THE COMPOSITION AND ANTIMICROBIAL ACTIVITY OF LEAF ESSENTIAL OILS OF SELECTED *AGATHOSMA* SPECIES (RUTACEAE)

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Declaration

I, Carla Fourie declare that this research report is my own work. It is being submitted for the degree of MSc (Med) Pharmaceutical Affairs in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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.....14th.....day of .....November....., 2003

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Abstract

This project was conducted to investigate the antimicrobial properties and to record the essential oil profiles of a selection of species belonging to the genus *Agathosma.* Plants have been used for many years by the local South Africans to treat various infections and illnesses. This knowledge has largely been untapped. Buchu is one of the plant species that are used extensively by the San and Khoi people. It is remarkable that of the ca. 150 *Agathosma* species indigenous to South Africa only two species (*Agathosma crenulata* and *Agathosma betulina*) have been investigated for biological activity. The genus *Agathosma* is traditionally used for the following conditions; stomach ailments, fever, coughs, colds, flu, urinary tract and kidney infections, haematuria, prostatitis, rheumatism, gout, bruises and for antiseptic purposes.

The antimicrobial activity and leaf essential oil chemistry were investigated for *A. arida, A. capensis, A. lanata, A. mundtii, A. ovalifolia, A. ovata, A. recurvifolia, A. serpyllaceae* and *A. zwartbergensis.* The phytochemistry of the essential oils was analyzed by using gas chromatography-mass spectrometry. The antimicrobial properties were analyzed by using disc diffusion assays, MIC/microplate assays and TLC bioautographic assays.

All the species showed some degree of antimicrobial activity. The minimum inhibitory concentrations of *A. capensis, A. ovata* and *A. recurvifolia* were determined. A TLC bioautographic assay for *A. zwartbergensis* indicated that citronellal could be responsible for the antimicrobial activity.

1. Introduction:

1.1 History

Historically, plants have been a source of inspiration to scientists searching for novel drug compounds and have made large contributions to human health. Natural compounds from plants may become a natural blue print for the development of new drugs or they may be used in the crude form for the treatment of disease based on their application by traditional societies from different parts of the world. Today it is estimated that plant products have contributed to 50% of Western drugs. The primary benefits of using plant-derived medicines are that they are relatively safe, offer profound therapeutic benefits and are more affordable (Iwu *et al.*, 1999).

Microbiologists have two reasons to be interested in the topic of antimicrobial plant extracts. Firstly, it is likely that these phytochemicals may be used, by physicians, as antimicrobial drugs and secondly, the public is becoming increasingly aware of the problems with over prescribing and misuse of antibiotics. Another driving factor for the renewed interest in plant antimicrobials has been the rapid rate of plant species extinction. The use of essential oils for healing purposes has been known in traditional medicine. It is still of interest today because of the trend back to natural drugs and therapies in medicine (Cowan, 1999).

Aromatherapy has been practiced for centuries. The Greeks, Romans and Egyptians all used aromatherapy oils. Hippocrates, the father of modern medicine, used aromatherapy baths and scented massage. The modern era of aromatherapy dawned in 1930 when the French chemist Rene Maurice Gattefosse used the term aromatherapy for the therapeutic use of essential oils. He began his research into the healing powers of essential oils after an accident in his laboratory when he burned his hand. He immersed his injured hand in a vat of lavender oil (containing linalool as major component) and was quite impressed with the healing results. He started investigating further healing properties of essential oils (www.naturalaromatherapy.com).

Commonly known herbs like cinnamon, ylang-ylang, basil, lemongrass, marjoram and rosemary are all essential oil containing plants. The antimicrobial and antioxidant

properties of these herbs together with lemon fruit were studied by Baratta *et al.* (1998). Generally all the oils, exhibited antibacterial activity.

One of the more recent success stories of essential oil research is that of Tea Tree oil obtained from *Melaleuca alternifolia*. For thousands of years the native Aborigines of Australia have used the leaves of the Tea Tree to cure various ailments. Early in this century, doctors and scientists began to realize that the natural oil contained in the leaves has healing properties. Today Tea Tree oil is recognized as an extremely effective curative for a wide range of common medical conditions (Combest, 2000).

1.2 Essential oils

Plants have a limitless ability to synthesize aromatic substances. Essential oils are volatile in steam and are generally mixtures of hydrocarbons and oxygenated compounds. The oxygenated compounds determine the odor and taste of volatile oils (Evans, 1996). These oils are secondary metabolites (compounds) based on an isoprene structure. These oils are called terpenes and occur as monoterpenes (C_{10}), sequiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}) and tetraterpenes (C_{40}), as well as hemiterpenes (C_5). When these compounds contain additional elements, usually oxygen, they are termed terpenoids (Cowan, 1999). Terpenoids comprise the largest organic chemical group, not only in essential oils, but also in natural products. Essential oils also contain other classes of molecules including phenolics, aromatics, cyclic and acyclic compounds, acetonides, and sulphur- and nitrogen-containing compounds, depending on the plant and the extraction method (Nakatsu *et al.,* 2000). Terpenes are active against bacteria and fungi. The mechanism of action is not fully understood but it might involve membrane disruption by the lipophilic compounds (Cowan, 1999). In general, gram-negative bacteria have been found to be more resistant to essential oils than gram-positive bacteria. Mangena and Muyima (1999) state a reason in an article on comparative evaluation of the antimicrobial activities of essential oils of *Artemisia afra*, *Pteronia incana* and *Rosmarinus officinalis* on selected bacteria and yeast strains, and can be attributed to the lipopolysaccharide cell wall of the gram-negative bacteria.

Essential oils (they are called essential because of the previous belief that each oil represented the essence of the original plant) of aromatic plant species are used in

industry for the production of soaps and perfumes. In view of the increasing use of essential oils in the food, cosmetic and pharmaceutical industries, it is important to examine the oils from indigenous plants for antimicrobial activities. Essential oils have been used as medicine since ancient times and form part of traditional folk medicine. Therefore they are considered to be the most widely used natural products (Nakatsu *et al.*, 2000).

1.3 Microbiological activity of essential oils

Testing and evaluating antimicrobial activity of essential oils is difficult because of their volatility, their water insolubility and complexity. Janssen *et al.* (1986) mentions that the following factors may change the composition of essential oils: the botanical source, the provenience of the plant material, the condition of the plant material (fresh or dried) and the isolation technique (steam distillation or hydrodistillation). These factors must be taken into consideration when evaluating the antimicrobial activity of essential oils. The following factors also influence the results of antimicrobial susceptibility tests of essential oils: methodology, the microorganisms and essential oils used.

Buchbauer (1993), states that essential oils are heterogeneous mixtures of single substances. Hence one has to be aware that biological actions are due to this complex mixture of various aromatic chemicals. Each constituent contributes to the biological effect in a complicated synergistic or antagonistic way.

Articles published by researchers in the last ten years, in the field of essential oil biological activity, focussed on the activity to inhibit microorganisms. Usually the essential oil inhibition on bacteria and fungi is determined followed by an investigation of the activity of the essential oil components. The most common method used for antimicrobial assays of essential oils is the disc diffusion method. The most frequently effectively used method to separate and identify essential oil components are by using gas chromatography coupled to mass spectrometry (GC-MS). This method makes use of database libraries of both retention indices and mass spectral fragmentation patterns (Nakatsu *et al.*, 2000).

The biological activity of spices and herbs has re-emerged as an area of interest. *Thymus* species is a common spice that has been extensively studied. The essential oil

that is isolated from this spice is active against gram-positive and gram-negative bacteria. The major constituent is thymol and has been implicated to be the molecule responsible for the activity. *Salvia* species or more commonly known as sage shows activity towards fungi and the major component that is responsible for the activity is cineole. Rosemary and *Origanum* species show antimicrobial activity. Anethole, limonene and fenchone found in fennel show antifungal activity. The major constituent of mint is menthol that plays a significant role in its activity towards bacteria and fungi. Eugenol commonly found in clove inhibits the growth of yeast (Nakatsu *et al.*, 2000).

The chemical composition and antimicrobial activity of the essential oil of *Calamintha nepeta* was investigated by Flamini *et al.* (1999). They found that the essential oil showed strong activity against *Salmonella* species and noteworthy effectiveness against mycetes parasites like *Aspergillus niger* (which can be pathogenic for man both by its spores an by the production of mycotoxins). In their investigations they also verified those constituents, which may be responsible for the strong activity against *Salmonella* species. The main constituents limonene, menthone, pulegone, and menthol were determined by using GC and of these the only one showing antibacterial activity was pulegone.

The essential oil of *Crysanthemum viscidehirtum* consists mainly of limonene, β-farnesene and many oxygenated sesquiterpenes. According to Khallouki *et al.* (2000) this essential oil exhibits activity against particularly *Salmonella typhi* and *Proteus mirabilis.*

Cobos *et al.* (2001) investigated the chemical composition and antimicrobial activity of the essential oil of *Baccharis notosergila.* The chemical analysis was done by using GC-MS and the main components were determined as α-pinene, limonene, β-caryophyllene and spathulenol. The antimicrobial activity was determined by using two techniques: the well diffusion and the paper disc diffusion method. The more susceptible strains were the gram-positive bacteria. *Proteus mirabilis* was the only gram-negative bacteria that were inhibited by the essential oil and *Candida albicans* demonstrated good sensitivity. They came to the conclusion that essential oils containing monoterpenes like limonene are more active against gram-positive

organisms and fungi than gram-negative organisms. They also found that spathulenol was found to be active against *Candida albicans* and *Proteus mirabilis.*

Mangena and Muyima (1999), compared the antimicrobial activity of *Artemisia afra, Pteronia incana* and *Rosmarinus officinalis*, *A. afra* had a broad-spectrum antibacterial activity and the main chemical constituents were α-thujone and β-thujone. *P. incana* displayed a fairly broad-spectrum antibacterial activity and the main constituents were β-pinene and α-pinene. *R. officinalis* had the same activity as *A. afra* but the main constituents differ, being camphor, 1,8-cineole and α-pinene for *R. officinalis. A. afra* seemed to produce larger zones of inhibition than *P. incana* and *R. officinalis* when the oils were tested on yeasts.

Composition and activity of *Salvia ringens* essential oil was studied by Tzakou *et al.* (2001) and the major constituents were 1,8-cineole, bornyl acetate, β-pinene and α-pinene. Their antibacterial results showed that *S. ringens* appeared to be inactive against gram-positive bacteria (*S. aureus* and *S. epidermidis*) while it showed strong activity towards gram-negative bacteria. They also screened the pure monoterpenoids α-pinene and 1,8-cineole to compare the results with the investigated oil. They came to the conclusion that the activity can be attributed to these two constituents especially 1,8-cineole.

1.4 The Rutaceae

The genus *Agathosma* belongs to the citrus family (Rutaceace). Trees, shrubs or shrublets belonging to the family Rutaceace are usually aromatic plants. The family is distributed in both temperate and tropical countries, but particularly abundant in South Africa and Australia. Glands containing essential oils are present in the leaves and other parts. The flowers are usually in cymes with 4-5 sepals, 4-5 petals, 8-10 stamens and a superior ovary. The fruits are of various types. Constituents of the Rutaceace include a wide variety of alkaloids, volatile oils, rhamno-glucosides, coumarins and terpenoids (Evans, 1996).

1.5 The genus *Agathosma*

The Cape region of South Africa has veld-types with arguably the richest composition of indigenous aromatic plant species in the whole of South Africa. Among these aromatic plants is the genus *Agathosma* that is restricted to this region. These shrubs are typical of the fynbos vegetation and are found in mountainous areas in the Cape (van Wyk and Gericke, 2000).

Agathosma species or commonly known as buchu, are perennial shrubs with woody branches and small, flat, gland dotted leaves. The flowers are star-shaped and open. The flowers contain five petals, five stamens, a five-lobbed ovary and the leaves have a characteristic smell when crushed (van Wyk *et al.*, 1997). The name buchu is applied by the Hottentots to any aromatic herb or shrub. The Buchuberg in Namaqualand does not derive its name from the genus *Agathosma* but from other aromatic plants. In Griqualand West *Othonna* species are known as "buchu" and is used as a cosmetic. *Empleurum* species commonly referred to as false buchu and berg buchu has also been referred to in trade as "buchu". The genus *Diosma* is often referred to as "wild buchu" and is used only when true buchu is not available (Watt and Breyer-Brandwijk, 1962).

Finding healing powers in plants is an ancient idea and so Buchu is an important part of the Khoi culture in the Cape and is still used as a general tonic and medicine throughout South Africa. It is also well documented for its cosmetic purposes.

A summary of the traditional uses of the genus *Agathosma* is listed below (Watt and Breyer-Brandwijk, 1962);

- To cause febrifuge (profuse perspiration).
- As an antispasmodic.
- As an antipyretic.
- A cough remedy, as well as for colds and flu.
- Kidney and urinary tract infections, as well as for hematuria and protatitis.
- To relief rheumatism, gout and bruises.
- As a diuretic and for genito-urinary system infections.
- Has been used as a liniment or embrocation.
- For relief of calculus.

- Is used for treatment of cholera and other stomach ailments.
- Has been used for antiseptic purposes.

Agathosma species have been used for cosmetic purposes and as an antibiotic. The leaves were used in a variety of preparations. The leaves were chewed or prepared in a tincture containing brandy to relieve stomach complaints. A mixture of buchu and vinegar is still being used today to clean wounds (van Wyk *et al.,* 1997). Boiling water is poured over 1g buchu leaves, covered and allowed to stand for 10 minutes before being strained. A cup of the infusion is drunk several times a day as a diuretic (Bisset, 1994).

Of the 150 species, *Agathosma betulina* (round-leaf buchu) and *A. crenulata* (oval-leaf buchu) are mainly used for medicinal purposes. These two species are important sources of valuable essential oils (van Wyk and Gericke, 2000). The essential oil of *A. betulina* is a dark yellow-brown oil with a minty-camphoraceous odour. *Agathosma betulina* and *A. crenulata* are cultivated and are in the process of being developed as crop plants. These two species are the only two that have been used commercially. This is due to the limited availability of the wild plant material (van Wyk and Gericke, 2000). Buchu forms part of about 10 prepared herbal teas, including Buccotean^{R} Tee, Buccosperin^{R} Tee, Uron-Tee tea bags and is a constituent of the UK product Potter's Kas-bah Herb. Other preparations available in the UK containing buchu are: Potter's Diuretictabs, Antitis Tablets, Backache Tablets, Stomach Mixture, Gerard House Herbal Powder and Buchu Compound Tablets (Bisset, 1994).
Agathosma serratifolia (narrow-leaf buchu) a willow-like small tree, *A. pulchella* and *A. ovata* (false buchu, Figure 1) a small rounded shrub with pink flowers, have also been used for medicinal purposes among the Cape people (van Wyk and Gericke, 2000).

1.6 Previous research on *Agathosma*

Buchu oil is a rarely studied oil and Lawrence (1976) summarized the different studies, in Progress In Essential Oils, reprinted from Perfumer & Flavorist, until 1976. In 1961 Fluck and coworkers succeeded in identifying pulegone and diophenol as

constituents of buchu oil. The first comprehensive analysis of buchu oil was published in 1968 by Klein and Rojahn in which they isolated and characterized seventeen compounds. Lamparsky and Schudel isolated 8-mercapto-*p*-menthan-3-one from Buchu oil in 1971 and found that this sulphur containing terpene was very important for the flavour and aroma of the oil.

Figure 1: *Agathosma ovata* (George Botanical Garden).

The most detailed and thorough study was by Kaiser *et al.* (1975) on the analysis of Buchu leaf oil. They identified more than 120 constituents including the already known pulegone, diosphenol and 8-mercapto-*p*-menthan-3-one in Buchu leaf oil of commercial origin. Their study was conducted to determine its aromatic important components. They determined that characteristic of the oil is the occurrence of bifunctional monoterpene ketones which can be classified as sulphurated and oxygenated derivatives of *p*-menthan-3-one. The mixture of 2-acetoxypulegones gives buchu oil a minty, hay-like odour. They also acknowledge that sensoric properties differ when comparing oils from different producers and materials of different botanical origin. In their study they compared an oil distilled in South Africa originating from *Barosma betulina*, an oil distilled in South Africa originating form *Barosma crenulata* and a so-called "English distilled" oil. They concluded that the more important aromatic 8-mercapto-*p*-menthan-3-one is present in larger quantities in *Barosma betulina* than in *Barosma crenulata* (*Barosma* is an old synonym of *Agathosma*).

Since then only a few studies have appeared. Two articles were published in the Journal of Essential Oil Research (1996). The one article, by Posthumus *et al.* (1996)

was on the chemical composition of the essential oils of *A. betulina*, *A. crenulata* and a Buchu hybrid. Chemical investigation was done by means of chromatographic and spectroscopic methods and their ultimate aim was to recognize plants with a specific chemical composition. The results indicated that buchu contains a few common monoterpenes and a number of rare bi- and trifunctionalized monoterpenes. These compounds included two diosphenols, hydroxylated diosphenols, several hydroxymenthones and some acetates thereof. They made use of the publication by Kaiser *et al.* (1975) that contained the peak order and the chromatogram and they used the highly characteristic mass spectra of these constituents to identify them. Further they identified in total 40 known compounds. According to their study *A. betulina* is characterized by 31% of (iso)menthone, 41% (ψ)-diosphenol and 3% *cis*- and *trans*-8-mercapto-*p*-menthane-3-ones, *A. crenulata* contains very high quantities of pulegone (54%) and the hybrid showed a high concentration of (iso)menthone (55%) and otherwise a intermediate composition. Collins *et al.* (1996) reported on the chemotaxonomy of commercial Buchu species. The oils of *A. betulina* and *A. crenulata* and their hybrids were analyzed to determine if they could be distinguished by their monoterpene content. *Agathosma betulina* and *A. crenulata* are mainly distinguished by their leaf form were *A. betulina* has more round leaves and is also known as round leaf buchu and *A. crenulata* has more oval leaves. Hybridization has occurred and has led to confusion in identification. Their study resulted in that all three taxa contained the same constituents but in different percentages. *Agathosma betulina* had high percentages of limonene, menthone, isomenthone, (ψ)-diosphenol, diosphenol, *cis*-8-mercapto-*p*-menthan-3-one, 4-hydroxy-diosphenol and 1-hydroxy-diosphenol. *Agathosma crenulata* had a very high percentage of pulegone and isopulegone isomers and a higher percentage of 8-acetylthio-p-menthan-3-one isomers than *A. betulina.* The hybrids had the simultaneous presence of high concentration of pulegone and diosphenol.

Round-leaf buchu contains a valuable essential oil rich in isomenthone and diosphenol. Oval-leaf buchu yields a less desirable oil because of the high levels of pulegone, a potentially toxic substance (van Wyk and Gericke, 2000).

Other components of the essential oil of *Agathosma betulina* are limonene, (-)-isomenthone, (+)- menthone, (-)-pulegone, terpinen-4-ol and *p*-menthan-3-on-8-thiol. *p*-Menthan-3-on-8-thiol is mainly responsible for the odour of this species.

Diosphenol separates in its crystalline form at room temperature, which is called buchu camphor. Unlike the round, serrulate leaves of *A. betulina*, the leaves of other *Agathosma* species contain little or no diosphenol. TLC and GLC can be used to distinguish between the leaves of *A. betulina* and *A. crenulata* seeing that the diosphenol zone or peak will be absent with the latter species (Bisset, 1994).

The main objective of this study is to investigate the possible antimicrobial properties of a selection of species belonging to the genus *Agathosma* (Rutaceae) and to record the essential oil profiles. An attempt will be made to correlate the antimicrobial activity to the essential oil composition.

2. Material and Methods

2.1 Collection of plant material

Agathosma species are indigenous to the Cape Province of South Africa. All specimens were collected from the wild and voucher specimens and are housed in the Bolus Herbarium, University of Cape Town and at the National Herbarium in Pretoria.

Table 1: List of study samples and percentage oil yield after distillation.

Species	Locality	Voucher	Yield (%)
A. arida	Rooiberg	TTS-241	0.61
A. capensis	Gamka	TTS-243	1.76
A. capensis	Rooiberg	JEV-5	0.74
A. capensis	Mossel Bay	JEV-7	0.68
A. lanata	Rooiberg	TTS-242	0.37
A. mundtii	Rooiberg	TTS-238	0.27
A. ovalifolia	Droekloof	TTS-260	1.02
A. ovata	Gamka	TTS-246	0.22
A. ovata	Anysberg	TTS-263	0.21
A. recurvifolia	Rooiberg	TTS-240	0.14
A. serpyllaceae	Heuningberg	JEV-11	0.31
A. zwartbergensis	Swartberg	TTS-257	1.78

Mr. Trinder-Smith, from the University of Cape Town, who is currently completing a PhD on the taxonomy of the genus *Agathosma,* confirmed the identity of all specimens.

2.1.1 Extraction of essential oils

Conventional hydrodistillation (Figure 2) was carried out using a Clevenger-type apparatus to selectively extract the essential oils from the plant material. The plant material is weighed before distillation and the amount of oil is weighed after distillation to determine the yield of oil. The essential oils are stored and refrigerated in amber vials to minimize stability problems often encountered with essential oils.

2.2 Antimicrobial testing

2.2.1 Test organisms

Selection of the test organisms took place by taking the traditional uses of *Agathosma* into consideration. *Agathosma* is used traditionally to relieve stomach complaints and for minor digestive disturbances, therefore *E. faecalis, E. coli, B. cereus* and *S. typhimurium* were selected. Buchu is used for washing and cleaning wounds, therefore *S. aureus* and *P. aeruginosa* were selected. Buchu is also used to treat kidney and urinary tract infections, hence *C. albicans* and *E. coli* were selected as pathogens.

Bacterial test organisms were as follow: *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739), *Bacillus cereus* (ATCC 11778) and *Salmonella typhimurium* (clinical strain).
Fungal test organisms were as follow: *Candida albicans* (ATCC 10231), *Cryptococcus neoformans* (ATCC 90112) and *Aspergillus niger* (clinical strain).

2.2.2 Disc diffusion assay

Four large Mueller-Hinton (Oxoid) agar plates were prepared containing 100 ml of agar and 100 ml of overlayed agar. Overnight broth cultures of the following bacteria; *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Enterococcus faecalis*, with an inocculum size $1x10^6$ was used to seed the agar. The bacterial spore suspensions were incorporated into the top agar layer. Smaller agar plates (Figure 3) were prepared for *Salmonella typhimurium* and *Bacillus cereus*. The smaller round petri dishes contained 15 ml of agar and 15 ml of agar seeded with inoculum.

Aseptic techniques were used to saturate discs (Figure 4) with essential oils and then placed onto the seeded agar plates. A disc containing Neomycin 30 µg, (Oxoid) was used as a positive bacterial control. The screening plates were refrigerated for 1 hour to allow pre-diffusion and then incubated at 37 °C for 24 hours, after which zones of inhibition were measured.

Figure 2: Charging the stills with plant material.

Figure 3: Preparation of plates for disc diffusion method by pouring base layer and proceeding with inoculated top layer.

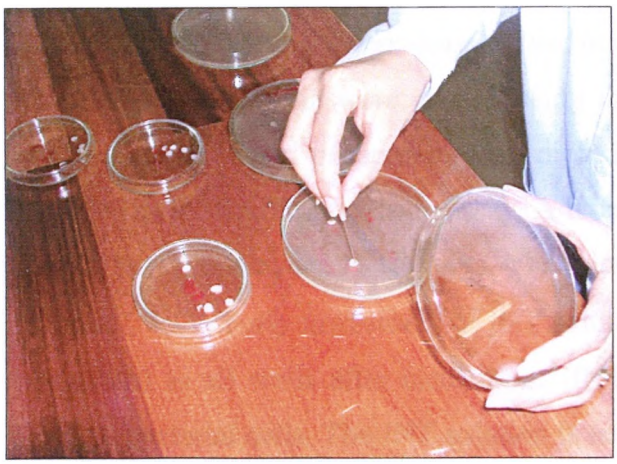


Figure 4: Aseptic transfer of discs with *Agathosma* essential oil onto seeded agar plate.

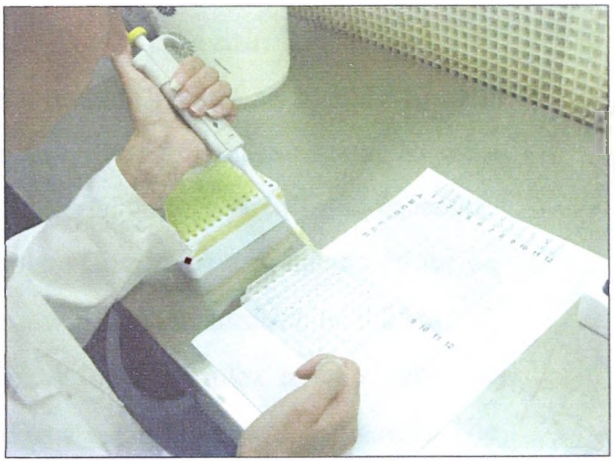


Figure 5: Preparing the MIC plate by performing doubling dilutions of *Agathosma* oil.

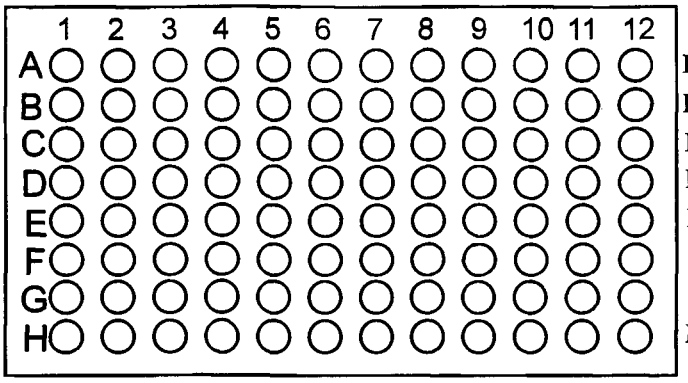
A second series of fungal test organisms namely *Candida albicans, Cryptococcus neoformans* and *Aspergillus niger* were also studied. A disc containing Nystatin (100 lμ, Oxoid) was used as a positive control. Tryptone Soya (Oxoid) agar was used for the fungi and the same process as described above was followed to prepare the agar and the saturated disc.

2.2.3 MIC/microplate bioassay method

After antimicrobial activity was recorded by the disc diffusion assay, the minimum inhibitory concentrations (MIC) of the most active oils were determined by using the *p*-iodonitrotetrazolium violet (INT) microplate method. A fixed bacterial culture yielding an inoculum size of $1x10^6$ was added to all wells. Further more, this method involves dilution of the essential oils in microplate test conditions. An essential oil concentration of 32 mg/ml was prepared in the first row of wells in the microplate (Figure 5). Serial dilutions were performed (Figure 6). The addition of the oils to the microplate as well as the serial dilutions took place under laminar airflow to minimize contamination.

Minimum inhibitory concentration (MIC) of the following essential oils; *A. ovata* (Gamka and Anysberg), *A. capensis* (Gamka) and *A. recurvifolia,* were determined for *Staphylococcus aureus, Enterococcus faecalis* and *Escherichia coli.*

These oils and bacteria were chosen using the screening results as a guide to their positive antimicrobial properties. Overnight broth cultures of these organisms were prepared as for the disc diffusion assay. A fixed bacterial culture yielding an inoculum size of $1x10^6$ was added to all wells. The microplate (Figure 5) was incubated for 24 hours at 37°C. Forty microliters of INT solution, with a concentration of 0.2 mg/ml, was added to all wells after incubation. INT binds in a complex manner with the DNA in bacterial cells. The INT solution is used as a bacterial growth indicator. The pink colour change was observed after 30 minutes, 2 hours and 24 hours. The minimum inhibitory concentration, of each sample of hydrodistilled oil, was calculated. Minimum inhibitory concentrations were determined presumptively as the first well, in ascending order, which did not produce a colour change.



Row A: Concentration of essential oil = 32 mg/ml
Row B: Concentration of essential oil = 16 mg/ml
Row C: Concentration of essential oil = 8 mg/ml
Row D: Concentration of essential oil = 4 mg/ml
Row E: Concentration of essential oil = 2 mg/ml
Row F: Concentration of essential oil = 1 mg/ml
Row G: Concentration of essential oil = 0.5 mg/ml
Row H: Concentration of essential oil = 0.25 mg/ml

Column 1: *A. capensis* (Gamka) and *Escherichia coli*
Column 2: *A. capensis* (Gamka) and *Staphylococcus aureus*
Column 3: *A. capensis* (Gamka) and *Enterococcus faecalis*
Column 4: *A. ovata* (Gamka) and *Escherichia coli*
Column 5: *A. ovata* (Gamka) and *Staphylococcus aureus*
Column 6: *A. ovata* (Gamka) and *Enterococcus faecalis*
Column 7: *A. ovata* (Anysberg) and *Escherichia coli*
Column 8: *A. ovata* (Anysberg) and *Staphylococcus aureus*
Column 9: *A. ovata* (Anysberg) and *Enterococcus faecalis*
Column 10: *A. recurvifolia* and *Escherichia coli*
Column 11: *A. recurvifolia* and *Staphylococcus aureus*
Column 12: *A. recurvifolia* and *Enterococcus faecalis*

Figure 6: Serial dilutions of the essential oils of *A. capensis* (Gamka), *A. ovata* (Gamka), *A. ovata* (Anysberg) and *A. recurvifolia.*

2.3 Analytical chemistry

2.3.1 Thin layer chromatography (TLC)

Silica thin layer plates were used together with toluene-ethyl acetate (93:7) as mobile phase. Detection was made possible with the use of spray reagents namely vanillin-sulphuric acid and anisaldehyde-sulphuric acid.

After development, the TLC plates were air-dried and sprayed with one of the reagents. Colour development took place after a short heating period.

2.3.2 Gas chromatograph-Mass Spectrometry (GC-MS)

Essential oils were analyzed using the following operating 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.). Helium as carrier gas.

0.9 μl of hexane with 0.1 μl of the essential oil was injected. Identification took place with the use of TBAM's database libraries by matching both retention indices and mass spectral fragmentation patterns. This part of the project was completed by myself at the Medicinal and Aromatic Plant and Drug Research Centre, Anadolu University, Turkey.

2.3.3 TLC bioautographic assay

The hydrodistilled oil of *Agathosma zwartbergensis* was chosen for the TLC bioautographic assay. A *Bacillus cereus* spore suspension with an inoculum size of $1x10^6$ was incorporated into Mueller Hinton agar. TLC plates of the essential oil and of a standard of the main compound were developed and placed directly onto the prepared agar plate. The TLC plates were developed by using toluene-ethyl acetate (93:7) as the mobile phase and vanillin-sulphuric acid spray reagent to detect the compounds of the oil. The TLC overlay-agar plate was incubated for 24 hours.

3. Monographs of *Agathosma* species studied

Agathosma arida P.A. Bean

1. Botanical description

Single-stemmed, rounded shrublet to 40cm, sweetly herb-scented. Flowers in terminal clusters, pink or violet. Fruits: 3-chambered. Ovary: usually 3-lobed.

2. Distribution

Gravelly loam, karoo-fynbos ecotone. This species is restricted to the Little Karoo, specifically the northern slopes of Langeberg and Outeniqua Mountains (Goldblatt and Manning, 2000).

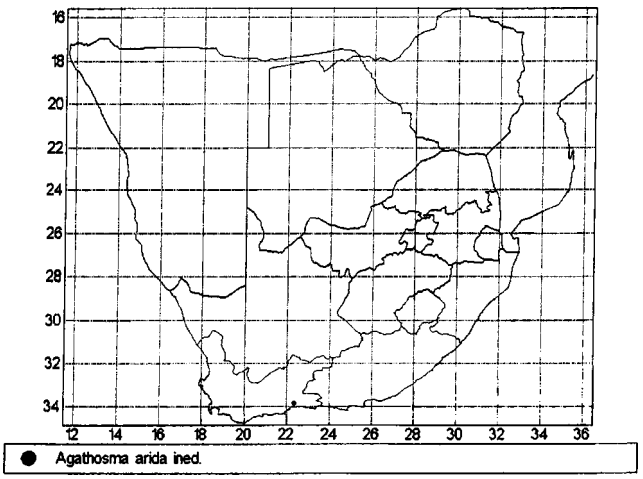


Figure 7: Geographical distribution of *A. arida.*

3. Essential oil composition

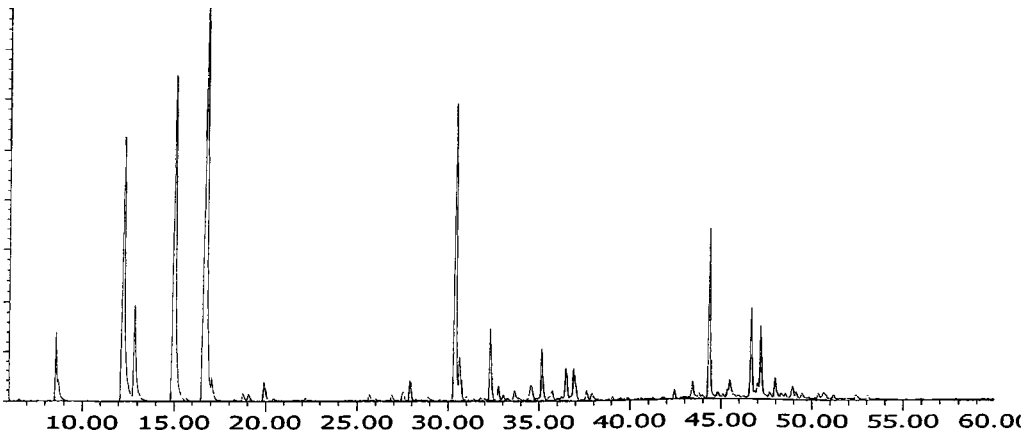


Figure 8: Gas chromatography profile of *A. arida.*

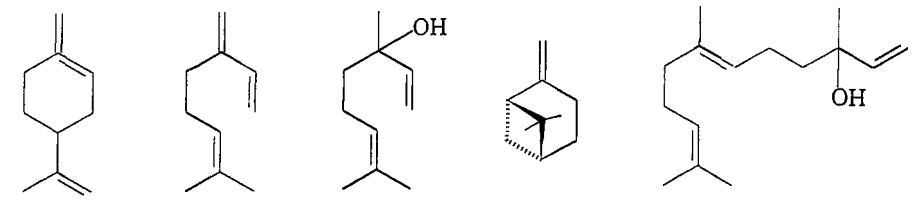
Table 2: GC-MS results of *A. arida.*

RRI*	Retention time (minutes)	Area percentage	Compound
1032	8.57	1.80	α-pinene
1035	8.70	0.10	α-thujene (not integrated)
1076	10.30	0.01	camphene
1118	12.20	**12.28**	**β-pinene**
1132	12.86	3.98	sabinene
1174	14.98	**16.35**	**myrcene**
1188	15.65	0.10	α-terpinene
1203	16.57	**21.79**	**limonene**
1218	17.00	0.83	β-phellandrene
1246	18.32	0.07	(Z)-β-ocimene
1255	18.76	0.29	γ-terpinene
1266	19.07	0.25	(E)-β-ocimene
1280	19.91	0.82	p-cymene
1290	20.42	0.10	terpinolene
1319	21.50	0.04	(E)-2,6 dimethyl 1,3,7 nonatriene
1337	22.20	0.09	geijerene
1429	25.97	0.07	perillen
1430	26.17	0.03	α-thujone
1451	26.88	0.21	β-thujone
1468	27.52	0.38	*trans*-1,2-limonene epoxide
1495	28.27	0.06	bicycloelemene
1490	28.58	0.02	isogeijerene C
1506	28.91	0.15	decanal
1541	29.91	0.01	benzaldehyde
1553	30.40	**11.02**	**linalool**
1562	30.63	1.74	isopinocamphone
1571	31.01	0.15	*trans*-p-menth-2-en-1-ol
1586	31.38	0.07	pinocarvone (not pure)
1594	31.77	0.07	*trans*-β-bergamotene (not pure)
1611	32.33	2.49	terpinen-4-ol
1626	32.75	0.47	2-methyl-6-methylene-3,7-octadien-2-ol
1639	33.02	0.15	*trans*-p-menth-2,8-dien-1-ol
1645	33.27	0.08	*cis*-isodihydrocarvone
1657	33.65	0.35	umbellulone
1678	34.32	0.04	*cis*-p-menth-2,8-dien-1-ol
1687+	34.56	0.76	α-humulene+methylchavicol
1700	34.86	0.03	p-mentha-1,8-dien-4-ol
1706	35.15	1.75	α-terpineol
1722	35.72	0.46	dodecanal (not pure)
1755	36.44	1.22	bicyclogermacrene
1763	36.76	0.09	naphthalene
1772+	36.89	1.43	citronellol+decanol(major)
1804	37.91	0.20	myrtenol
1845	39.06	0.09	*trans*-carveol
1857	39.24	0.01	geraniol
1864	39.42	0.02	p-cymen-8-ol
1882	39.89	0.04	*cis*-carveol
1896	40.44	0.01	*cis*-p-mentha-1(7)8 dien-2-ol
1945	41.78	0.03	neo-isodihydrocarveol
1973	42.43	0.28	dodecanol
2008	43.38	0.54	caryophyllene oxide (not pure)
2030	43.75	0.41	methyleugenol
2050	44.32	**5.55**	**(E)-nerolidol**
2071	44.82	0.14	humulene epoxide II
2096+2096	45.46	0.58	elemol+(E)-methyl cinnamate
2113	45.96	0.07	cumin alcohol
2144	46.62	3.00	spathulenol
2148	47.00	0.27	dictamnol

RRI*	Retention time (minutes)	Area percentage	Compound
2247	48.96	0.39	*trans*-α-bergamotol
2255	49.14	0.17	α-cadinol
2316	50.54	0.01	caryophylladienol I
2324	50.65	0.10	caryophylladienol II
2389	51.89	0.04	caryophyllenol I
2392	52.39	0.18	caryophyllenol II (not pure)
	Total	**94.33**	

RRI*-relative retention indices calculated against n-alkanes

The main compounds of *A. arida* are the four monoterpenes; limonene (21.79%), myrcene (16.35%), β-pinene (12.28%), linalool (11.02%) and the sequiterpene alcohol (E)-nerolidol (5.55%).



limonene myrcene linalool β-pinene (E)-nerolidol

Figure 9: Chemical structures of the major compounds identified in *A. arida* essential oil.

Agathosma capensis (L.) Dummer

1. Common name

Boegoe.

2. Botanical description

Resprouting shrub to 90 cm, sweetly spice-scented. Flowers in lax terminal clusters, white, pink and purple. Fruits: 3-chambered. Ovary: usually 3-lobed.

3. Distribution

Slopes and flats on shale, granite or coastal sands, less then often on acid sand. This species is distributed form Namaqualand to Port Elizabeth (Goldblatt and Manning, 2000).

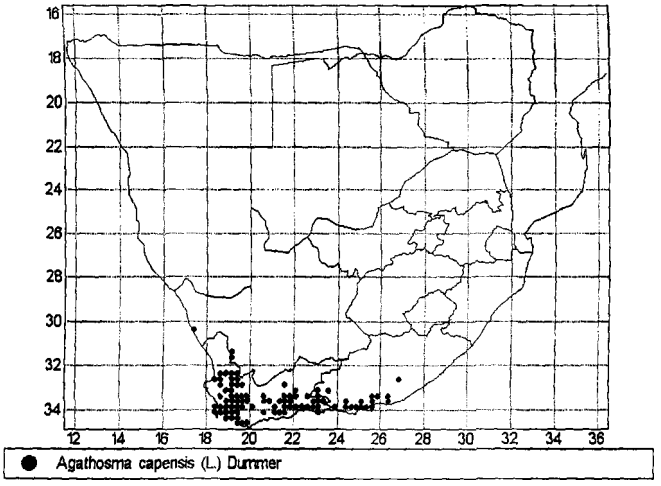


Figure 10: Geographical distribution of *A. capensis.*

4. Essential oil composition

Three different samples of *A. capensis* were analyzed by GC-MS. One sample was harvested from the Gamka Mountains, the second sample from the Rooiberg region in the Cape Province and the third species was collected from Mossel Bay.

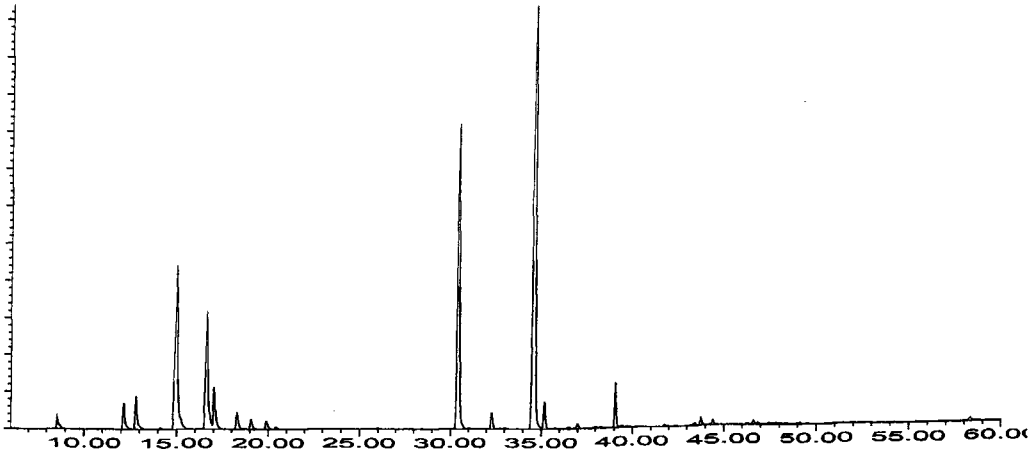


Figure 11: Gas chromatography profile of *A. capensis* (Gamka).

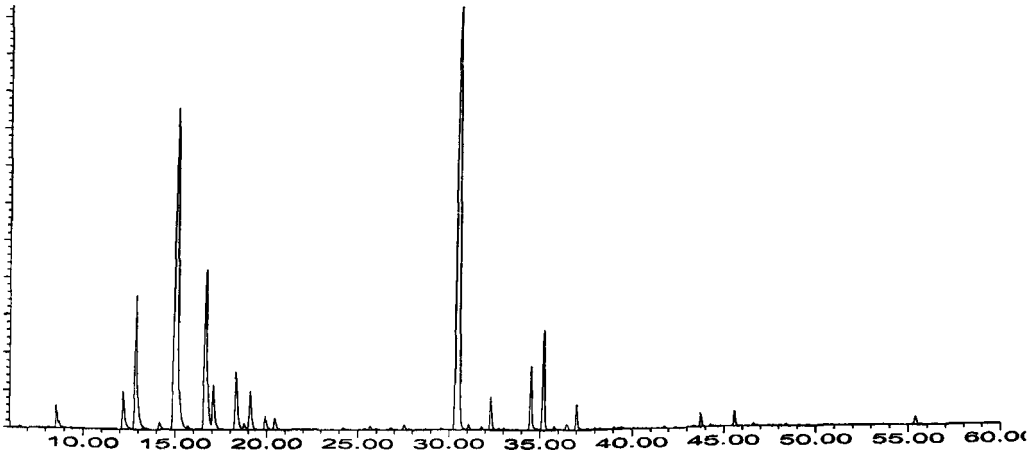


Figure 12: Gas chromatograpy profile of *A. capensis* (Rooiberg).

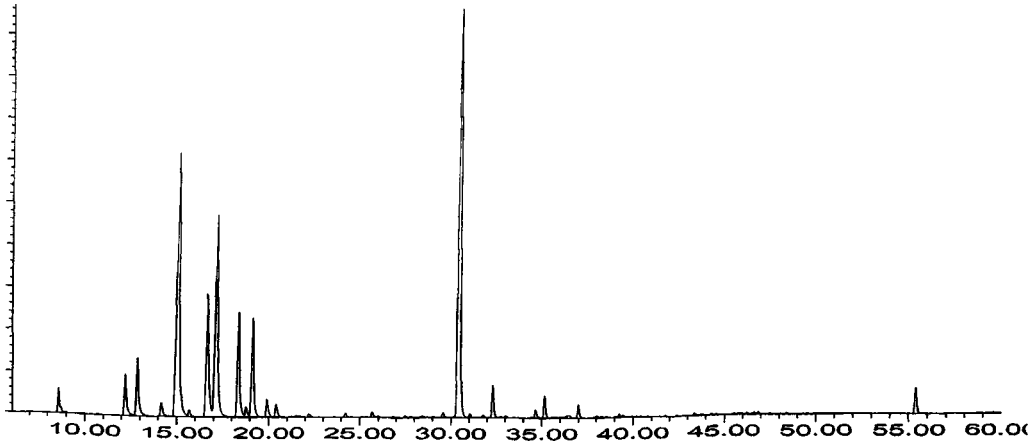


Figure 13: Gas chromatography profile of *A. capensis* (Mossel Bay).

Table 3: GC-MS results of *A. capensis* (Gamka) – A, *A. capensis* (Rooiberg) – B and *A. capensis* (Mossel Bay) – C.

RRI*	Retention time	Area percentage (A)	Area percentage (B)	Area percentage (C)	Compound
1018	8.15	-	0.01	-	methyl-2-methyl-buteryte
1032	8.57	0.64	0.76	0.95	α-pinene
1035	8.71	0.10	0.20	0.20	α-thujene (not integrated)
1076	10.30	-	0.01	0.01	camphene
1118	12.17	1.81	2.08	2.44	β-pinene
1132	12.86	2.20	**7.43**	3.40	**sabinene**
1159	14.14	0.12	0.39	0.86	δ-3-carene
1174	15.03	**14.13**	**25.31**	**20.88**	**myrcene**
1188	15.68	-	0.14	0.30	α-terpinene
1195	16.15	-	0.01	-	dehydro-1,8-cineole
1203	16.65	**9.43**	**9.62**	**8.23**	**Limonene**
1218	17.04	2.97	2.40	**13.36**	**β-phellandrene**
1246	18.23	1.19	3.08	**6.39**	**(Z)-β-ocimene**
1255	18.74	0.04	0.29	0.53	γ-terpinene
1266	19.04	0.67	2.03	**5.88**	**(E)-β-ocimene**
1280	19.89	0.58	0.71	1.07	p-cymene
1290	20.42	-	0.62	0.74	terpinolene
1319	21.48	-	-	0.02	(E)-2,6-dimethyl-1,3,7-nonatriene
1337	22.20	0.02	0.01	0.18	geijerene
1382	24.21	0.02	0.09	0.22	*cis*-alloocimene
1391	24.51	-	-	0.02	(Z)-3-hexenol
1429	25.98	0.12	-	0.07	perillen
1450	26.79	0.05	0.10	0.02	*trans*-linalool oxide (furanoid)
1469	27.49	-	-	0.13	*trans*-1,2-limonene epoxide
1474	27.53	0.03	0.29	-	*trans*-sabinene hydrate
1476	27.74	-	-	0.01	(Z)-β-ocimene epoxide
1478	27.81	0.02	-	-	*cis*-linalool oxide (furanoid)
1498	28.46	0.04	0.02	0.04	(E)-β-ocimene epoxide
1553	30.43	**21.54**	**31.71**	**26.74**	***linalool***
1571	31.02	0.07	-	0.17	*trans*-p-menth-2-en-1-ol
1571	31.07	-	0.19	-	methyl-citronellate
1591	31.79	-	0.11	0.12	myrcenone
1600	31.97	-	-	0.11	β-elemene
1611	32.30	-	1.43	1.58	terpinen-4-ol
1626	32.76	0.03	0.05	0.02	2-methyl-6-methylene-3,7-octadien-2-ol
1638	33.02	0.07	0.04	0.11	*cis*-p-menth-2-en-1-ol
1664	33.94	-	-	0.01	*trans*-pinocarveol
1678	34.32	-	0.01	-	*cis*-p-menth-2,8-dien-1-ol

RRI*	Retention time	Area percentage (A)	Area percentage (B)	Area percentage (C)	Compound
1682	34.36	-	-	0.01	δ-terpineol
1687	34.64	**36.87**	2.57	-	**methyl-chavicol**
1690	34.65	-	-	0.36	cryptone
1700	34.90	-	Trace	-	p-mentha-1,8-dien-4-ol
1706	35.15	1.47	4.14	1.03	α-terpineol
1726	35.75	0.03	0.14	0.02	germacrene D
1743	36.07	-	0.01	-	α-cadinene
1744	36.21	-	-	0.02	phellendral
1748	36.33	-	0.01	0.01	piperitone
1755	36.47	-	0.28	-	bicyclogermacrene
1755	36.47	0.04	-	-	bicyclogermacrene+ carvone
1758	36.85	-	0.01	-	*cis*-piperitol
1772	36.99	0.31	1.10	0.58	citronellol
1802	37.90	-	-	0.03	cumin aldehyde
1808	38.02	-	0.07	-	nerol
1830	38.59	0.02	0.03	0.05	2,6-dimethyl-3(E),5(E),7-octatriene-2-ol
1845	39.04	-	0.09	0.03	*trans*-carveol
1845	39.06	2.24	-	-	(E)-anethole
1857	39.24	-	0.03	0.14	geraniol
1864	39.41	0.10	0.11	0.09	p-cymen-8-ol
1868	39.67	0.02	0.01	-	(E)-geranyl acetone
1860	39.71	-	-	0.01	8-epi-dictamnol
1949	41.75	0.09	0.08	-	(Z)(3)-hexenyl-nonanoate
2030	43.74	0.40	0.51	0.06	methyleugenol
2050	44.30	-	0.07	0.03	(E)-nerolidol
2053	44.39	0.31	-	-	anisaldehyde
2069	44.82	-	0.01	-	germacren-D-4-ol
2096	45.47	-	0.01	-	elemol
2096	45.57	-	0.51	-	(E)-methyl-cinnamate
2113	45.93	0.03	0.01	0.05	cumin alcohol
2144	46.62	0.22	0.11	0.06	spathulenol
2148	46.84	0.08	-	0.11	dictamnol
2187	47.70	Trace	-	-	t-cadinol
2200	47.87	0.05	-	-	*trans*-methyl isoeugenol
2209	48.05	0.01	-	-	t-muurolol
2219	48.39	0.01	0.06	0.07	dimyrcene-II a
2255	49.11	0.09	0.03	-	α-cadinol
2269	49.53	-	0.02	-	dimyrcene-II b
2353	51.41	0.04	-	-	chavicol
	Total	**98.32**	**99.16**	**97.57**	

RRI* - relative retention indices calculated against n-alkanes

The major compounds of *A. capensis* (Gamka) were determined as the four monoterpenes methyl-chavicol (36.87%), linalool (21.54%), myrcene (14.13%) and limonene (9.43%). The major compounds of *A. capensis* (Rooiberg) were determined as the four monoterpenes linalool (31.71%), myrcene (25.31%), limonene (9.62%) and sabinene (7.43%). The six monoterpenes identified in *A. capensis* (Mossel Bay) are linalool (26.74%), myrcene (20.88%), β-phellandrene (13.36%), limonene (8.23%), (Z)-β-ocimene (6.39%) and (E)-β-ocimene (5.88%).

All three samples had the following similar major compounds: linalool, myrcene and limonene. The differences further are the major compound of *A. capensis* (Gamka), methyl-chavicol is not a major compound of *A. capensis* (Rooiberg) and *A. capensis* (Mossel Bay). Likewise sabinene is a major compound for *A. capensis* (Rooiberg) but not for *A. capensis* (Gamka) and *A. capensis* (Mosel Bay), and β-phellandrene and(E)-β-ocimene are major compounds for *A. capensis* (Mossel Bay) but not for *A. capensis* (Gamka) and *A. capensis* (Rooiberg).

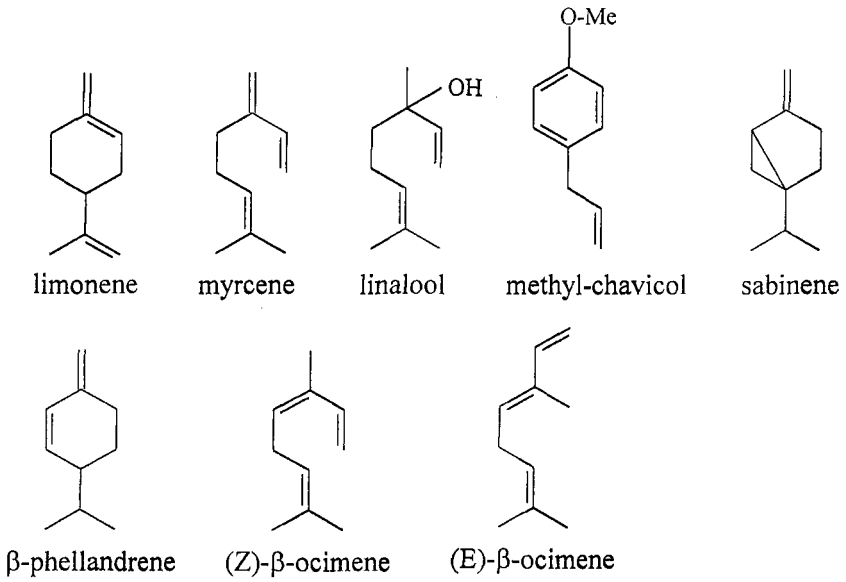


Figure 14: Chemical structures of the major compounds identified in *A. capensis* essential oils.

Agathosma lanata P.A. Bean

1. Botanical description

Dense, harsh, rounded shrub to 80cm, branching profusely at ground level, herb-scented. Flowers in dense, woolly terminal clusters, white. Fruits: 3-chambered. Ovary: usually 3-lobed.

2. Distribution

Dry rocky upper slopes. Rooiberg and Outeniqua Mountains (Goldblatt and Manning, 2000).

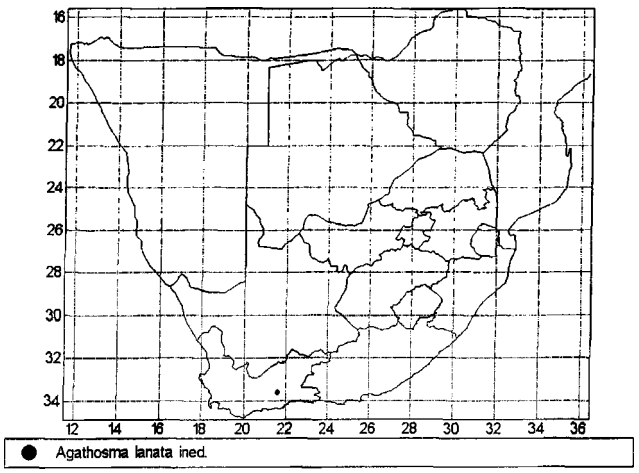


Figure 15: Geographical distribution of *A. lanata.*

3. Essential oil composition

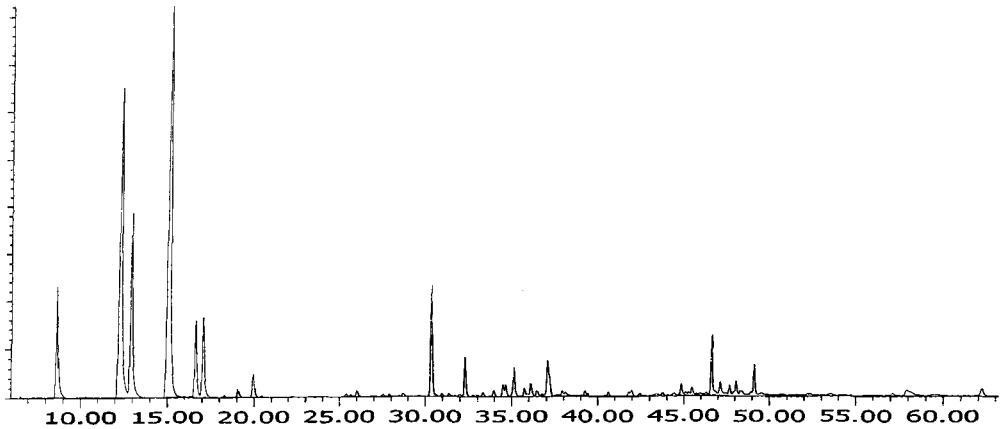


Figure 16: Gas chromatography profile of *A. lanata.*

4. GC-MS results

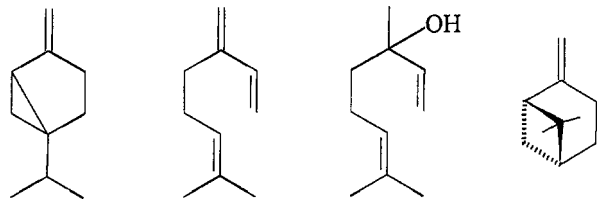
Table 4: GC-MS results of *A. lanata.*

RRI*	Retention time (minutes)	Area percentage	Compound
1032	8.57	4.01	α-pinene
1035	8.70	1.00	α-thujene (not integrated)
1076	10.30	0.03	camphene
1118	12.20	**21.57**	**β-pinene**
1132	12.86	**10.22**	**sabinene**
1174	14.98	**30.70**	**myrcene**
1203	16.57	4.39	limonene
1218	17.00	4.52	β-phellandrene
1246	18.32	0.13	(Z)-β-ocimene
1255	18.76	0.01	γ-terpinene
1266	19.07	0.44	(E)-β-ocimene
1280	19.91	1.31	p-cymene
1290	20.42	0.06	terpinolene
1337	22.20	0.05	geijerene
1429	25.97	0.33	perillen
1451	26.88	0.02	β-thujone
1466	27.42	0.03	α-cubebene
1474	27.55	0.10	*trans*-sabinene hydrate
1495	28.27	0.01	bicycloelemene
1498	28.47	0.04	(E)-β-ocimene epoxide
1497	28.69	0.17	α-copane
1535	29.64	0.04	β-bourbonene
1553	30.40	**5.21**	**linalool**
1571	31.01	0.05	*trans*-p-menth-2-en-1-ol
1586	31.36	0.13	pinocarvone
1600	31.97	0.11	β-elemene
1611	32.30	1.73	terpinen-4-ol
1626	32.75	0.06	2-methyl-6-methylene-3,7-octadien-2-ol
1638	33.02	0.15	*cis*-p-menth-2-en-1-ol
1648	33.31	0.19	myrtenal
1664	33.94	0.28	*trans*-pinocarveol
1687	34.47	0.51	methyl chavicol
1690	34.65	0.58	cryptone
1706	35.15	1.41	α-terpineol
1726	35.75	0.42	germacrene D
1740	36.10	0.61	α-muurolene
1755	36.50	0.30	bicyclogermacrene (not integrated)
1763	36.76	0.08	naphthalene
1773	37.06	1.51	δ-cadinene
1776	37.30	0.60	gamma-cadinene (not integrated)
1804	37.91	0.20	myrtenol
1810	38.10	0.12	3,7-guaiadiene
1830	38.58	0.03	2,6 dimethyl 3(E),5(E),7 octatriene-2-ol
1845	39.06	0.01	*trans*-carveol
1853	39.24	0.21	*cis*-calamenene
1864	39.42	0.09	p-cymen-8-ol
1860	39.71	0.02	8-epi-dictamnol
1893	40.42	0.05	dodecyl acetate
1900	40.62	0.20	epi-cubebol
1941	41.52	0.01	α-calacorene-1
1957	41.90	0.10	cubebol (not integrated)
1973	42.43	0.10	dodecanol
2030	43.75	0.12	methyleugenol
2069	44.84	0.50	germacrene D-4-ol
2080	45.10	0.09	cubenol

RRI*	Retention time (minutes)	Area percentage	Compound
2088	45.27	0.04	1-epi-cubenol
2096	45.47	0.35	elemol
2113	45.93	0.06	cumin alcohol
2144	46.62	2.42	spathulenol
2187	47.70	0.37	t-cabinol
2209	48.07	0.52	t-muurelol
2219	48.31	0.07	δ-cadinol
2219	48.40	0.10	dimyrcene II a (not integrated)
2247	48.96	0.09	*trans*-α-bergamotol
2255	49.14	0.17	α-cadinol
2269	49.52	0.04	dimyrcene II b
	Total	**99.18**	

RRI*-relative retention indices calculated against n-alkanes

The major compounds of *A. lanata* are the two linear monoterpenes myrcene (30.70%) and linalool (5.21%) and the two biciclic monoterpene hydro carbons β-pinene (21.57%) and sabinene (10.22%).



sabinene myrcene linalool β-pinene

Figure 17: Chemical structures of the major compounds identified in *A. lanata* essential oil.

Agathosma mundtii Cham. and Schltdl

1. Common name

Jakkalspisbos.

2. Botanical description

Single-stemmed; sometimes resprouting, finely velvetly, wiry shrub to 1m, foetid. Flowers in terminal or axillary clusters, white. Fruits: 2-chambered, flatsided. Ovary: usually 1- or 2-lobed.

3. Distribution

Middle to upper dry rocky slopes. Distributed from the Witteberg to Humansdorp (Goldblatt and Manning, 2000).

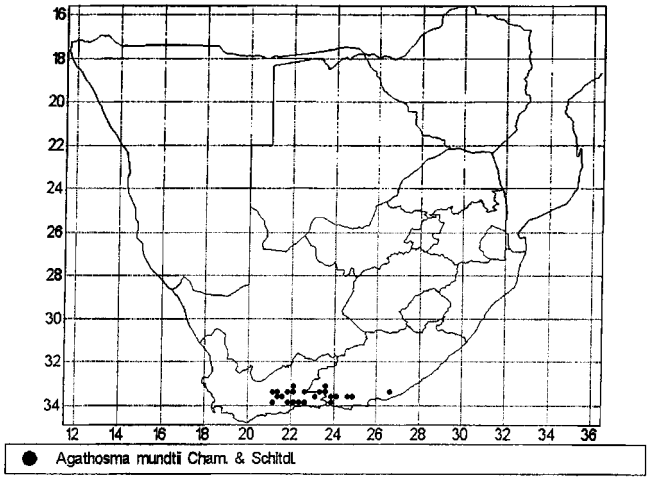


Figure 18: Geographical distribution of *A. mundtii.*

4. Essential oil composition

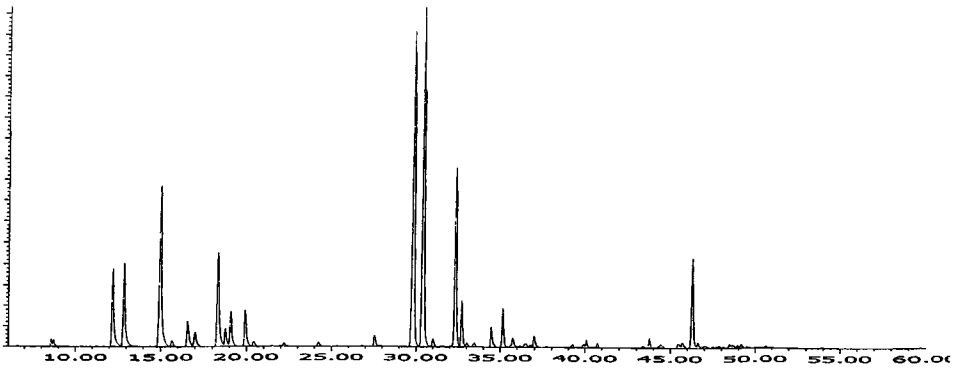


Figure 19: Gas chromatography profile of *A. mundtii.*

Table 5: GC-MS results of *A. mundtii.*

RRI*	Retention time (minutes)	Area percentage	Compound
1032	8.57	0.26	α-pinene
1035	8.71	0.28	α-thujene
1118	12.20	**4.62**	**β-pinene**
1132	12.86	**4.79**	**sabinene**
1174	14.98	**10.28**	**myrcene**
1188	15.65	0.35	α-terpinene
1203	16.57	1.55	limonene
1218	17.00	0.99	β-phellandrene
1246	18.32	**5.84**	**(Z)-β-ocimene**
1255	18.76	1.01	γ-terpinene
1266	19.07	2.11	(E)-β-ocimene
1280	19.91	2.22	p-cymene
1290	20.42	0.27	terpinolene
1319	21.50	0.04	(E)-2,6 dimethyl 1,3,7 nonatriene
1327	21.96	0.02	3 methyl-2-butenol
1337	22.20	0.15	geijerene
1382	24.22	0.24	*cis*-alloocimene
1413	25.34	0.05	rosefuran
1429	25.97	0.01	perillen
1450	26.80	0.05	*trans*-linalool oxide (furanoid)
1474	27.55	0.61	*trans*-sabinene hydrate
1478	27.85	0.04	*cis*-linalool oxide (furanoid)
1487	28.20	0.01	citronellal
	29.82	**20.00**	**BP 69 M+ 172 (unidentified)**
1553	**30.40**	**19.19**	**linalool**
1571	31.01	0.41	*trans*-p-menth-2-en-1-ol
1604	32.01	0.03	thymolmethylether
1611	32.33	**9.62**	**terpinen-4-ol (not pure)**
1638	33.02	0.20	*cis*-p-menth-2-en-1-ol
1687	34.49	1.09	methyl-chavicol
1700	34.86	0.02	p-mentha-1,8-dien-4-ol
1706	35.15	1.97..	α-terpineol
1722	35.64	0.05	2-undecanol
1726	35.75	0.48	germacrene D
1755	36.44	0.06	bicyclogermacrene
1758	36.50	0.10	*cis*-piperitol (not integrated)
1763	36.76	0.08	naphtalene
1772	36.99	0.62	citronellol
1797	37.70	0.06	benzyl-isobutyrate
1845	39.05	0.01	(E)-anethole
1854	39.24	0.15	germacrene B
1864	39.42	0.01	p-cymen-8-ol
1880	40.09	0.31	benzyl -2-methylbutyrate
1902	40.72	0.21	benzyl-isovalerate
1916	40.96	0.04	α-agarofuran
1949	41.75	0.02	(Z)(3)-hexenyl-nonanoate
1988	42.86	0.02	2-phenylethyl-2-methylbutyrate
2008	43.38	0.13	caryophyllene oxide
2030	43.75	0.41	methyleugenol
2050	44.32	0.06	(E)-nerolidol
2057	44.42	0.10	ledol
2096	45.46	0.28	elemol
2103	45.72	0.28	guaiol
2127	46.28	4.47	10-epi-γ-eudesmol
2144	46.62	0.20	spathulenol
2157	47.04	0.06	5-epi-7-epi-α-eudesmol
2184	47.48	0.03	*cis*-p-menth-3-en-1,2-diol
2209	48.09	0.03	t-muurolol

RRI*	Retention time (minutes)	Area percentage	Compound
2232	48.62	0.06	bulnesol
2247	48.96	0.10	*trans*-α-bergamotolol
2257	49.13	0.16	β-eudesmol
2282	50.64	0.04	(Z)-isoeugenol
	Total	**96.95**	

RRI*-retention indices calculated against n-alkanes

Various GC-MS libraries could not identify the major compound of *A. mundtii.* This compound had a base peak of 69 and a molecular mass of 172. 76.95% (61 compounds) of the essential oil composition was identified with the following six monoterpenes as major compounds: linalool (19 %), myrcene (10 %), terpinen-4-ol (9.62%), (Z)-β-ocimene (5.84%), sabinene (4.79%) and β-pinene (4.62%).

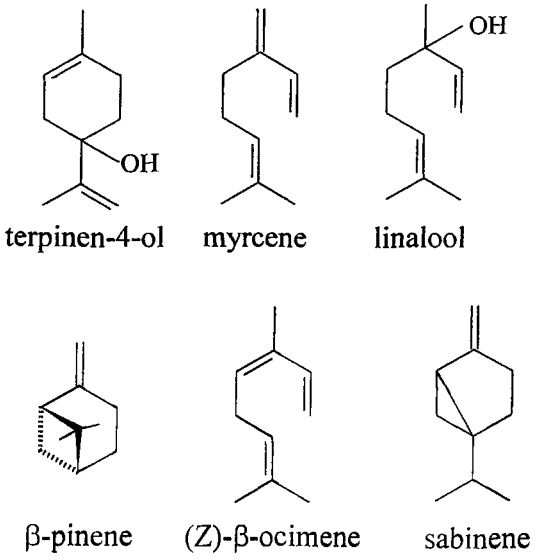


Figure 20: Chemical structures of the major compounds identified in *A. mundtii* essential oil.

Agathosma ovalifolia Pillans

1. Botanical description

Single-stemmed, rounded shrub to 1.5m, acrid or spice-scented. Flowers in lax terminal clusters white, red-dotted. Fruits: 2-chambered. Ovary: usually 1- or 2-lobed.

2. Distribution

Rocky quartzitic upper slopes. This species is distributed from the Swartberg Mountains to Willowmore (Goldblatt and Manning, 2000).

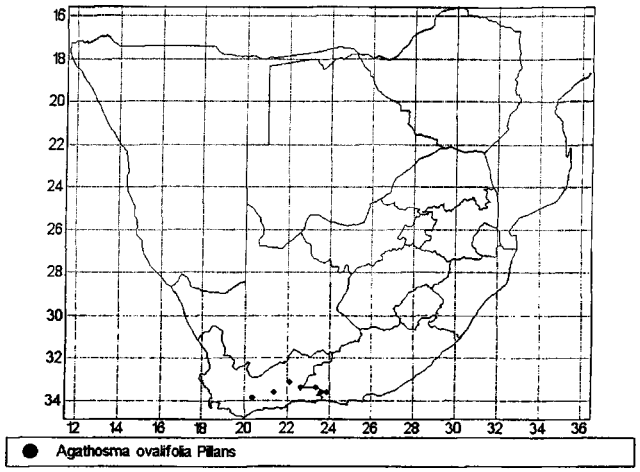


Figure 21: Geographical distribution of *A. ovalifolia.*

3. Essential oil composition

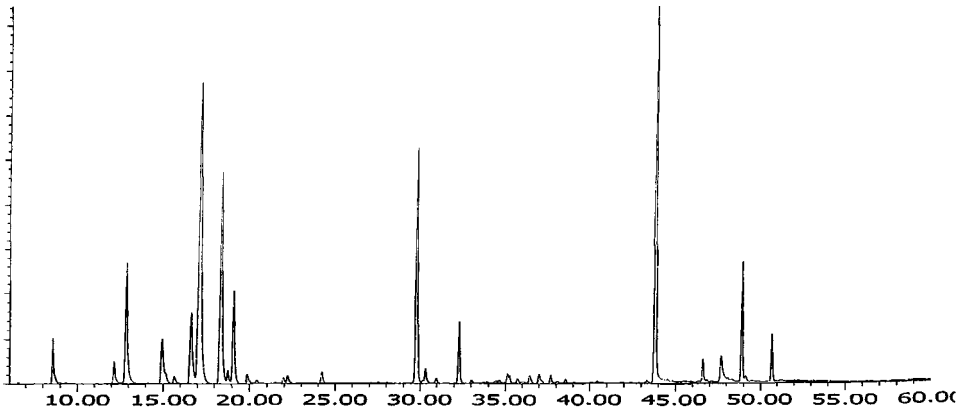


Figure 22: Gas chromatography profile of *A. ovalifolia.*

Table 6: GC-MS results of *A. ovalifolia.*

RRI*	Retention time	Area percentage	Compound
1032	8.58	1.55	α-pinene
1035	8.70	0.20	α-thujene (not integrated)
1118	12.18	1.18	β-pinene
1132	12.87	**6.36**	**sabinene**
1159	14.13	0.03	δ-3-carene
1174+1176	14.93	2.57	myrcene+α-phellandrene
1183	15.10	0.10	pseudo-limonene
1188	15.64	0.39	α-terpinene
1203	16.59	**4.36**	**limonene**
1218	17.16	**20.86**	**β-phellandrene**
1246	18.38	**12.23**	**(Z) β-ocimene**
1255	18.78	0.62	γ-terpinene
1266	19.11	**4.84**	**(E) β-ocimene**
1280	19.91	0.53	p-cymene
1290	20.41	0.17	terpinolene
1319	21.49	0.06	(E)-2,6-dimethyl-1,3,7-nonatriene
1327	21.98	0.25	3-methyl-2-butenol
1337	22.19	0.44	geijerene
1382	24.21	0.59	*cis*-alloocimene
1451	26.87	0.03	β-thujone
1460	27.07	0.06	2,6-dimethyl-1,3-(E),5(E),7octatetraene
1474	27.54	0.07	*trans*-sabinene hydrate
1487	28.04	0.07	isoneroloxide I
1495	28.24	0.01	bicycloelemene
1498	28.45	0.01	(E)-β-ocimene epoxide
1553	30.55	0.67	linalool
1571	31.04	0.25	*trans*-p-menth-2-en-1-ol
1591	31.48	0.02	pregeijerene
1611	32.30	2.69	terpinen-4-ol
1638	33.02	0.13	*cis*-p-menth-2-en-1-ol
1655	33.67	0.01	2,6-dimethyl-5-hepten-1-ol
1661	33.81	0.03	*trans*-pinocarvyl acetate
1682	34.38	0.05	δ-terpineol
1687	34.40	0.10	methyl-chavicol (not integrated)
1690	34.63	0.12	cryptone
1706	35.15	0.30	α-terpineol
1720	35.30	0.20	*trans*-sabinol
1726	35.73	0.19	germacrene D
1755	36.44	0.40	bicyclogermacrene
1773+1772	37.01	0.46	δ-cadinene+citronellol
1797	37.68	0.36	benzyl isobutyrate
1815	38.07	0.03	2,6-dimethyl-3(E),5(Z),7-octatriene-2-ol
1830	38.55	0.18	2,6-dimethyl-3(E),5(E),7-octatriene-2-ol
1845	39.04	0.03	*trans*-carveol
1880	40.07	0.03	benzyl 2-methylbutyrate
1896	40.43	0.06	phenyl ethyl isobutyrate
2030	43.83	**16.84**	**methyleugenol**
2098	45.49	0.04	globulol
2144	46.62	0.91	spathulenol
2148	46.80	Trace	dictamnol
2186	47.65	0.60	eugenol
2248	48.878	**4.45**	**elemicine**
	Total	***86.69***	

RRI*-relative retention indices calculated against n-alkanes

The major compounds of *A. ovalifolia* are the five monoterpenes β-phellandrene (20.86%), (Z)-β-ocimene (12.23%), sabinene (6.36%), (E)-β-ocimene (4.84%) and limonene (4.36%). The major compounds also include methyleugenol (16.84%) and elemicine (4.45%).

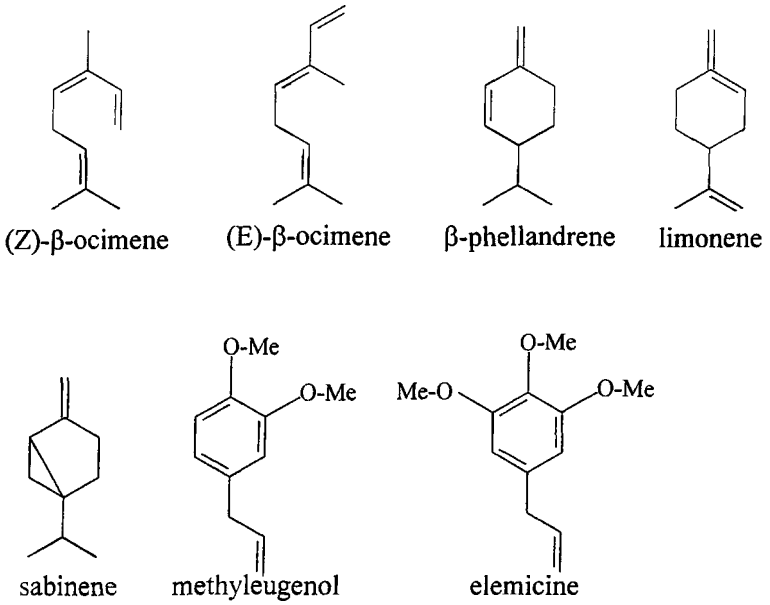


Figure 23: Chemical structures of the major compounds identified in *A. ovalifolia* essential oil.

Agathosma ovata Thunb

1. Common name

Basterboegoe.

2. Botanical description

Leafy shrub, usually single-stemmed to 3m, herb-scented. Flowers auxiliary, white, pink or purple. Fruits: 5-chambered. Ovary: usually 4- or 5-lobed.

3. Distribution

Rocky sandstone and silcrete on open slopes and forest margins. This species is distributed from the Witteberg to Lesotho (Goldblatt and Manning, 2000).

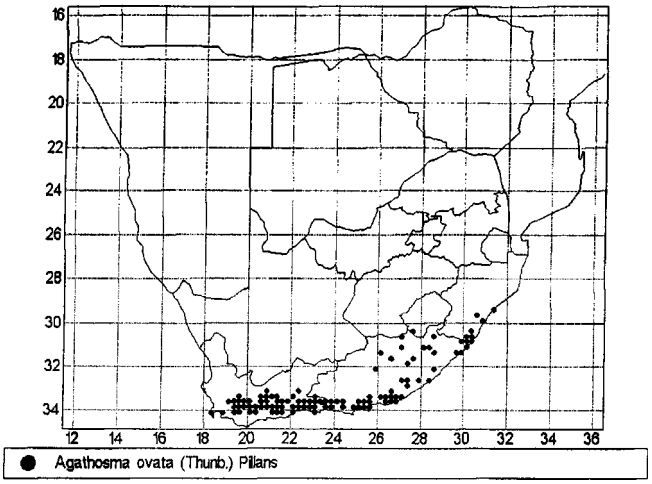


Figure 24: Geographical distribution of *A. ovata.*

4. Essential oil composition

Two different samples of *A. ovata* were analyzed by GC-MS. The one sample was collected form the Gamka Mountains and the other was collected from the Anysberg region in the Cape.

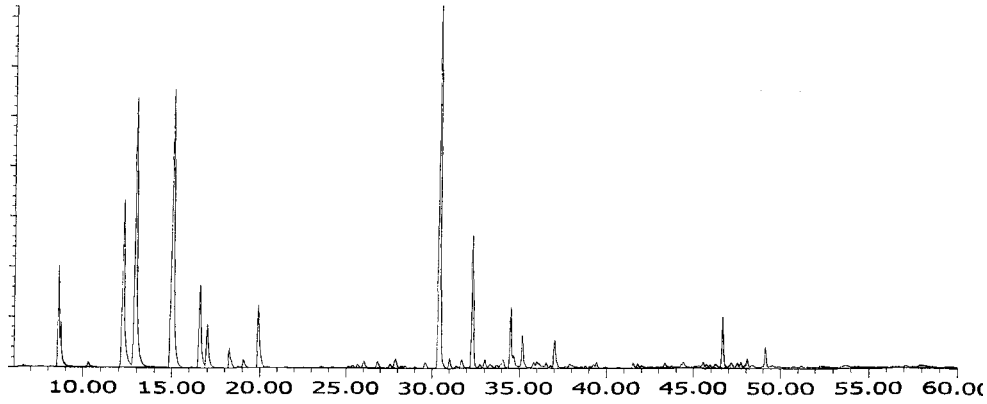


Figure 25: Gas chromatography profile of *A. ovata* (Gamka).

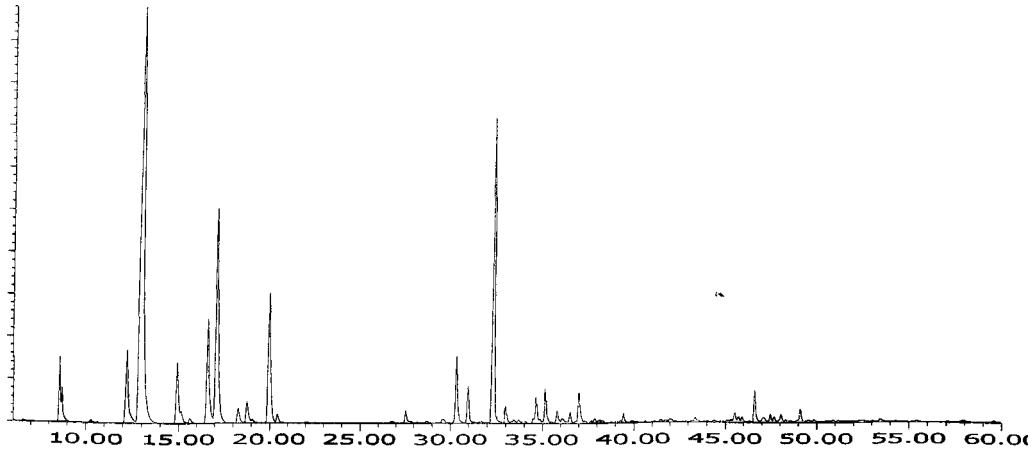


Figure 26: Gas chromatography profile of *A. ovata* (Anysberg).

Table 7: GC-MS results of *A. ovata* (Gamka) – A and *A. ovata* (Anysberg) – B.

RRI*	Retention time	Area percentage (A)	Area percentage (B)	Compound
1017	8.13	0.01	-	4-methyl-2-pentanone
1032	8.58	3.39	2.00	α-pinene
1035	8.70	1.82	1.29	α-thujene
1076	10.30	0.22	0.11	camphene
1118	12.24	**9.47**	4.12	**β-pinene**
1132	12.87	**15.68**	**31.43**	**sabinene**
1159	14.17	0.02	0.01	δ-3-carene
1174	15.03	**17.18**	2.89	**myrcene**
1176	15.20	-	0.30	α-phellandrene (not integrated)
1188	15.63	-	0.23	α-terpinene
1195	16.15	0.03	0.01	dehydro-1,8-cineole
1203	16.67	4.58	**5.64**	**limonene**
1218	17.05	2.32	**12.06**	**β-phellandrene**
1246	18.31	1.02	0.72	(Z)-β-ocimene
1255	18.76	0.04	1.05	γ-terpinene
1266	19.08	0.39	0.15	(E)-β-ocimene

RRI*	Retention time	Area percentage (A)	Area percentage (B)	Compound
1280	19.91	3.40	**6.91**	**p-cymene**
1290	20.42	0.03	0.40	terpinolene
1319	21.49	0.02	0.02	(E)-2,6-dimethyl-1,3,7-nonatriene
1382	24.22	0.03	0.01	*cis*-alloocimene
1424	25.63	-	0.01	o-methylanisol
1429	26.02	0.33	-	perillen
1452+1450	26.72	-	0.04	α-p-dimethylstyrene + *trans*-linalool oxide (furanoid)
1450	26.80	0.36	-	*trans*-linalool oxide (furanoid)
1466	27.40	-	0.48	α-cubebene
1474	27.54	0.16	0.16	*trans*-sabinene hydrate
1476	27.72	0.02	-	(Z)-β-ocimene epoxide
1478	27.88	0.50	-	*cis*-linalool oxide (furanoid)
1487	28.23	0.05	-	citronellal
1498	28.45	0.10	-	(E)-β-ocimene epoxide
1497	28.71	0.03	-	α-copaene
1505	28.71	-	0.05	dihydroedulene II
1532	29.59	0.36	0.27	camphor
1553	30.55	**17.81**	3.08	**linalool**
1571	31.00	0.43	1.60	*trans*-p-menth-2-en-1-ol
1586	31.37	0.04	-	pinocarvone
1597	31.70	0.41	0.11	bonyl acetate
1611	32.30	**5.64**	**13.64**	**terpinen-4-ol**
1626	32.76	0.14	-	2-methyl-6-methylene-3,7-octadien-2-ol
1638	33.02	0.35	0.66	*cis*-p-menth-2-en-1-ol
1664	33.06	0.08	-	*trans*-pinocarveol
1648	33.34	0.17	0.04	myrtenal
1651	33.45	-	0.12	sabina ketone
1668	34.07	0.37	-	citronellyl acetate
1687	34.49	2.57	0.15	methyl chavicol
1690	34.65	-	1.04	cryptone
1690	34.70	0.50	-	cryptone (not integrated)
1700	34.84	-	0.10	p-menth-1,8-dien-4-ol
1706	35.15	1.40	1.44	α-terpineol
1729	35.80	0.27	0.59	*cis*-1,2-epoxy-terpin-4-ol
1733	35.99	0.13	-	neryl acetate
1743	36.07	0.10	-	α-cadinene (not integrated)
1740	36.09	-	0.16	α-muurolene
1758	36.85	0.20	0.42	*cis*-piperitol
1763	36.77	0.10	-	naphthalene
1772+1773	36.99	1.45	1.58	citronellol+ δ-cadinene
1802	37.88	-	0.11	cumin aldehyde
1804	37.91	0.14	-	myrtenol
1830	38.59	0.06	-	2,6-dimethyl-3(E),5(E),7-octatriene-2-ol
1845	39.04	0.06	0.08	*trans*-carveol
1853	39.24	-	0.08	*cis*-calamenene
1857+1853	39.24	0.15	-	geraniol+*cis*-calamenene

RRI*	Retention time	Area percentage (A)	Area percentage (B)	Compound
1864	39.44	0.23	0.34	p-cymen-8-ol
1900	40.61	0.01	0.06	epi-cubebol
1949	41.75	0.13	-	(Z)(3)-hexenyl-nonanoate
2008	43.38	-	0.13	caryophyllene oxide
2030	43.74	0.06	-	methyleugenol
2050	44.30	0.10	-	(E)-nerolidol
2080	45.10	-	0.05	cubenol
2088	45.26	0.02	0.02	1-epi-cubenol
2113	45.95	0.07	0.16	cumin alcohol
2144	46.62	2.04	1.23	spathulenol
2184	47.50	0.13	0.24	*cis*-p-menth-3-en-1,2-diol
2187	47.70	0.19	0.16	t-cadinol
2209	48.07	0.32	0.26	t-muurolol
2219	48.31	0.06	0.05	δ-cadinol
2247	48.97	0.03	0.03	*trans*-α-bergamotol
2255	49.12	0.82	0.48	α-cadinol
	Total	**98.34**	**98.57**	

RRI* - relative retention indices calculated against n-alkanes

Two samples of *A. ovata* were analyzed and some differences were noted in their composition. *Agathosma ovata* (Gamka) contains the five monoterpenes linalool (17.81%), myrcene (17.18%), sabinene (15.68%), β-pinene (9.47%) and terpinen-4-ol (5.64%) as main compounds. *Agathosma ovata* (Anysberg) contains the five monoterpenes sabinene (31.43%), terpinen-4-ol (13.64%), β-phellandrene (12.06%), p-cymene (6.91%) and limonene (5.64%) as main compounds.

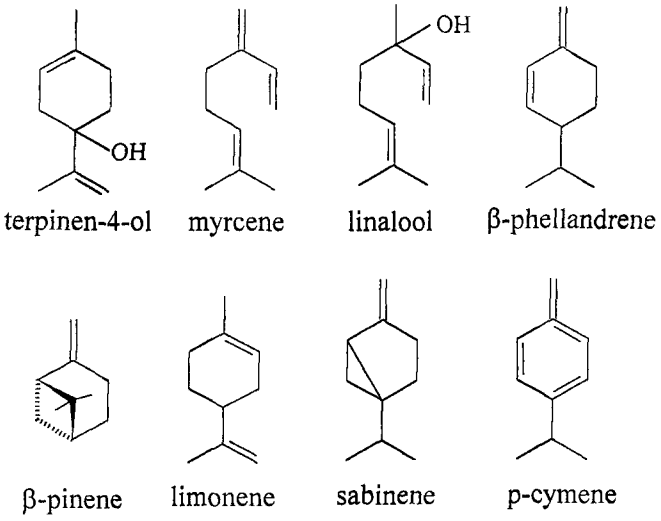


Figure 27: Chemical structures of the major compounds identified in *A. ovata* essential oil.

Agathosma recurvifolia Sond

1. Common name:

Kanferboegoe.

2. Botanical description

Single-stemmed, stiff, spreading shrublet to 1.5m, turpentine-scented. Leaves recurving, with hyaline margins. Flowers in terminal clusters, white. Fruits: 2-chambered. Ovary: usually 1- or 2-lobed.

3. Distribution

Dry middle to upper slopes and valley bushveld ecotone. Distributed from Rooiberg and Swartberg Mountains to Uitenhage (Goldblatt and Manning, 2000).

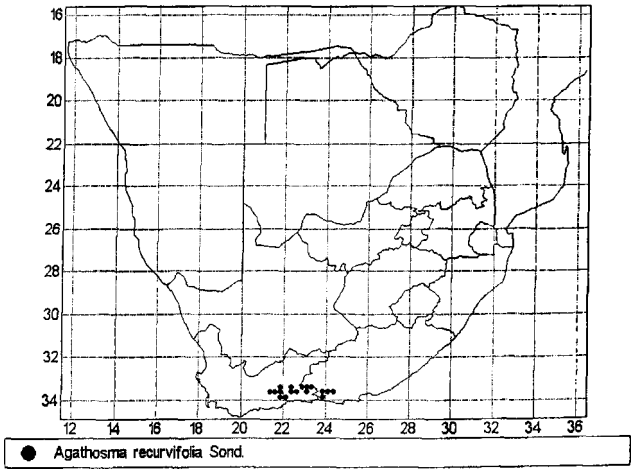


Figure 28: Geographical distribution of *A. recurvifolia.*

4. Essential oil composition

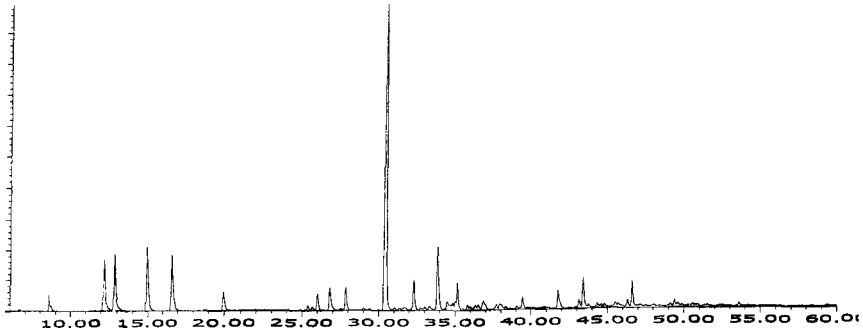


Figure 29: Gas chromatography profile of *A. recurvifolia.*

Table 8: GC-MS results of *A. recurvifolia.*

RRI*	Retention time (minutes)	Area percentage	Compound
1032	8.58	1.03	α-pinene
1035	8.70	0.41	α-thujene
1048	8.98	0.07	2-methyl-3-buten-2-ol
1118	12.18	**5.27**	**β-pinene**
1132	12.87	**5.89**	**sabinene**
1174	14.93	**6.97**	**myrcene**
1203	16.59	**6.55**	**limonene**
1280	19.91	1.98	p-cymene
1327	21.98	0.07	3-methyl-2-butenol
1348	22.79	0.01	6-methyl-5-hepten-2-one
1398	24.88	0.09	2-nonanone
1429	26.01	1.59	perillen
1450+	26.81	2.58	*trans*-linalool oxide (furanoid)+*cis*-1,2-limonene epoxide (0.3%)
1474	27.54	0.26	*trans*-sabinene hydrate
1478	27.84	2.13	*cis*-linalool oxide (furanoid)
1498	28.45	0.02	(E)-β-ocimene epoxide
1521	29.38	0.16	2-nonanol
1553	**30.55**	**35.17**	**linalool**
1571	31.04	0.18	*trans*-p-menth-2-en-1-ol
1586	31.38	0.15	pinocarvone
1600	31.99	0.04	β-elemene
1611	32.30	2.80	terpinen-4-ol
1638	33.02	0.19	*cis*-p-menth-2-en-1-ol
1648	33.32	0.38	myrtenal
1658	33.86	**6.50**	**sabinyl acetate**
1678	34.32	0.06	*cis*-p-menth-2,8-dien-1-ol
1687	34.49	0.66	methyl-chavicol
1698	35.01	0.45	myrtenyl acetate
1706	35.15	2.45	α-terpineol
1729	35.81	0.33	*cis*-1,2-epoxy-terpin-4-ol
1733	35.98	0.14	neryl acetate
1750	36.32	0.18	*cis* linalool oxide (pyranoid)
1751	36.51	0.35	carvone
1765	36.85	0.89	geranyl acetate
1772	36.99	0.30	citronellol (not integrated)
1797	37.69	0.28	p-methyl acetophenone
1804	37.91	0.30	myrtenol
1804	39.04	0.22	*trans*-carveol
1857	39.24	0.08	geraniol
1864	39.44	1.00	p-cymen-8-ol
1882	39.90	0.06	*cis*-carveol
1949	41.75	1.88	(Z)(3)-hexenyl-nonanoate
2001	43.14	0.61	isocaryophyllene oxide
2008	43.41	2.65	caryophyllene oxide
2030	43.74	0.24	methyleugenol
2050	44.30	0.23	(E) nerolidol
2071	44.80	0.16	humulene epoxide II
2144	46.62	1.98	spathulenol
2219	48.39	0.06	dimyrcene-II a
2255	49.11	0.16	α-cadinol
	Total	**96.21**	

RRI*-relative retention indices calculated against n-alkanes

The following five monoterpenes, linalool (35.17%), myrcene (6.97%), limonene (6.55%), sabinene (5.89%) and β-pinene (5.27%) form part of the major compounds in the essential oil of *A. recurvifolia.* Sabinyl acetate (6.50%) was also one of the major compounds.

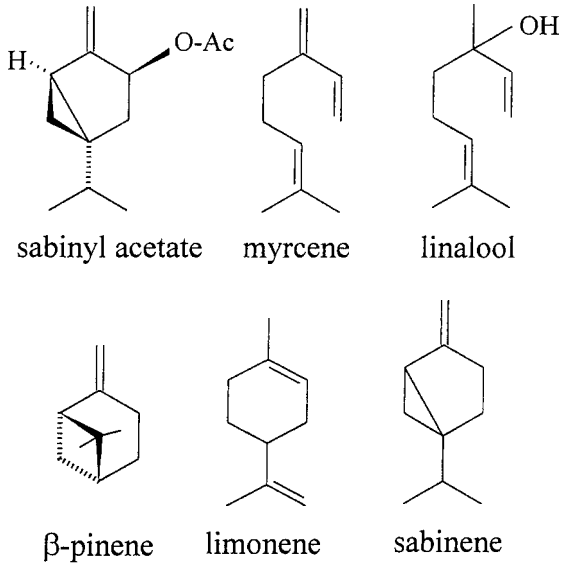


Figure 30: Chemical structures of the major compounds identified in *A. recurvifolia* essential oil.

Agathosma serpyllacea Licht. ex. Roem. and Schult

1. Botanical description

Single-stemmed rounded shrublet. Leaves narrow, swollen behind tip and slightly twisted. Flowers in many, lax terminal clusters, white, pink or purple. Fruits: 3-chambered.

2. Distribution

Coastal or inland sand or limestone flats and slopes. Piketberg to Humansdorp.

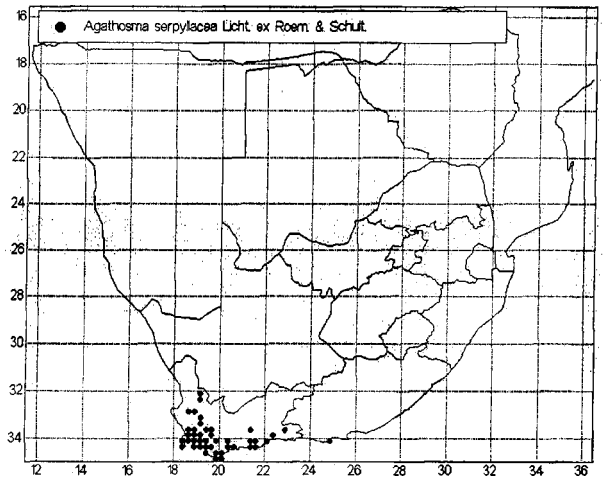


Figure 31: Geographical distribution of *A. serpyllacea.*

3. Essential oil composition

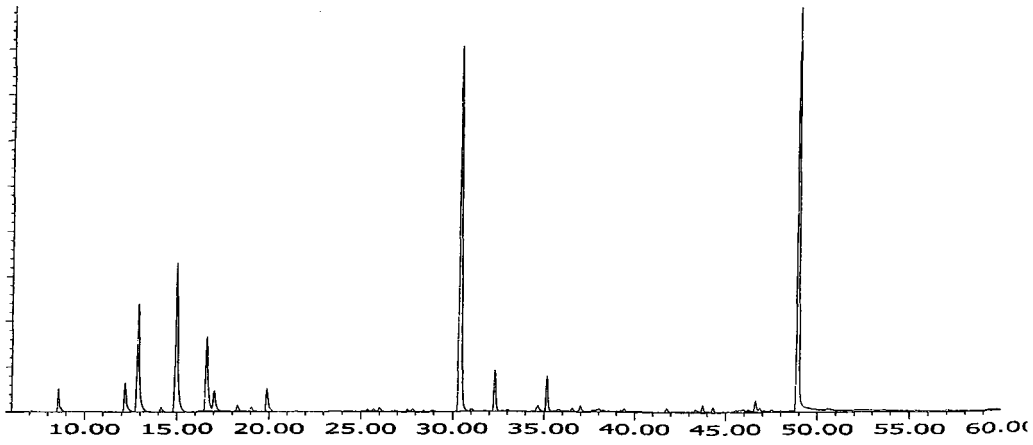


Figure 32: Gas chromatography profile of *A. serpyllacea.*

Table 9: GC-MS results of *A. serpyllacea.*

RRI*	Retention time (minutes)	Area percentage	Compound
1032	8.57	1.10	α-pinene
1035	8.70	0.20	α-thujene (not integrated)
1118	12.20	2.18	β-pinene
1132	12.86	**8.10**	**sabinene**
1159	14.17	0.33	δ-3-carene
1174	14.98	**11.82**	**myrcene**
1203	16.57	**5.81**	**limonene**
1218	17.02	1.71	β-phellandrene
1225	17.65	0.02	(Z) 3-hexenal
1246	18.32	0.48	(Z)-β-ocimene
1266	19.07	0.34	(E)-β-ocimene
1280	19.91	1.85	p-cymene
1290	20.42	0.01	terpinolene
1319	21.48	0.05	(E),2,6 dimethyl-1,3,7-nonatriene
1337	22.20	0.02	geijerene
1360	23.26	0.02	hexanol
1382	24.22	0.01	*cis*-alloocimene
1424	25.68	0.20	o-methylanisol
1429	25.97	0.29	perillen
1450	26.79	0.17	*trans*-linalool oxide (furanoid)
1474	27.56	0.18	*trans*-sabinene hydrate
1478	27.84	0.21	*cis*-linalool oxide (furanoid)
1498	28.47	0.09	(E)-β-ocimene epoxide
1506	28.92	0.08	decanal
1553	30.43	**26.33**	**linalool**
1571	31.02	0.16	*trans*-p-menth-2-en-1-ol
1611	32.30	2.50	terpinen-4-ol
1626	32.75	0.03	2-methyl-6-methylene-3,7-octadien-2-ol
1638	33.02	0.14	*cis*-p-menth-2-en-1-ol
1648	33.28	0.01	myrtenal
1678	34.34	0.02	*cis*-p-menth-2,8-dien-1-ol
1690	34.65	0.43	cryptone
1706	35.15	2.12	α-terpineol
1758	36.52	0.20	*cis*-piperitol (not pure)
1772	36.99	0.38	citronellol
1797	37.69	0.02	p-methyl acetophenone
1804	37.90	0.06	myrtenol
1830	38.58	0.03	2,6 dimethyl 3(E),5(E),7 octatriene-2-ol
1845	39.04	0.05	*trans*-carveol
1857	39.27	0.09	geraniol
1864	39.42	0.20	p-cymen-8-ol
1949	41.76	0.21	(Z)-3-hexenyl nonanoate
2030	43.75	0.39	methyleugenol
2050	44.31	0.28	(E) nerolidol
2144	46.62	0.69	spathulenol
2148	46.85	0.17	dictamnol
2248	48.97	**28.84**	**elemicine**
	total	**98.60**	

RRI*-relative retention indices calculated against n-alkanes

The main compounds of *A. serapyllacea* were identified as the four monoterpenes linalool (26.33%), myrcene (11.82%), sabinene (8.10%) and limonene (5.81%). Elemicine (28.84%) was also one of the main compounds.

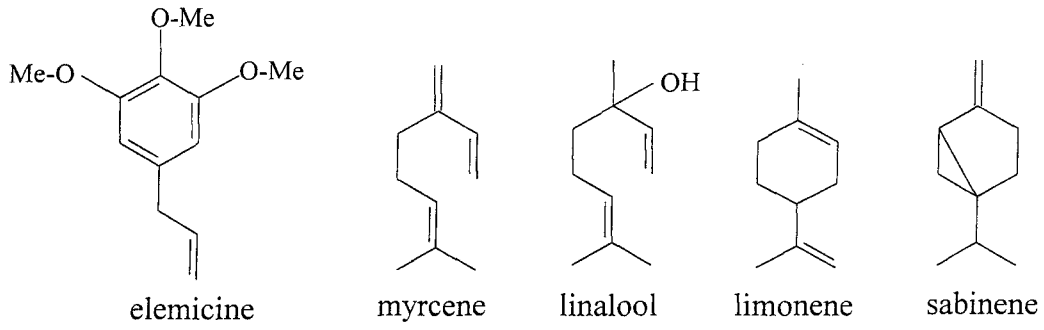


Figure 33: Chemical structures of the major compounds identified in *A. serapyllacea* essential oil.

Agathosma zwartbergensis Pillans

1. Botanical description

Single-stemmed, tangled dwarf shrublet to 20cm, lemon-scented. Flowers 2-4 in terminal clusters, pink. Fruits: 5-chambered. Ovary: usually 4- or 5-lobed.

2. Distribution

Upper sandstone slopes. This species is restricted to the Swartberg and Kammanassie Mountains (Goldblatt and Manning, 2000).

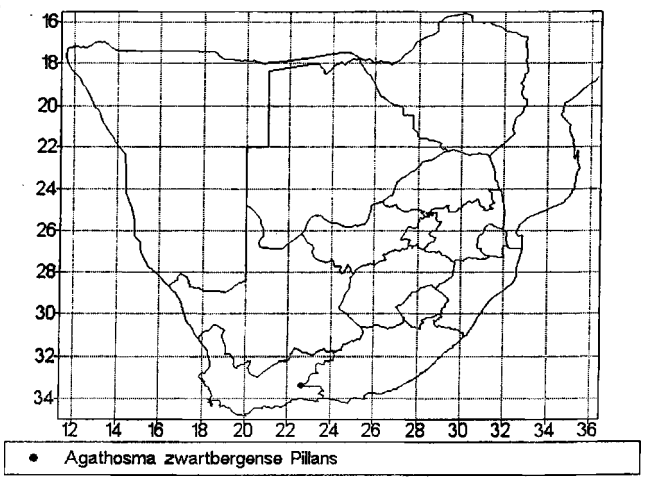


Figure 34: Geographical distribution of *A. zwarbergensis.*

3. Essential oil composition

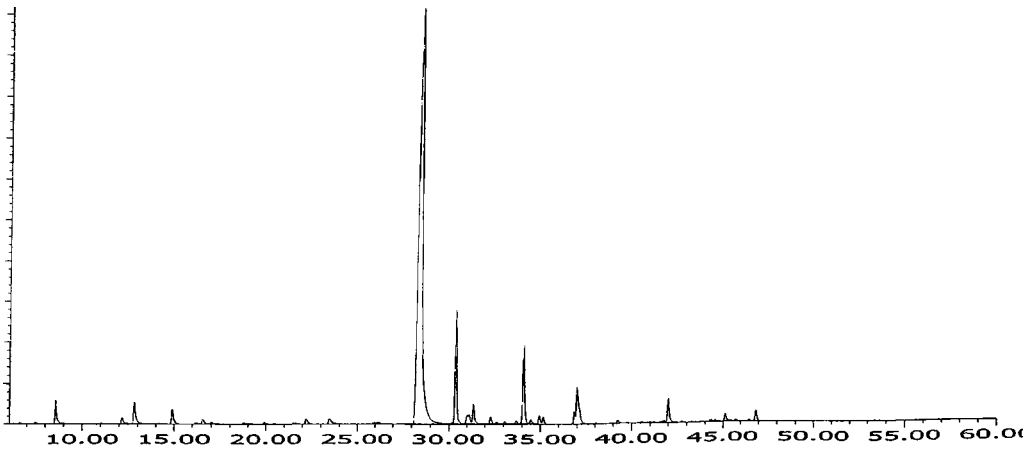


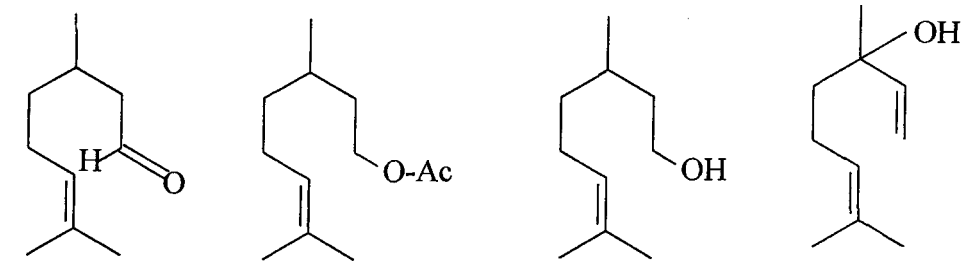
Figure 35: Gas chromatography profile of *A. zwartbergensis.*

Table 10: GC-MS results of *A. zwartbergensis.*

RRI*	Retention time (minutes)	Area percentage	Compound
1032+1035	8.58	1.48	α-pinene+α-thujene (0.2%)
1118	12.20	0.51	β-pinene
1132	13.05	1.82	sabinene
1159	14.17	0.02	δ-3-carene
1174	15.03	1.30	myrcene
1203	16.67	0.41	limonene
1218	17.05	0.13	β-phellandrene
1255	18.76	0.11	γ-terpinene
1266	19.08	0.09	(E)-β-ocimene
1280	19.94	0.17	p-cymene
1290	20.42	0.05	terpinolene
1337	22.22	0.44	geijerene
1365	23.48	0.62	melonal
1450	26.81	0.02	*trans*-linalool oxide (furanoid)
1487	28.50	**64.72**	**citronellal**
1553	30.55	7.95	linalool
1571	31.00	0.54	methyl citronellate
1583	31.35	1.45	isopulegol
1611	32.30	0.55	terpinen-4-ol
1641	33.06	0.21	methylbenzoate
1655	33.70	0.24	2,6-dimethyl-5-hepten-1-ol
1668	34.10	5.70	citronellyl acetate
1687	34.49	0.25	methyl chavicol
1706	35.15	0.48	α-terpineol
1740	36.31	0.03	geranial
1765	36.87	0.74	geranyl acetate
1772	36.99	3.82	citronellol (not pure)
1860	39.74	0.06	8-epi-dictamnol
1973	42.44	0.05	dodecanol
2050	44.30	0.16	(E)-nerolidol
2148	46.85	0.65	dictamnol
	Total	**95.24**	

RRI*-relative retention indices calculated against n-alkanes

The main compound of the essential oil of *A. zwarbergensis* is citronellal (64.72%).



citronellal citronellyl acetate citronellol linalool

Figure 36: Chemical structures of the major compounds identified in *A. zwartbergensis* essential oil.

4. Results and Discussion

4.1 Antimicrobial activity

The antimicrobial results are summarized in Tables 11, 12 and 13. Antibacterial and antifungul screening was done followed by the determination of the minimum inhibitory concentration (MIC) for selected oil samples showing positive antimicrobial activity in the disc diffusion assay.

Table 11 shows the disc diffusion antibacterial screening results of all the *Agathosma* species studied. The results were taken after 24 hours of incubation. Zones of inhibition were measured in millimeters from the edge of the disc containing the essential oil. All the species studied showed a variable degree of antibacterial activity. *Agathosma capensis* (Mossel Bay) showed activity against *E. coli*, *E. faecalis* and *S. aureus*. Figure 37 shows a broadscreening of *E. coli* on all *Agathosma* oils. *Agathosma lanata* and *A. zwartbergensis* only showed antibacterial activity against *B. cereus*. Figure 38 shows the zones of inhibition on selected oil samples (34 – *A. capensis* (Gamka); 36 – *A. ovata* (Gamka); 38 – *A. zwartbergensis*; 40 – *A. ovalifolia*) for *B. cereus*. The *A. capensis* and the *A. ovata* samples that were both collected from the Gamka Mountains in the Cape and *Agathosma serpyllacea* showed activity against all test organisms except *P. aeruginosa*. The *A. capensis* (Rooiberg) sample showed little activity against *E. coli*, *S. typhimurium* and *S. aureus*; *A. recurvifolia* showed activity against *E. coli*, *E. faecalis* and *S. aureus*; *A. ovalifolia* showed activity against *E. faecalis* and to a lesser extend *S. aureus* and *A. arida* showed activity against *S. typhimurium* and *B. cereus*. *Agathosma mundtii* showed activity against *E. faecalis*, *S. typhimurium*, *S. aureus* and some initial activity against *B. cereus*. The activity of *A. mundtii* against *B. cereus* was difficult to determine as initial zones were detected after 24 hours but regrowth was noted after 48 hours, thus indicating that the essential oil probably evaporated.

The *A. ovata* (Anysberg) sample has minimal broad-spectrum antibacterial activity and was the only species that showed activity against *P. aeruginosa*.

The largest zone of inhibition was 7.0 mm from the disc and was the result of the essential oil of *A. recurvifolia*'s activity against *E. faecalis*. Neomycin 30 µg, (Oxoid)

was used as a positive control and measured 3.0 mm from the disc, therefore the inhibition of the essential oil of *A. recurvifolia* was greater than that of the control.

The antifungal results were similar to the antibacterial results. A small range of fungi were however tested. Table 12 shows the antifungal screening results of all *Agathosma* species studied. All the species showed activity against *C. neoformans*, with *A. arida* exhibiting the largest zone (3.0 mm) of inhibition. No activity was noted for both *C. albicans* and *A. niger* for *A. arida.* The following species showed antifungal activity against *C. albicans*: *A. ovata* (Gamka), *A. zwartbergensis, A. ovalifolia, A. recurvifolia* and *A. serpyllacea. Agathosma ovalifolia* was the only species showing some minimal activity against *A. niger.*

The minimum inhibitory concentration (MIC) of the essential oils on the test bacteria was determined by using the *p*-iodonitrotetrazolium violet (INT) microplate method. The MIC of *A. ovata* (Gamka and Anysberg samples), *A. recurvifolia* and *A. capensis* (Gamka sample) were determined on *E. coli, S. aureus* and *E. faecalis*. The results as summarized in Table 13 and in Figure 39. These results reflect that the concentration of *A. capensis* (Gamka) oil needed to inhibit the growth of *E. coli* is 16 mg/ml, *S. aureus* is 32 mg/ml and *E. faecalis* is 32 mg/ml. The concentration of *A. ovata* (Gamka) oil needed to inhibit the growth of *E. coli* is 16 mg/ml, *S. aureus* is 8 mg/ml and *E. faecalis* is 16 mg/ml. The concentration of *A. ovata* (Anysberg) oil needed to inhibit the growth of *E. coli* is 8 mg/ml, *S. aureus* is 8 mg/ml and *E. faecalis* 16 mg/ml. The MIC of *A. recurvifolia* is 8 mg/ml for *E. coli*, 8 mg/ml for *S. aureus* and 16 mg/ml for *E. faecalis*. Well 10E on the microplate showed no INT colouring. This can be attributed to the fact that well 10E was not inoculated with culture (*E. coli*). The MIC for *A. recurvifolia* for *E. c*oli however stays 8 mg/ml.

The bacteriostatic and fungistatic activities of the essential oils were evaluated by using the undiluted oils of the *Agathosma* species in the screening tests. Quantitative results were determined by calculating the minimum inhibitory concentration (MIC), using the serial dilution method. *Agathosma ovata* (Gamka) had zones of inhibition of less than 1.0 mm on *E. coli*, 5.0 mm on *E. faecalis* and less than 1.0 mm on *S. aureus*. The final values taken after 24 hours MIC, for *A. ovata* (Gamka) on the same bacteria were 16 mg/ml, 16 mg/ml and 8 mg/ml. *Agathosma ovata* (Anysberg) had

zones of inhibition of 3.0 mm on *E. coli*, 2.0 mm on *E. faecalis* and 1.0 mm on *S. aureus*. The MIC for *A. ovata* (Anysberg) on the same bacteria were 8 mg/ml, 16 mg/ml and 8 mg/ml. *Agathosma capensis* (Gamka) had zones of inhibition of 1.0 mm on *E. coli*, 3.0 mm on *E. faecalis* and 2.0 mm on *S. aureus*. The MIC's for *A. capensis* (Gamka) on the same bacteria were 16 mg/ml, 32 mg/ml and 32 mg/ml respectively. *Agathosma recurvifolia* had zones of inhibition of 1.0 mm on *E. coli*, 7.0 mm on *E. faecalis* and 1.5 mm on *S. aureus*. The MIC for *A. recurvifolia* on the same bacteria was 8 mg/ml, 16 mg/ml and 8 mg/ml respectively. The MIC results therefore correlate with what was observed in the disc diffusion screening results.

The TLC bioautographic assay (Figure 40) with the hydrodistilled oil of *A. zwartbergensis* showed one zone of inhibition. A TLC bioautographic assay of pure citronellal was done simultaneously with the assay of the hydrodistilled oil. As indicated on figure 40, the main compound of *A. zwartbergensis,* the yellow compound (Rf = 0.79) on the TLC vanillin-sulphuric plate, correlates with the citronellal standard. This compound was also identified with GC-MS as being citronellal and could be the antimicrobial factor of the essential oil.

Table 11: Antibacterial screening results as expressed in the disc diffusion assay (mm from disc edge).

Species	*Escherichia coli*	*Enterococcus faecalis*	*Pseudomonas aeruginosa*	*Salmonella typhimurium*	*Bacillus cereus*	*Staphylococcus aureus*
Neomycin 30 µg, (Oxoid)	5.0	3.0	2.0	4.0	10.0	10.0
A. arida	R*	R*	R*	< 1.0	2.0	R*
A. capensis (Gamka)	1.0	3.0	R*	1.0	1.0	2.0
A. capensis (Rooiberg)	1.0	R*	R*	1.0	R*	1.5
A. capensis (Mosel Bay)	< 1.0	2.0	R*	R*	R*	2.0
A. lanata	R*	R*	R*	R*	1.0	R*
A. mundtii	R*	1.0	R*	<1.0	R*	1.0
A. ovalifolia	R*	2.0	R*	R*	R*	<1.0
A. ovata (Gamka)	<1.0	5.0	R*	<1.0	2.0	< 1.0
A. ovata (Anysberg)	3.0	2.0	1.0	< 1.0	1.0	1.0
A. recurvifolia	1.0	7.0	R*	R*	R*	1.5
A. serpyllacea	<1.0	3.0	R*	<1.0	2.0	2.5
A. zwartbergensis	R*	R*	R*	R*	3.0	R*

***R = Resistant**

Figure 37: Disc diffusion plate of *E. coli* on the essential oils of *Agathosma* species studied.

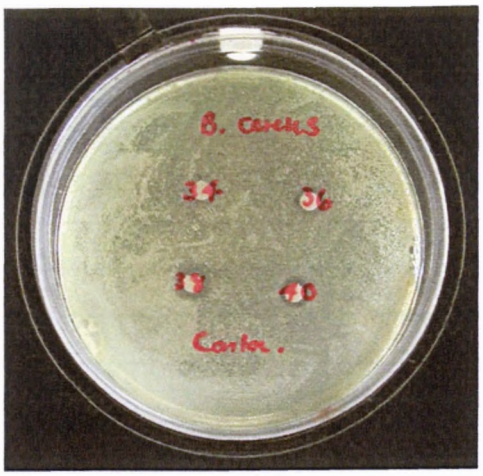


Figure 38: Disc diffusion plate of *B. cereus* on essential oils of (34 – *A. capensis* (Gamka); 36 – *A. ovata* (Gamka); 38 – *A. zwartbergensis*; 40 – *A. ovalifolia*).

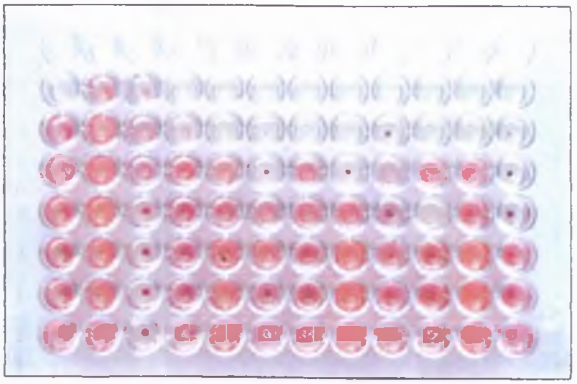


Figure 39: MIC results after 24 hours.

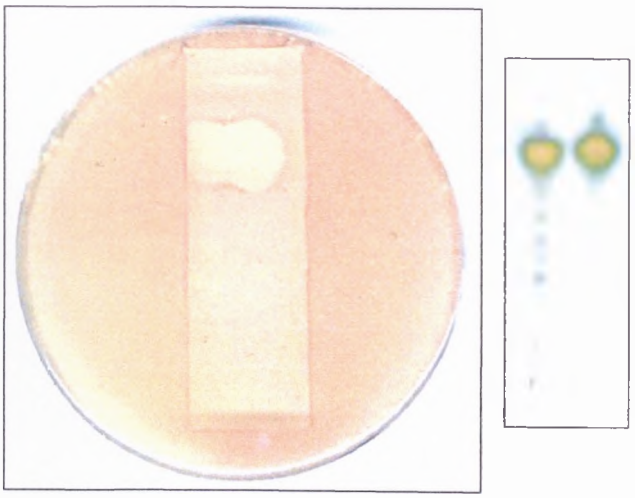


Figure 40: TLC bioautographic assay.

Table 12: Antifungal disc diffusion screening results (expressed as mm from disc edge).

Species	*Candida albicans*	*Cryptococcus neoformans*	*Aspergillus niger*
Nystatin (100 iu, Oxoid).	7.0	5.0	7.0
A. arida	R*	3.0	R*
A. capensis (Gamka)	R*	< 1.0	R*
A. capensis (Rooiberg)	R*	< 1.0	R*
A. capensis (Mossel Bay)	R*	2.0	R*
A. lanata	R*	2.0	R*
A. mundtii	R*	<1.0	R*
A. ovalifolia	2.0	1.0	1.0
A. ovata (Gamka)	1.0	2.0	R*
A. ovata (Anysberg)	R*	2.0	R*
A. recurvifolia	2.0	2.0	R*
A. serpyllacea	2.0	2.0	R*
A. zwartbergensis	3.0	2.0	R*

***R = Resistant**

Table 13: MIC results after 30 minutes, 2 hours and 24 hours.

Microplate column	Test organism	Species	MIC (mg/ml) after 30 min	MIC (mg/ml) after 2 hours	MIC (mg/ml) after 24 hours
1	*Eschericha coli*	*A. capensis* (Gamka)	8	8	16
2	*Staphylococcus aureus*	*A. capensis* (Gamka)	8	16	32
3	*Enterococcus faecalis*	*A. capensis* (Gamka)	No colouring	16	32
4	*Eschericha coli*	*A. ovata* (Gamka)	4	8	16
5	*Staphylococcus aureus*	*A. ovata* (Gamka)	4	4	8
6	*Enterococcus faecalis*	*A. ovata* (Gamka)	2	8	16
7	*Eschericha coli*	*A. ovata* (Anysberg)	4	4	8
8	*Staphylococcus aureus*	*A. ovata* (Anysberg)	2	4	8
9	*Enterococcus faecalis*	*A. ovata* (Anysberg)	2	8	16
10	*Eschericha coli*	*A. recurvifolia*	4	4	8
11	*Staphylococcus aureus*	*A. recurvifolia*	2	4	8
12	*Enterococcus faecalis*	*A. recurvifolia*	1	8	16

4.2 Analytical chemistry

TLC plates of all 10 essential oils were developed and detection was made possible with the use of spray-reagents namely vanillin-sulphuric acid (Figure 41) and anisaldehyde-sulphuric acid (Figure 42). For the TLC plate where vanillin-sulphuric acid was used, the following similarities were seen on the TLC plates of the individual essential oils. *Agathosma mundtii* and *A. ovalifolia* have similar compounds (coloured blue after development of the plate) with Rf = 0.90. There is a consistency between all 10 samples in the middle region of the TLC plate. This indicates similar compounds in the different essential oils. *Agathosma capensis* (Gamka) contains a unique compound (coloured brown after development of the plate) with Rf = 0.90. *Agathosma zwartbergensis* contains a unique compound (coloured yellow-brown after development) with Rf = 0.79. *A. ovalifolia* contains a unique compound (colour yellow on TLC plate) with Rf = 0.59 and *Agathosma* species contains a unique compound (colour pink) with Rf = 0.52. Similar results were obtained with the anisaldehyde-sulphuric acid colour reagent. Thin layer chromatography is however not very useful when working with complex mixtures such as essential oils. The purpose of this study was merely to also produce a fingerprint of the species studied (e.g. *A. zwartbergensis*) and to visually summarize the immense variation between the selected species.

The GC-MS results are tabulated under the monographs of each species and the major compounds are clearly indicated. Three samples of *A. capensis* were analyzed by GC-MS. The one sample was harvested from the Gamka Mountains, the other sample was harvested from the Rooiberg region in the Cape Province and the last sample was collected from Mossel Bay. There are very interesting differences between the composition of the three samples and these results support the fact that external factors may influence the chemical compositions of species. All three samples had the following same major compounds: linalool, myrcene and limonene. Methyl-chavicol is a major compound of *A. capensis* (Gamka) but not for *A. capensis* (Rooiberg) and *A. capensis* (Mossel Bay). Sabinene is a major compound for *A. capensis* (Rooiberg) but not for *A. capensis* (Gamka) and *A. capensis* (Mossel Bay). β-phellandrene, (Z)-β-ocimene and

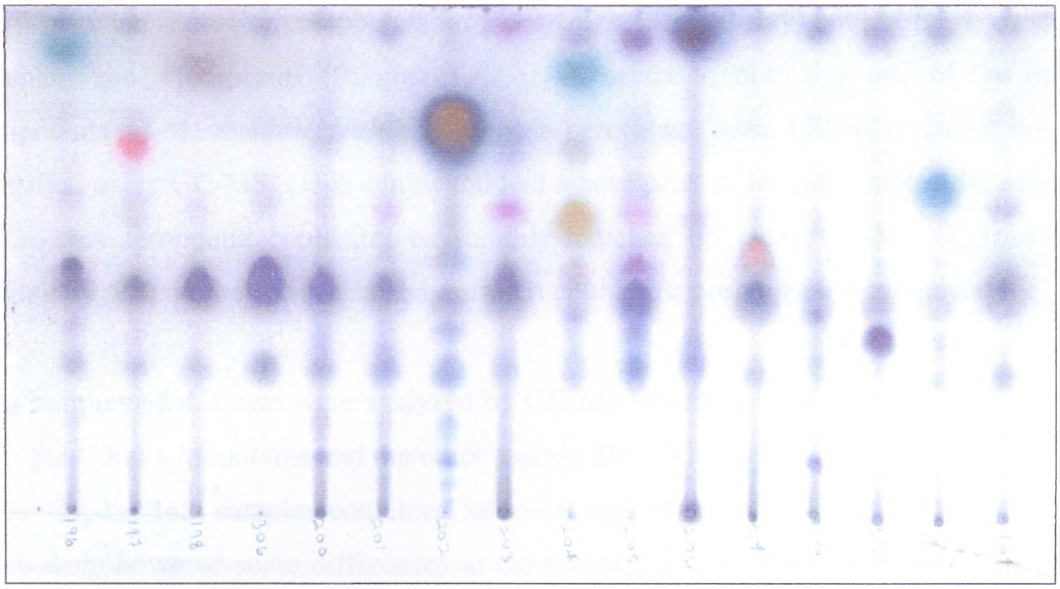


Figure 41: TLC plate of *Agathosma* essential oils. The plate has been treated with vanillin-sulphuric acid.

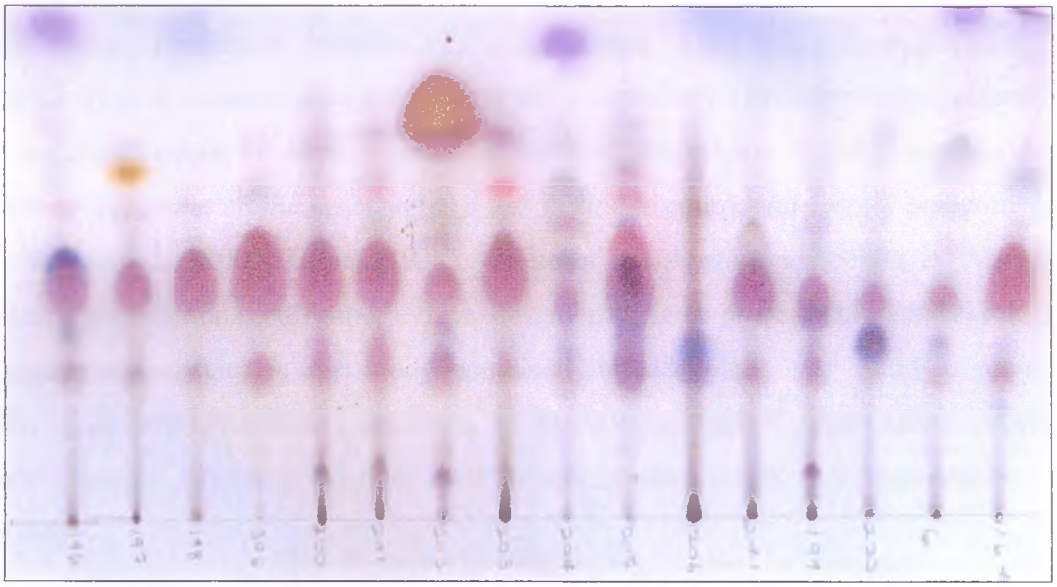


Figure 42: TLC plate of *Agathosma* essential oils. The plate has been treated with anisaldehyde-sulphuric acid.

(E)-β-ocimene are major compounds for *A. capensis* (Mossel Bay) but not for *A. capensis* (Gamka) and *A. capensis* (Rooiberg). It is important to note that one of the major compounds of *A. mundtii* with the highest percentage area (20.00%) could not be identified using GC-MS. This compound had a base peak of 69 and a molecular mass of 172. This compound could not be suitably matched on many GC-MS libraries and judging by the comprehensive database used it could be a possible new compound.

Two samples of *A. ovata* were analyzed by GC-MS where the one sample was collected from the Gamka Mountains and the other sample was collected form the Anysberg region in the Cape. Both samples contained sabinene and terpinen-4-ol as major compounds. There were however some differences in the compositions of the two samples that may be attributed to external factors. Myrcene and linalool are major compounds of *A. ovata* (Gamka) but not for *A. ovata* (Anysberg). Limonene, p-cymene and β-phellandrene are major compounds of *A. ovata* (Anysberg) but not for *A. ovata* (Gamka).

Previous analytical chemistry research has been done on commercial *Agathosma* species. These species include *A. betulina* and *A. crenulata.* The major compounds in the essential oil of *A. betulina* are isomenthone and diosphenol. Other compounds identified in *A. betulina* include limonene, menthone, pulegone, terpinen-4-ol and *p*-menthan-3-on-8-thiol. *Agathosma crenulata* contains a less desirable compound namely pulegone (van Wyk and Gericke, 2000; Bisset, 1994). None of the *Agathosma* species in this study contained isomenthone, pulegone, diosphenol, menthone or *p*-menthan-3-on-8-thiol. All the *Agathosma* species in this study contained limonene that was found in previous studies of *A. betulina* and *A. crenulata.* All the species studied except the *A. capensis* (Gamka) sample contained terpinen-4-ol, which is also found in *A. betulina* and *A. crenulata.*

4.3 Antimicrobial activity of main compounds

All the *Agathosma* species studied showed some degree of antimicrobial activity. Literature studied on essential oil containing plant species show similarities in antimicrobial activity with the compounds found in some of the *Agathosma* species.

A study was done by Carson and Riley (1995) to examine the antimicrobial activity of eight individual components of Tea Tree oil. Tea Tree oil is commonly used to treat skin disorders such as cuts, burns, insect bites and athlete's foot. Tea Tree oil contains 1,8-cineole, 1-terpinen-4-ol, p-cymene, linalool, α-terpinene, γ-terpinene, α-terpineol and terpinolene. The test organisms included *Candida albicans, Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus.* The results of the study indicated that terpinen-4-ol was active against all test organisms. Linalool and α-terpineol were active against all the tested organisms except *Pseudomonas aeruginosa.* In this study the *Agathosma* species containing linalool and α-terpineol as main compounds were also active against all the test organisms except *Pseudomonas aeruginosa. Agathosma mundtii* and *A. ovata* (Gamka and Anysberg) contain terpinen-4-ol as a main compound. *Agathosma ovata* (Anysberg) showed activity towards all the test organisms, *A. mundtii* showed activity towards all test organisms except *E. coli* and *P. aeruginosa* and *A. ovata* (Gamka) showed activity towards all test organisms except *P. aeruginosa.* Terpinen-4-ol, p-cymene, linalool and α-terpineol are present, not necessarily as major components, in all the *Agathosma* species studied.

Cimanga *et al.* (2002) indicates that *Eucalyptus, Aframomum, Ocimum, Cympobogon* and *Monodora* species, from the Democratic Republic of the Congo, contain the essential oil compounds 1,8-cineole, α-pinene and β-pinene, p-cymene, myrcene, γ-terpinene, α-terpineol, limonene, β-terpineol, citronellal, cryptone, phellandrene and thymol. The results published in the article indicated that these essential oils showed inhibition of selected bacterial growth. They compared the chemical composition of the essential oils of *Eucalyptus camadulensis* and *Cympobogon citratus* with their antibacterial activity and found that their activity is related to the high levels of 1,8-cineole, geranial and neral.

Similar bacteria were tested in this study of the antimicrobial activity of buchu and include *E. coli, P. aeruginosa* and *S. aureus.* The *Agathosma* species studied contain similar essential oil compounds as the plant species from the Congo. All the *Agathosma* species studied contain α-pinene, β-pinene, p-cymene, myrcene, α-terpineol and limonene as major or minor compounds.

The essential oil of *Phlomis lanata* contains the major compounds; α-pinene, limonene and *trans*-caryophyllene. Couladis *et al.* (2000) on the antimicrobial activity and chemical composition of *Phlomis lanata,* indicates that the oil had moderate activity against the bacteria tested and strong activity against the test fungi. Pure limonene and α-pinene were tested on the same cultures and the results in the article suggest that the activity of the oil could be largely attributed to these two main compounds of the oil. *Agathosma capensis* (Gamka and Rooiberg), *A. ovata* (Anysberg), *A. recurvifolia* and *A. ovalifolia* contain limonene as a major compound. *Agathosma mundtii, A. ovata* (Gamka) and *A. recurvifolia* contain α-pinene as a major compound. The essential oils of these *Agathosma* species with the same major compounds as the essential oil of *Phlomis lanata* showed similar activity towards similar test organisms (*E. coli, S. aureus, P. aeruginosa* and *C. albicans). Agathosma arida* also contains limonene and α-pinene but did not show activity towards these test organisms. It is noteworthy that all the buchu species studied contain limonene and α-pinene either as a minor or a major compound.

Cobos *et al.* (2001) investigated the chemical composition and antimicrobial activity of the essential oil of *Baccharis notosergila.* The major compounds were α-pinene, limonene, β-caryophyllene and spathulenol. They came to the conclusion that essential oils containing monoterpenes like limonene are more active against gram-positive organisms and fungi than gram-negative organisms. *Agathosma capensis* (Gamka and Rooiberg), *A. ovata* (Anysberg), *A. recurvifolia* and *A. ovalifolia* contain limonene, a monoterpene, as a major compound and showed activity towards the gram-positive bacteria *Enterococcus faecalis, Bacillus cereus* and *Staphylococcus aureus.*
The antibacterial activity of *Eucalyptus* essential oils is due to the synergy of citronellol and citronellal (Zakarya *et al.,* 1993). *Agathosma zwartbergensis* contains citronellal as a

main constituent and citronellol as a minor constituent. *Agathosma mundtii, A. capensis* (Gamka and Rooiberg), *A. ovata* (Anysberg), *A. recurvifolia* and *A. arida* contain citronellol as a minor constituent. *Agathosma ovata* (Gamka) contains citronellol and citronellal as minor constituents.

The positioning of functional groups (terpinen-4-ol compared to α-terpineol), level of ring saturation (carvone compared to dihydrocarvone), type of functional group present and the level of chain saturation in an acyclic terpenoid (geraniol compared to citronellol) determine the antibacterial activity of monoterpenes. Small changes in molecular properties affect the permeation through bacterial outer membranes and therefore the antibacterial activity (Griffin *et al.,* 2001). Griffin *et al.* (1999) examined the structure-activity relationships of terpenoids. Low water solubility, mainly essential oil compounds containing hyrocarbons and acetates, attribute to the relative inactivity of essential oils. Furthermore the antimicrobial activity of oxygenated terpenoid containing essential oils is associated with hydrogen bonding. It is important to note that water solubility and hydrogen bonding does not account for all the trends in the activity of essential oils. The activity of geraniol, nerol and linalool is largely determined by the presence of the alcohol functional group on the carbon skeleton of these acyclic terpenoids. The hydrogen-bonding capacity and hence activity is illustrated when comparing the activity of citronellol, inactive towards *E. coli*, and geraniol, active towards *E. coli.* Citronellal, the corresponding aldehyde to citronellol, was also inactive towards *E. coli* and can be attributed to the lower solubility (less hydrogen bonding) than geraniol, linalol and nerol (Griffin *et al.*, 1999).

When comparing the main constituents of the *Agathosma* species studied with the main constituents of other species showing antibacterial activity, the compounds present in the greatest proportions are not necessarily responsible for the greatest share of the total activity. It is important to consider the less abundant constituents and the possibility of synergy between components.

5. Conclusion

The main objectives of this study were to investigate the possible antimicrobial properties of a selection of species belonging to the genus *Agathosma* and to record the essential oil profiles of these species.

The presence of antibacterial and antifungal activity of the various *Agathosma* species were proven in this study. The activity varied amongst the studied species. *Agathosma recurvifolia* for *E. faecalis* had an activity greater than the positive control (Neomycin 30 μg (Oxoid) used. After comparing the results with antimicrobial results of other essential oil containing plant species, it was noted that it is important to consider the less abundant compounds of the essential oils. The compounds in the greatest proportion are not necessarily responsible for the antimicrobial activity and the possibility of synergy between compounds should be considered. A TLC bioautographic assay was conducted to determine the antimicobial factor of *A. zwarbergensis*. Results indicated that citronellal could possibly be responsible for the observed antimicrobial activity.

The twelve oils were subjected to GC-MS and the profiles were recorded. For all twelve oils more than 90% of the compounds were identified. However the main compound of *A. mundtii* could not be identified and is most probably a new terpenoid. Each species still has a unique qualitative and quantitative composition. The differences amongst and within species could possibly be attributed to some external factors that include the botanical source, the condition of the plant material (fresh or dried) and the isolation technique (steam distillation or hydrodistillation).

The results of this report can support the use of these medicinal plants, generally referred to as 'Buchu', as traditional remedies for selected infectious diseases.

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