inhalant fumes of squeezed leaf • Tea or enema	Jacobs *et al.* 1995 Watt & Breyer-Brandwijk, 1962 Watt & Breyer-Brandwijk, 1962
Leaf	Colic, digestive disorders	• Infusion	Van Wyk *et al.* 1997

OBJECTIVES

- To record the variation of essential oil composition within and between populations.
- To determine the antimicrobial activity of the essential oils and thus providing a scientific basis for the traditional use of *A. afra*.
- To emphasise the need to standardize favourable clones (in terms of efficacy and safety) for phytomedicinal purposes.

MATERIALS AND METHODS

Analysis of essential oil

Four natural plant populations were selected; **Setibeng** (Lesotho), **Giant's Castle** (Kwa-Zulu Natal), **Phuthaditshaba** (Free State) and **Kliprivérsberg** (Gauteng). Three plants were selected in each population except from Kliprivérsberg, where seven plants were collected. The essential oils were obtained through hydro-distillation in a Clevenger apparatus for 3 hours.

The essential oils were analyzed using thin layer chromatography and GC/MS.

A cluster analysis was done using the NTSYSpc (Rholf, 1997).

Antimicrobial Evaluation

Qualitative (disc diffusion) and quantitative (MIC) methods were used to evaluate the antimicrobial activity.

Death kinetic assays were performed on *Klebsiella pneumonia* (NCTC 9633), a respiratory pathogen associated with lung infections. Oil concentrations 0.063%, 0.125%, 0.25%, 0.5% and 0.75% with inoculum 1 X 10^6 was incubated at 37°C. At time intervals ranging from 0 min – 24 hrs, samples were taken and death kinetics expressed in a log_{10} reduction time-kill plot. A control was included in the study having the same broth formulation but without the oil.

RESULTS

Essential oil chemistry

The aerial part of *A. afra* is rich in essential oil. The GC-MS results indicate that the main chemical constituents, which appear frequently in the essential oil is α-thujone, β-thujone, camphor, borneol and 1.8-cineole. However, these compounds show immense quantitative variation within and between populations.

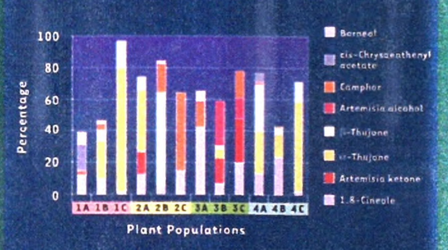

Figure 4: Quantitative variation in chemical constituents of *Artemisia afra*.

The figure above represents the variation of some main chemical constituents within and between the plant populations. Plant 1A from Setibeng is chemically very different from 1C collected from the same population. Plant 1C (Setibeng) is more similar to 4C (Qwa-qwa) collected in a distant population. These two plants accumulate α- and β-thujone, a potentially toxic substance (Watt & Breyer-Brandwijk, 1962).

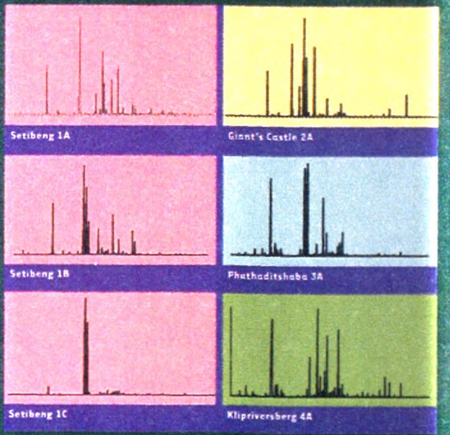

Figure 5: GC/MS profile of three plants from Setibeng population and one plant from Kliprivérsberg, Giant's Castle and Qwa-qwa populations

CLUSTER ANALYSIS

Figure 6: Dendrogram constructed on quantitative GC-MS data for seventeen samples of *Artemisia afra* collected from four natural populations.

Antimicrobial activity

Table 2: The zones of inhibition measured for seventeen samples (including the 6 mm of the disc).

[illegible]

(Setibeng: 1 A, B, C; Giant's Castle: 2 A, B, C; Kliprivérsberg: 3 A, B, C, D, E, F, G; Qwa-qwa: 4 A, B, C)

Time kill studies on *Klebsiella pneumonia*.

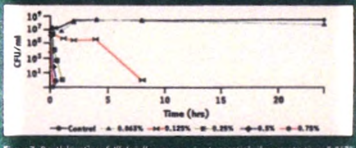

Figure 7: Death kinetics of *Klebsiella pneumonia* at essential oil concentrations 0.063%-0.75% over a 24 hr period.

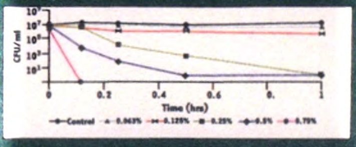

Figure 8: Death kinetics study of *Klebsiella pneumonia* showing activity within one hour.

CONCLUSIONS

- With reference to essential oil composition, *A. afra* is a highly variable species.
- *Artemisia afra* displays chemical variation both within and between natural populations.
- The variation is erratic and not correlated to geographical distribution.
- The variation in essential oil composition is reflected in the varying antimicrobial results. It is difficult to correlate the essential oil composition to the observed antimicrobial activity.
- The use of the essential oil of *Artemisia afra* to treat respiratory ailments is
- validated by the bactericidal activity on *Klebsiella pneumonia*.
- Selection of favourable chemotypes should be based on efficacy (i.e. wide spectrum anti-microbial activity) and safety (e.g low thujone content).

REFERENCES