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A PHYTOCHEMICAL AND PHARMACOLOGICAL INVESTIGATION OF INDIGENOUS *AGATHOSMA* SPECIES

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Master of Pharmacy

Johannesburg, South Africa, 2005.

DECLARATION

I, Aneesa Moolla, declare that this dissertation is my own work. It is being submitted for the Degree of Master of Pharmacy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Signature: ……

Date: ……...

DEDICATION

To my parents Hassim Ahmed Moolla and Sara Bibi Moolla.

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University of the Witwatersrand, and finally all the people who helped me in ways I cannot detail here.

ABSTRACT

As part of an investigation of the biological activities of South African plants and due to their extensive traditional use and lack of scientific evidence, a phytochemical and pharmacological investigation was performed on 17 indigenous *Agathosma* species (19 samples). The chemical composition of the essential oils was determined using gas chromatography coupled to mass spectroscopy (GC-MS). Analysis resulted in the identification of 333 compounds. To evaluate the chemical similarities and differences, cluster analysis was used to assess the essential oil composition of the samples. The results showed qualitative and quantitative differences amongst the taxa. The essential oils of *Agathosma hirsuta* and *A. zwartbergense* are particularly rich in citronellal, hence they are tightly clustered in the dendrogram obtained from the cluster analysis. Linalool, myrcene and limonene are the major constituents of both *A. capensis* (Gamka) and *A. capensis* (Besemfontein). Qualitative and quantitative differences are noted in the chemical compositions of the leaf oils of *Agathosma capensis* (Gamka) and *A. capensis* (Besemfontein). *Agathosma arida* and *A. lanata* are united in a single cluster due to the compounds β-pinene, linalool and spathulenol being major components in both species. The presence of 1,8-cineole in large quantities in both *Agathosma namaquensis* (23.5%) and *A. ovalifolia* (9.7%), unites them in a single cluster. A wide chemical variability for the essential oils of indigenous *Agathosma* species has been demonstrated.

There was considerable variation in the percentage oil yield of the essential oils. *Agathosma hirsuta* produced the highest yield (1.15%) whilst *A. ovalifolia* produced the lowest yield (0.16%).

Previous studies have revealed that the coumarin and flavonoid components of *Agathosma* species are responsible for their biological activities. High performance liquid chromatography (HPLC) was used to document the non-volatile composition of *Agathosma* species and to establish if phenolic patterns were present amongst the species. All species were found to be rich in flavonoids (i.e. flavones and flavonols). Many of the compounds detected were common to most of the species. A pure coumarin, puberulin, was identified in the diethyl ether extract of *Agathosma ovata* (round-leaf) and detected in the dichloromethane and methanol (1:1) extract of *A. namaquensis*.

Agathosma species have been used traditionally to treat a wide variety of infections. They has been used as a cough remedy, for the treatment of colds and flu, kidney and urinary tract infections, for the treatment of cholera and other stomach ailments. Based on the extensive use and lack of scientific evidence, a study was embarked upon to determine its bioactivity. Using the disc diffusion assay as a preliminary screening and thereafter the minimum inhibitory concentration (MIC) assay, the antimicrobial activity of the essential oils and non-volatile compounds was assessed on two Gram-positive bacteria, *Staphylococcus aureus* and *Bacillus cereus*, one Gram-negative bacterium, *Klebsiella pneumoniae*, and one yeast, *Candida albicans*. All of the extracts proved to be active against the four pathogens tested with the exception of *Agathosma bathii* which showed poor activity against *Klebsiella pneumoniae* (MIC value of 32mg/ml). The extracts exhibited stronger activity against the pathogens as compared to the essential oils. Both the essential oils and extracts exhibited higher activity towards the Gram-positive bacteria than the Gram-negative bacterium, with the extract of *Agathosma ovata* (round-leaf) displaying the greatest

activity against *Staphylococcus aureus* (MIC value of 0.156mg/ml) and *Bacillus cereus* (MIC value of 0.125mg/ml). The extract of *Agathosma parva* displayed the greatest activity against *Candida albicans* and *Klebsiella pneumoniae* (MIC value of 1.5mg/ml). Amongst the essential oils, *Agathosma pungens* proved to be the most active against the Gram-positive pathogen, *Bacillus cereus* (MIC value of 3mg/ml). *Agathosma collina* was the most active against *Candida albicans* (MIC value of 3mg/ml) whilst *A. zwartbergense* proved to be the least active against most of the tested pathogens. The antimicrobial activity of the essential oils may be ascribed to oxygenated constituents, such as 1,8-cineole, linalool and carvacrol. The activity of the extracts may be ascribed to constituents such as flavonoids, coumarins and alkaloids.

Due to the availability and accessibility of *Agathosma ovata*, a seasonal variation study was performed on the chemical composition of the essential oils and how this may impact on the antimicrobial activity. Furthermore, this species has recently been earmarked for commercial development by the flavour and fragrance industry and information on variability is required to establish the harvesting protocol. Ten samples were harvested in total. There was a substantial variation in the oil yield throughout the year, ranging from 0.23% in early Spring to 0.85% in late Autumn. A higher yield was observed during the flowering season as compared to the non-flowering season. Oil yields were low during Summer (0.44%-0.48%) which may have been due to the low oil content in stems and higher proportion of stems after flowering. The proportion of oil-rich green leaves also decreased markedly, hence affecting the yield. Overall the yields were dependant on the season harvested and proportion of plant parts distilled.

The chemical composition of the essential oils was determined using GC-MS and resulted in the identification of 145 compounds in 10 of the samples. All samples contained a large number of common monoterpenes and had very similar compositions, with minor quantitative variation. Some components common to all samples include: sabinene, *p*-cymene, β-pinene, α-pinene, α-thujene, myrcene, limonene, linalool and terpinen-4-ol. Sabinene was found to be the most dominant component in all samples, ranging between 25.6% and 44.4%. Myrcene levels dropped sharply between the beginning of Spring and end of Summer, from 14.9% to 1.0%. β-pinene followed a similar trend, peaking during Spring and decreasing during the Summer months. The lowest levels of linalool (4.3%), myrcene (1.0%), β-pinene (3.9%), limonene (1.9%) and sabinene (25.6%), occurred during the Summer months when the temperatures were high. There was a Springtime increase in the levels of β-pinene, terpinen-4-ol, linalool, sabinene, limonene and *p*-cymene in the non-flowering *Agathosma ovata*. These changes may have been due to the higher proportion of young leaves during Spring, which may have oil compositions slightly different to those of mature leaves. A rare thiol derivative (tr) that could not be identified was detected in the March sample. Many of the changes were associated with flowering and the results obtained reveal that the chemical composition of the essential oil of *Agathosma ovata* is subject to seasonal variation.

Using the MIC assay, the antimicrobial activity of the essential oils was assessed on two Gram-positive bacteria, *Staphylococcus aureus* and *Bacillus cereus*, one Gram-negative bacterium, *Klebsiella pneumoniae*, and one yeast, *Candida albicans*. The study demonstrated differences in the potency of antimicrobial activity of the essential oils distilled each month. The Winter samples were more active against *Bacillus cereus*, *Staphylococcus aureus* and *Klebsiella pneumoniae*. Activity in mid Spring

was greater against *Staphylococcus aureus* (MIC value of 3mg/ml) and *Klebsiella pneumoniae* (MIC value of 3mg/ml), whilst activity decreased in Summer. There was a correlation between the concentrations of the active compounds each month and the oils antimicrobial activity. The results reveal that the antimicrobial activity of the essential oil of *Agathosma ovata* may not depend on the level of one component but rather the ratio of several components.

'Buchu' has been used traditionally as a general tonic and medicine. Tonics generally have a high anti-oxidant content in order to promote the overall well-being of the user. The anti-oxidant properties of the essential oils and non-volatile compounds was investigated using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays. Only the non-volatile compounds exhibited activity. Their activities may be ascribed to the flavonoid components. Most of the species portrayed moderate to poor activity in the DPPH assay with the exception of *Agathosma capensis* (Gamka) (IC_{50} value of 24.08 $\pm$ 4.42µg/ml) and *A. pubigera* (IC_{50} value of 35.61 $\pm$ 0.86µg/ml) which were two of the most active species, although their activities were inferior when compared to vitamin C. The results from the ABTS assay differed from that of the DPPH assay. All extracts showed greater activity in this assay with *Agathosma namaquensis* (IC_{50} value of 15.66 ± 4.57µg/ml) and *A. capensis* (Besemfontein) (IC_{50} value of 19.84 ± 0.09µg/ml) being the most active species. This may be due to the ABTS assay having an additional reaction system.

'Buchu' has been used traditionally as an antipyretic, topically for the treatment of burns and wounds and for the relief of rheumatism, gout and bruises. The anti-

inflammatory activity of the essential oils and non-volatile compounds was assessed using the 5-lipoxygenase (LOX) assay. Only the essential oils exhibited activity. All proved to be active with the exception of *Agathosma stipitata* which was UV active and caused interference. This was due to its major compounds neral (39.9%) and geranial (10.1%) which absorbed strongly at 234 nm and hence rendered its spectrophotometric measurement impossible. The essential oil of *Agathosma collina* displayed the most promising activity (IC_{50} value of 25.98 ± 1.83µg/ml).

It is well known that many herbal medicines can have adverse effects, in which case it is necessary to evaluate the benefit-risk profile. The toxic effects of *Agathosma* species have been poorly studied and no information is available in this regard. Hence the toxicity profile of the non-volatile compounds and essential oils was assessed on transformed human kidney epithelium (Graham) cells using the microculture tetrazolium (MTT) cellular viability assay. The extracts of *Agathosma lanata* (IC_{50} value of 26.17 ± 9.58µg/ml) and *A. ovata* (round-leaf) (IC_{50} value of 25.20 ± 6.30µg/ml) proved to be the most toxic, whilst the extracts of *Agathosma bathii*, *A. capensis* (Besemfontein), *A. betulina*, *A. crenulata* and *A. namaquensis* did not prove to be toxic at the concentrations tested. Serial dilutions displayed different inhibitions of cell growth and the species proved to be toxic in a dose-dependant manner. The essential oils of all 19 species proved to be much more toxic (IC_{50} values < 0.0001µg/ml) than a plant-derived compound that is considered relatively safe, namely quinine (IC_{50} value of 136.06 ± 4.06µg/ml). The toxicities of the essential oils may be due to compounds like methyl chavicol, eugenol, methyl eugenol, pulegone and methyl salicylate whilst the toxicities of the extracts may be due to the alkaloid and coumarin components.

CONFERENCE AND PUBLICATIONS

- A. Moolla, A. M. Viljoen and S. F. van Vuuren. 2004. A phytochemical and pharmacological investigation of selected indigenous *Agathosma* species. Podium presentation at the Academy of Pharmaceutical Sciences Conference, Grahamstown. (13 September).

- A. Moolla, A. M. Viljoen, S. F. van Vuuren, R. L. van Zyl, K. H. C. Başer, B. Demirci and T. Özek. The biological activity and essential oil composition of selected indigenous *Agathosma* (Rutaceae) species. Journal of Essential Oil Research (Accepted for publication, November 2005).

- A. Moolla, A. M. Viljoen, S. F. van Vuuren, K. H. C. Başer, B. Demirci and T. Özek. A seasonal variation study of *Agathosma ovata* (Rutaceae). Journal of Essential Oil Research (To be submitted for publication).

- A. Moolla, A. M. Viljoen, S. F. van Vuuren and R. L. van Zyl (Rutaceae). The biological activity of the extracts of selected indigenous *Agathosma* (Rutaceae) species. Journal of South African Botany (To be submitted for publication).

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LIST OF ACRONYMS AND SYMBOLS

ABTS:	2, 2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)
$ABTS^{\cdot+}$:	2, 2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation
AIDS:	acquired immunodeficiency syndrome
ATCC:	American type culture collection
C:	carbon atom
°C:	degree Celsius
CAM:	complementary alternative medicine
CFU:	colony forming unit
CNS:	central nervous system
CO_2:	carbon dioxide
COX:	cyclo-oxygenase
DMSO:	dimethyl sulfoxide
DNA:	deoxyribonucleic acid
DPPH:	2, 2-diphenyl-1-picrylhydrazyl
FCS:	fetal calf serum
g:	gram
GC:	gas chromatography
GC-MS:	gas chromatography-mass spectroscopy
GLC:	gas-liquid chromatography
h:	hour
HCl:	hydrochloric acid
HIV-1:	human immunodeficiency virus-1
H_2O_2:	hydrogen peroxide

HP: Hewlett Packard

HPLC: high performance liquid chromatography

IC_{50}: inhibitory concentration 50%

INT: iodonitrotetrazolium

kg: kilogram

K_p: partition co-efficient

$K_2S_2O_8$: potassium persulphate

L: liter

LAC: luminal-amplified chemiluminescence

LC_{50}: lethal concentration 50%

LD_{50}: lethal dose 50%

LDL: low density lipoprotein

LOX: lipoxygenase

LT: leukotrienes

M: molar

max: maximum

mg: milligram

Mg: magnesium

MIC: minimum inhibitory concentration

min: minute

ml: milliliter

mm: millimeter

mM: millimol

MOP: methoxypsoralen

MTT: microculture tetrazolium

n: number of experimental runs

NADH: nicotinamide adenine dinucleotide (reduced form)

NADPH: nicotinamide adenine dinucleotide phosphate (reduced form)

NDGA: nordihydroguaiaretic acid

NHLS: National Health Laboratory Services

nm: nanometer

NMDA: N-methyl-D-aspartic acid

no: number

NSAID's: non-steroidal anti-inflammatory drugs

$O_2^{\cdot-}$: superoxide radical

$OH^{\cdot}$: hydroxyl radical

PDA: photodiode-array detector

pH: potential hydrogen

PPP: progressive pigmented purpura

$R^{\cdot}$: non-oxy free radical

ROI: reactive oxygen intermediates

rpm: revolutions per minute

R_t: retention time

SOD: superoxide dismutase

sp: species

spp: sub-species

TBA: thiobarbituric acid

TLC: thin layer chromatography

tr: trace

U: units

µg:	microgram
UK:	United Kingdom
µl:	microliter
UPGMA:	unweighted pair-group method with arithmetic average
UV:	ultraviolet
UV-VIS:	ultraviolet-visible
w/v:	weight per volume
°:	degree
$:	dollar
%:	percent

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CHAPTER 1: INTRODUCTION

1.1. Use of medicinal plants

Medicinal plants have always played an important role in therapy within the traditional health care system in South Africa. It is estimated that between 12 and 15 million South Africans still use traditional remedies from as many as 700 indigenous plants (Meyer and Afolayan, 1995), and that approximately 80% of the South African population use a traditional remedy at some stage in their life (Brandt and Muller, 1995). The use of traditional medicines remains widespread in developing countries while the use of complementary alternative medicine (CAM) is increasing rapidly in developed countries. Only a few African phytomedicines are available in the international market, however, locally medicinal plants play a key role in basic healthcare, particularly in rural areas due to their accessibility and affordability (Steenkamp, 2003). The sale of herbal medicines has increased considerably over the last 10 years in the industrialized countries. The growing trend to use herbal remedies to treat a wide range of problems (from insomnia, anxiety, obesity, bronchial asthma, constipation, gingivitis, Vincent's infection, eczema and varicosity to immunodeficiency syndrome) has been promoted by: (1) the development of new diseases, with severe complications, for which there is still no appropriate treatment; (2) the belief that herbal remedies are innocuous, in contrast to conventional drugs; (3) the idea that what is natural can only be good; (4) the special attention that ecological movements give to herbal medicines in Western countries; and (5) the belief that herbal medicines are naturally superior to synthetic drugs (Capasso *et al*., 2000).

The large continent of Africa has a variety of ethnic groups from north to south, and a similar variety of vegetation from bare deserts to tropical rain forests. Africans have utilized and continue to utilize plants in many ways. This knowledge about plant uses is passed on from generation to generation. Due to the large stretches of deserts in the continent, the number of species composing the flora is estimated at about 40,000 compared to about 80,000 in the Amazon (Kokwaro, 1995). Traditional medicine as practiced in Africa embraces a wide field of medicine and pharmacology, including pharmacognoscy. The traditional pharmacopoeia includes a recollection of where the plant is to be found as well as the identification and the preparation of decoctions, infusions, etc. (Kokwaro, 1995) . The indigenous people of South Africa have a long history of traditional plant usage for the treatment of various diseases and ailments including the uses of plants for the treatment of wounds (van Wyk *et al*., 1997). Apart from indigenous species, a number of introduced plants now have encroached upon traditional medicine. The aromatic leaves of a number of gum trees (*Eucalyptus* species) introduced from Australia, are used for treating influenza. *Citrus* species are used for the same purpose. Bark of Pride of India (*Melia azedarach*) is used in Zaire and west Africa as an antihelmintic, and the castor oil plant (*Ricinus communis*), which grows wildly throughout Africa, is used as a purgative (Kokwaro, 1995).

1.2. Ethnobotany and ethnopharmacology

Modern ethnobotany is concerned with the 'totality of the place of plants in a culture' (Ford, 1978). It is the study of plant human inter-relationships embedded in dynamic ecosystems of natural and social components (Alcorn, 1995). The term can also briefly be defined as folk botany, or the description of the various methods by which local people utilize plants (Kokwaro, 1995). The aims are two-fold: (1) to document

facts about plant use and plant management and (2) to elucidate the ethnobotanical text by defining, describing and investigating, ethnobotanical roles and processes. Changes in the interpretation of ethnobotany are depicted in Table 1.1.

Table 1.1: Changes in the interpretation of 'ethnobotany' (Cotton, 1997).

Date	Interpretation of ethnobotany	Source
1873	*Aboriginal botany* - the study of all forms of vegetation which aborigines used for commodities such as medicine, food textiles and ornaments.	Powers, 1873
1895	*Ethnobotany* - the use of plants by aboriginal people.	Harshberger, 1896
1916	Not just a record of plant use, but the traditional impressions of the total environment as revealed through custom and ritual.	Robbons *et al*., 1916
1932	Not only tribal economic botany, but the whole range of traditional knowledge of plants and plant life.	Gilmour, 1992
1941	The study of the relations which exist between humans and their ambient vegetation.	Schultes, 1941
1941	The study of the interrelations between 'primitive' humans and plants.	Jones, 1941
1981	The study of the *direct* relationships between humans and plants.	Ford, 1978
1990	The study of useful plants prior to commercialization and eventual domestication.	Wickens, 1990
1994	All studies (concerning plants) which describe local people's interaction with the natural environment.	Martin, 1995

In the field of medicine alone, between 25% and 50% of the modern drug armamentarium is derived from natural products, and most of these compounds were first used as medicines or poisons in a folk context (Davis, 1995; Holmstedt and Bruhn, 1995). Almost every class of drug includes a model structure derived from

nature, exhibiting the classical effects of that specific pharmacological category. A great number of these natural products have come to us from the scientific study of remedies traditionally employed by various cultures. Most of them are plant derived e.g. pilocarpine, vincristine, emetine, physostigmine, digitoxin, quinine, atropine and reserpine (Holmstedt and Bruhn, 1995). For much of Western history, ethnobotany was a strategy that sought to satisfy an economic imperative by yielding new natural products of economic potential. The greatest economic potential of ethnobotany lies in the area of folk-medicine. Annual worldwide sales of plant derived pharmaceuticals currently total over $20 billion and many of these drugs were first discovered by traditional healers in folk contexts (Davis, 1995).

Without the vast store of basic knowledge about plant uses, plant chemistry, and indigenous cultures, the present phase of ethnobotanical research could not begin. It is tragic that the inventory is far from complete, when the plant knowledge that indigenous people built up over thousands of years of interaction with their environment is being lost at an ever-increasing rate. Scientists will not be able to gather ecological data when indigenous groups have been acculturated. It is therefore urgent to consider what information we should gather from the remnants of the great indigenous cultures of the world, such as those of the South American Indians, the African Bushmen or the natives of New Guinea (Prance, 1995).

The term ethnopharmacology has been used to describe the observation, identification, description and experimental investigation of the ingredients and the effects of indigenous drugs (Holmstedt and Bruhn, 1995). Ethnopharmacologic research is based on botany, pharmacology and chemistry. Harmful practices can be

discouraged, such as the use of plants containing tumor-producing pyrrolizidine alkaloids. Ethnopharmacology is not just a science of the past utilizing an outmodelled approach. It constitutes a scientific backbone in the development of active therapeutics based upon traditional medicine of various ethnic groups (Holmstedt and Bruhn, 1995).

Two major challenges mark the future of ethnobotany and ethnopharmacology. Firstly, the longstanding task of documenting which plants are and are not important to a society and secondly the task of sifting through the immense repositories of folk belief for plants that may serve the needs of a society. Our ultimate aim should be the validation (or invalidation) of these traditional preparations, either through the isolation of active substances or through pharmacological findings.

1.3. Introduction to the family and genus

1.3.1. The family: Rutaceae

The Rutaceae is a predominantly tropical family of trees and shrubs containing genera of importance in horticulture (*Citrus*), silviculture (*Chloroxylon, Flindersia, Zanthoxylum*) and medicine (*Pilocarpus*, *Agathosma*) (Hutchinson, 1959). It is considered to consist of about 1600 species distributed between approximately 150 genera (Engler, 1964).

The family is distributed in both temperate and tropical countries, but particularly abundant in South Africa and Australia. Trees, shrubs or shrublets belonging to this family are usually aromatic. Glands containing essential oils are present in the leaves and other parts. The flowers are usually in cymes with four to five sepals, four to five

petals, eight to ten stamens and a superior ovary. The fruits are of various types. Constituents of the Rutaceae include a wide variety of alkaloids, volatile oils, rhamno-glucosides, coumarins and terpenoids (Evans, 1996).

Classification of the Rutaceae still rests largely upon the system adopted by Engler (1964) (Figure 1.1). Of the seven sub-families proposed by Engler, three (the Rutoideae, Toddalioideae and Aurantioideae), encompass the great majority of genera and species.

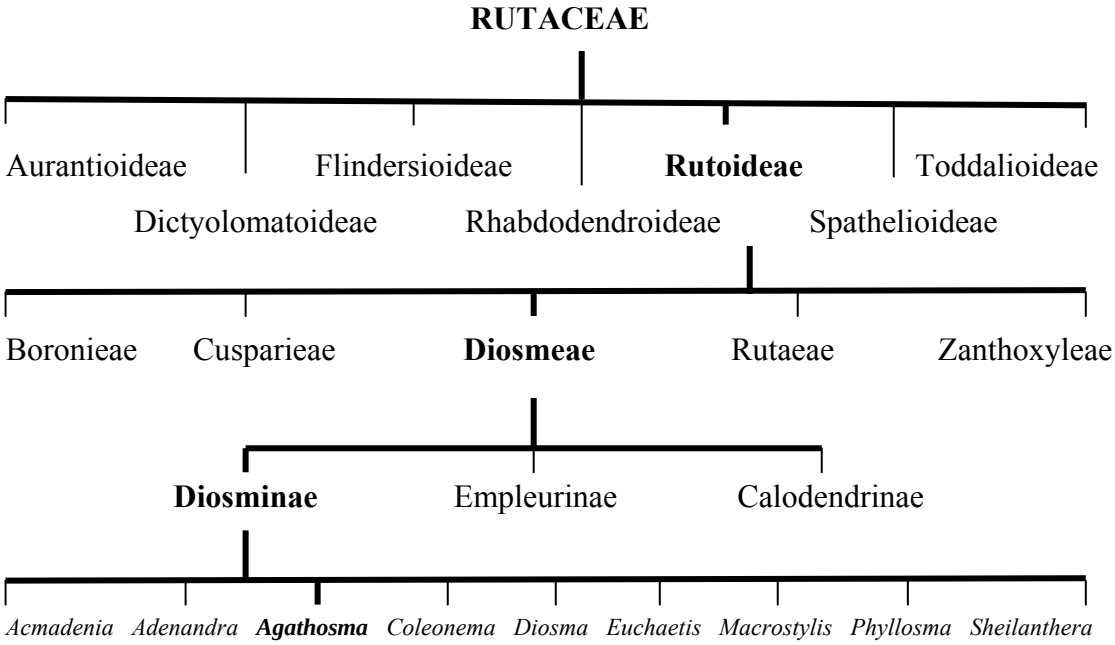


Figure 1.1: Taxonomic hierarchy of the genus *Agathosma* (Engler, 1964).

The Rutoideae is a sub-family of the Rutaceae. Description: carpels are usually four to five, seldom one to three or more, often united only by the common pistil and free below, at maturity more or less separated, opening inward by splitting the follicle (loculicidal), usually with a dehiscent endocarp and very seldom with one to four

fleshy drupes (Pitaviinae). The leaves, bark and twigs have schizolysigenous oil glands. They consist of five tribes, 17 sub-tribes and 86 genera (Swingle, 1943).

The Diosmeae, one of the five tribes of a sub-family of the Rutaceae, the Rutoideae, is mainly confined to the predominantly winter-rainfall fynbos of the south west and southern regions of South Africa. The tribe consists of 10 genera and approximately 260 shrubby and sub-shrubby species, more than half belonging to the genus *Agathosma* (Campbell *et al*., 1986). They consist mostly of perennial herbs and shrubs, seldom trees (*Calodendrum*), always with simple leaves. The flowers are almost always actinomorphic. They consist of three tribes and 12 genera (Swingle, 1943). The Diosminae are sclerophyllous shrubs and sub-shrubs, commonly known as 'buchus'.

1.3.2. The genus: *Agathosma*

The Cape region of South Africa has veldt-types with arguably the richest composition of indigenous aromatic plant species in the whole of South Africa. Amongst 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. They have a rue-like smell (Grieve, 1995). The flowers are star shaped and open and contain five petals, five stamens, a five-lobed ovary (Figure 1.2) and the leaves have a characteristic smell when crushed (van Wyk *et al*., 1997).



Agathosma ovata



Agathosma bathii



Agathosma collina



Fruits and flowers of *Agathosma* species

Figure 1.2: Flowers, fruit and leaves of some *Agathosma* species.

The Khoi and San people used the word 'buchu' for any fragrant plant that could be dried and powdered, so this name does not designate a single species. Many species (135) of the same family (*citrus*) are regarded as 'buchus' and are used, for example *Agathosma serpyllacea*, *A. riversdalensis*, *A. collina*, *A. dielsiana*, *A. cerefolium*, *A. imbricata*; *Adenandra obtusata*, *A. viscida*, *A. brachyphilla* and *Coleonema album.* 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 are used as a cosmetic. *Empleureum* species commonly referred to as 'false buchu' and 'berg buchu', have 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). Species of *Acmadenia*, *Diosma* and *Euchaetis* all have one thing in common, mainly that when you rub them you can smell a fresh citrus-liquorice smell, and even aniseed in some cases (Schwegler, 2003). *Agathosma serratifolia* ('narrow-leaf buchu') a willow-like small tree, *A. pulchella* and *A. ovata* ('false buchu') a small rounded shrub with pink flowers, have also been used for medicinal purposes amongst the Cape people (van Wyk and Gericke, 2000).

'Buchu' (*Agathosma betulina* and *A. crenulata*) is probably one of the best known South African herbs both locally and internationally. The indigenous people first introduced 'buchu' as a medicinal plant to the European settlers in the Cape. The use of the plant subsequently spread to Europe and America where it was extensively used as a medicine. Of the 150 species, *Agathosma betulina* ('round-leaf buchu') (Figure 1.4) 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 *Agathosma betulina* is dark yellow-brown in colour, with a minty-camphoraceous odour. Both species are cultivated, developed as crop plants and are used commercially. This is due to the limited availability of the wild plant material (van Wyk and Gericke, 2000).



Figure 1.3: 'Buchu' leaves.



Figure 1.4: *Agathosma betulina*.

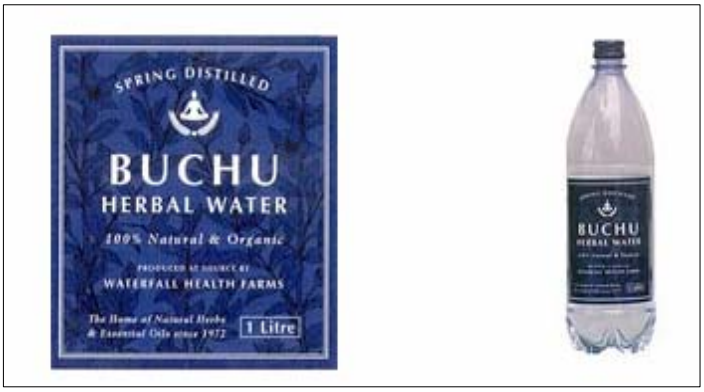


Figure 1.5: Commercial 'buchu' water.

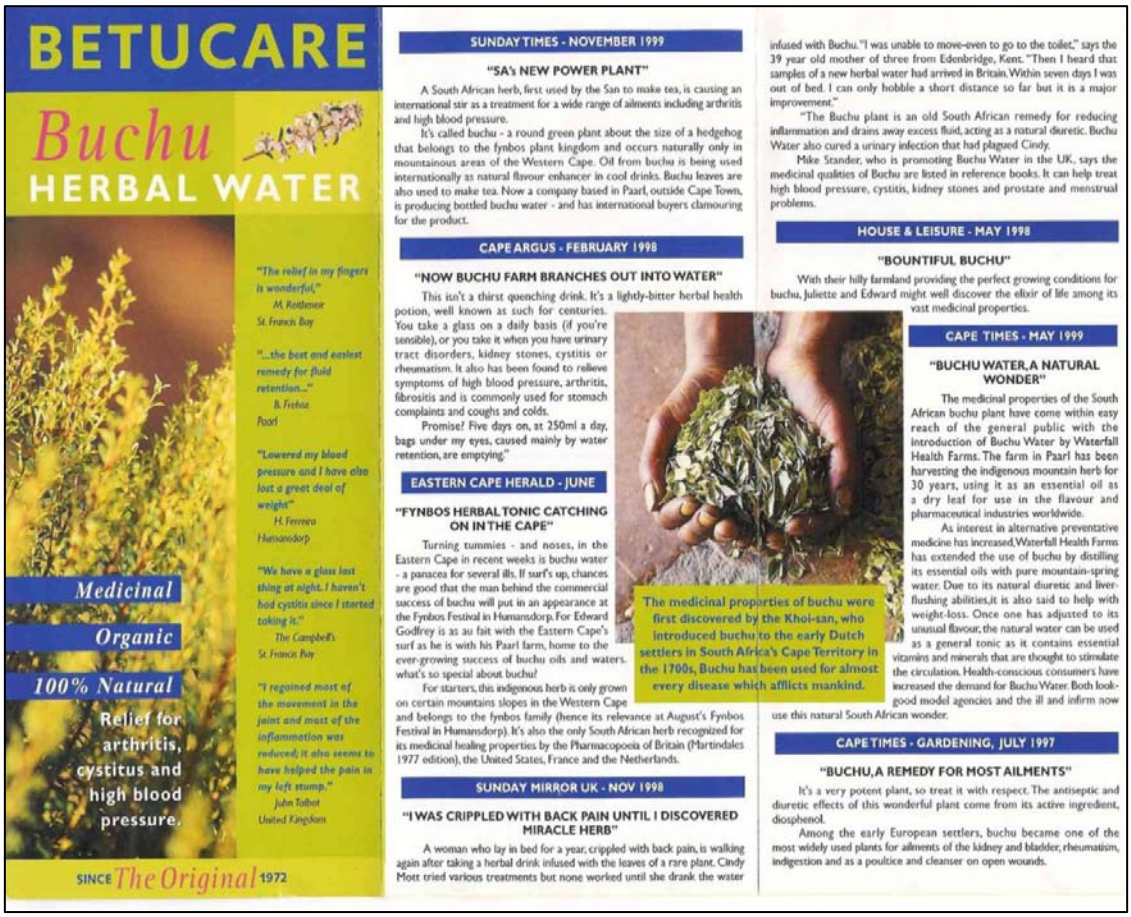


Figure 1.6: Uses of 'buchu' herbal water (www.betucare.com, 17 August 2004).

'Buchu' forms part of about 10 prepared herbal teas, including Buccotean Tee®, Buccosperin Tee®, Uron-Tee® and is a constituent of the UK product Potter's Kas-bah Herb®. 'Buchu' herbal water is also available (Figures 1.5 and 1.6). Other preparations available in the UK containing 'buchu' are: Potter's Diuretictabs®, Antitis tablets, Backache tablets and Gerard House Herbal Powder® (Bisset, 1994).

'Buchu' leaves (Figure 1.3) are collected while the plant is flowering and fruiting, and are then dried and exported from Cape Town. The Cape government exercises strict control over the gathering of 'buchu' leaves and has lately made the terms and conditions more onerous, in order to prevent the wholesale destruction of the wild

plants, no person being permitted to pick or buy ‘buchu’ without a license. Cultivation experiments with ‘buchu’ have been made from time to time by private persons, and experiments were also conducted at the National Botanical Gardens, Kirstenbosch (Cape Town), the results of which (given in the South African Journal of Industries, 1919, 2: 748) indicate that, under suitable conditions, the commercial cultivation of ‘buchu’ should prove a success; *Agathosma betulina* being the most valuable species to be grown (Grieve, 1995).

1.3.3. Traditional uses

Finding the healing powers in plants is an ancient idea. ‘Buchu’ is an important part of the San and Khoi culture in the Cape and is still used as a general tonic and medicine throughout South Africa. Some of the traditional uses of plants belonging to the genus *Agathosma* are listed below (Watt and Breyer-Brandwijk, 1962):

- An antispasmodic
- An antipyretic
- A liniment
- A cough remedy, as well as for the treatment of colds and flu
- A diuretic
- Treatment of kidney and urinary tract infections, as well as haematuria and prostatitis
- Treatment of cholera and other stomach ailments
- Relief of rheumatism, gout and bruises
- Relief of calculus
- For antiseptic purposes

- To cause febrifuge (profuse perspiration)

Agathosma species have been used for cosmetic purposes and as an 'antibiotic protectant'. The San used the aromatic plants lubricated with fat, to keep their skin soft and moist in the desert climate, as an antibacterial and antifungal agent, as an insect repellant, as a deodorant and to promote the general well-being of the body through the uptake of aromatic substances through the skin (Simpson, 1998). The leaves were used for a variety of preparations. They 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 infuse for 10 minutes before being strained. A cup of the infusion is drunk several times a day as a diuretic (Bisset, 1994).

1.3.4. Modern uses

The major use of 'buchu' is in the flavour industry, where it is used to enhance fruit flavours. It is particularly useful for black currant flavours. It is said to have a minty camphoraceous, sweet berry, catty, tropical guava, apricot and peach, green herbal taste. The oil is also used in perfumes and colognes. In the Pharmacopoeias, 'buchu' is categorized as a diuretic and urinary tract antiseptic. It is also used to treat arthritis, cellulite, cystitis, diarrhea, flatulence, kidney infections, nausea, rheumatism and wounds. 'Buchus' are natural deodorizers, and fishermen remove the fishy smell by rubbing the twigs of *Coleonema album* (Cape May or 'aasbossie') between their hands. They are also natural insect repellants, and campers can rub their bedding with them to keep ants and mosquitoes away (Schwegler, 2003).

1.4. Previous research

Studies have been performed previously in order to determine the chemical constituents of *Agathosma* essential oils. Fluck *et al*. (1961) performed a study to determine the chemical composition of 'buchu' leaf oil. They succeeded in identifying pulegone and diosphenol 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 (1971) discovered that two monoterpene thiols were responsible for the characteristic odour. They isolated 8-mercapto-*p*-menthan-3-one from 'buchu' oil and found that this sulphur containing terpene was very important for the flavour and the aroma of the oil. Kaiser *et al*. (1973) has performed the most detailed study on 'buchu' identifying more than 120 components including the already known pulegone, diosphenol and 8-mercapto-*p*-menthan-3-one, in the oils of two different 'buchu' species. The study was performed in order to determine its aromatic important components. *S*-prenyl-thioisobutyrate was detected in the oils of *Agathosma apiculata*, *A. clavisepala* and *A. puberula* (Rivett, 1974). The same compound was detected in large quantities in a similar study (Moran *et al*., 1975). Gas-liquid chromatography (GLC) and gas chromatography-mass spectroscopy (GC-MS) analysis revealed the presence and established the structures of three *S*-prenyl thioesters in the same species (Campbell *et al*., 1980).

Nijssen and Maarse (1986) investigated the usefulness of GC-MS in controlling the authenticity of fruit products. Sixteen black currant samples were investigated and it appeared that several of these commercially available products contained buchu oil to improve their quality, without any such indication on the label. The oil is used

because of one its constituents, 8-mercapto-*p*-menthan-3-one, has a catty odour that is similar to the odour of black currants. Campbell and Williamson (1991) identified *S*-prenyl thioesters in the essential oils of two Diosmeae species, *Agathosma rosmarinifolia* and *Empleurum fragrans*. In the same year they evaluated the composition of the essential oil of *Agathosma capensis* using gas chromatography (GC) and GC-MS. An investigation of 'buchu' oil with two-dimensional GC with sulphur specific chemiluminescence detection was performed by MacNamara *et al*. (1992). Another study was published to authenticate the natural origin of 'cassis' type fruit aromas (Köpke *et al*., 1994).

Collins and Graven (1996) performed a study to determine the chemotaxonomy of commercial 'buchu' species. In the same year Posthumus and van Beek (1996) performed a study to determine the chemical composition of the essential oils of *Agathosma betulina*, *A. crenulata* and an *A. betulina* x *A. crenulata* 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. Kramer *et al*. (1996) aimed at learning more about the sulphur chemistry of buchu leaf oil, hence their work entailed the isolation and structure elucidation of sulphur-bearing compounds present in trace levels in the essential oil of *Agathosma betulina*. They also determined the origins (natural biosynthesis or thermal processing) and flavour characteristics of the various sulphur compounds detected. An *in vitro* study on the mode of action and the antimicrobial activity of the essential oils of *Agathosma betulina* and *A. crenulata* was performed by Lis-Balchin *et al*. (2000), on the guinea pig ileum. It was found that at high concentrations the oils had an initial spasmogenic activity followed by spasmolysis. Very low antimicrobial activity was observed.

Another study involved stem feeding the young plants of *Agathosma crenulata* with aqueous solutions of $^{2}H_2$ and $^{18}O/^{2}H_2$ - labelled monoterpene precursors (Fuchs *et al*., 2001). The essential oil was extracted by solid phase microextraction and subsequently analysed with enantioselective multidimensional GC-MS. Both labelled pulegone precursors were converted into corresponding labelled menthone, isomenthone and menthofuran with different enantioselectivities (Fuchs *et al*., 2001).

Very few studies have been performed on the non-volatile fractions of *Agathosma* species. Waterman (1975) published an article on the distribution and systematic significance of alkaloids of the Rutaceae. Blommaert and Bartel (1976) performed a study, in which they measured the leaf form of buchu plants (*Agathosma betulina* and *A. crenulata*) from local plantings. They stated that this criterion was the only taxonomic basis for distinguishing the two species and that the method was reliable for the purposes of identification but did not hold true for hybrid buchu. Puberulin (6,8-dimethoxy-7-prenyloxycoumarin), a new prenyloxy-coumarin was discovered by Finkelstein and Rivett (1976) in *Agathosma puberula*. Campbell *et al*. (1986) investigated 24 species from the genera *Agathosma*, *Diosma* and *Empleureum* (tribe Diosmeae) for coumarins. Nine simple coumarins were isolated. The aerial parts of 42 taxa of the genera *Agathosma*, *Coleonema*, *Diosma*, *Empleureum* and *Phyllosma* (tribe Diosmeae) were screened in a study by Campbell *et al*. (1987) for alkaloids. Positive results were obtained for five *Agathosma* species and the compounds halfordamine and skimmianine were identified. Direct testing of ^{14}C-labelled aesculetin found it to be the intermediate between umbelliferone and scopoletin in the biosynthesis of puberulin by *Agathosma puberula* (Brown *et al*., 1988). Skimmianine and two new alkaloids were identified by means of spectral data in a study performed

by Campbell *et al*. (1990). Campbell and Bean (1996) detected quinoline alkaloids in *Agathosma barosmaefolia* and hence reinforced their previous proposals that alkaloids in the Diosmeae may be confined to advanced *Agathosma* species.

1.5. Rationale

Despite the large number of indigenous *Agathosma* species (154) and their traditional uses, it is surprising that research in the pharmaceutical domain has only been extensively performed on two *Agathosma* species, namely *A. betulina* and *A. crenulata*. Limited research has been performed on a few other species.

Guided by the previous mentioned medicinal uses of *Agathosma* species as well as the recorded ethnobotanical data for some species, this study sought to investigate the phytochemical and pharmacological properties of selected indigenous *Agathosma* species.

1.6. Objectives

- Determine the essential oil composition of selected indigenous *Agathosma* species
- Investigate the anti-oxidant, anti-inflammatory and antimicrobial activities of the selected species
- Determine the chemical profiles of the phenolic (non-volatile) fractions of the selected species
- Determine the toxicities of the selected species
- Establish the scientific rationale for the traditional use of *Agathosma* species.

CHAPTER 2: PLANT COLLECTION AND PREPARATION

2.1. Species

The 17 species (19 samples) used for the study were selected on the basis of traditional use and accessibility to the localities for collection of plant material (Table 2.1).

2.2. Collection of plant material

The study was performed on fresh plant material of indigenous *Agathosma* species collected from natural populations in the Cape (Figure 2.1). The taxonomy was confirmed by Mr Terry Trinder-Smith (Bolus Herbarium, University of Cape Town) and voucher specimens have been deposited in the Bolus Herbarium. Duplicate specimens are maintained in the Department of Pharmacy and Pharmacology, University of the Witwatersrand.

Table 2.1: List of indigenous *Agathosma* species studied, their localities and voucher information.

Species	Locality	Voucher information
A. arida	Rooiberg	TTS 241
A. bathii	Kleinplaas	AV 1013
A. betulina	Landmeterskop (Middelberg)	AV 852
A. capensis	Besemfontein	TTS 348
A. capensis	Gamka Mountains	JEV 164
A. collina	De Hoop	TTS 328
A. crenulata	Welbedacht, Tulbagh	AV 853

Species	Locality	Voucher information
A. hirsuta	Landdrostkop, Hottentots Holland Mountains	TTS 310
A. lanata	Rooiberg	TTS 242
A. namaquensis	Khamiesberg	TTS 289
A. ovalifolia	Droëkloof Mountains	TTS 240
A. ovata (hook-leaf)	Gamka Mountains	TTS 246
A. ovata (round-leaf)	Anysberg	TTS 263
A. parva	Die Galg, Riviersonderend Mountains	TTS 298
A. pubigera	Pakhuis	TTS 357
A. pungens	Khamanassie	TTS 253
A. roodebergensis	Rooiberg	TTS 237
A. stipitata	Die Galg, Riviersonderend Mountains	TTS 301
A. zwartbergense	Swartberg Range	TTS 257

The samples of *Agathosma capensis* were collected from two localities, i.e. Besemfontein and Gamka Mountains in order to determine chemotypic variation.



Figure 2.1: Collection of plant material in the Cape.

2.3. Preparation of samples

2.3.1. Essential oils

A known quantity of fresh plant material of each species was subjected to hydrodistillation in a Clevenger apparatus for three hours (Figure 2.2), either on the same day of harvesting or one day after harvesting. This technique is based on the evaporation of volatile compounds induced by steam. The essential oils were collected in amber vials, weighed, sealed and stored in the refrigerator until analysis. The percentage oil yield is based on the dry weight of the plant material.



Figure 2.2: Clevenger apparatus used for distillation.

2.3.2. Non-volatile compounds (phenolics)

A known quantity of fresh plant material of each species was dried, ground and thereafter kept at room temperature until analysis. Extraction of the non-volatile compounds was done by a solvent extraction method. A known quantity of ground plant material of each of the species was extracted for 24 h in a solvent system consisting of methanol and dichloromethane (1:1). The extraction procedure was performed three times. The extracts were filtered to remove all the debris and thereafter they were dried using a rotavaporator. The dry extracts were then weighed and stored in vials at room temperature until analysis.

CHAPTER 3: ESSENTIAL OIL COMPOSITION

3.1. Introduction

Essential oils are the odourous, volatile products of an aromatic plant's secondary metabolism, normally formed in special cells or groups of cells, found in many leaves and stems. They are commonly concentrated in one particular region such as leaves, bark or fruit, and when they occur in various organs in the same plant, they frequently have different chemical profiles (Araújo, 2002).

Volatile oils are very complex mixtures of compounds. The constituents of the oil are mainly monoterpenes and sesquiterpenes which are hydrocarbons with the general formula $(C_5H_8)n$. Oxygenated compounds derived from these hydrocarbons include alcohols, aldehydes, esters, ethers, ketones, phenols and oxides. It is estimated that there are more than 1000 monoterpene and 3000 sesquiterpene structures (Svoboda and Hampson, 1992). Other compounds include phenylpropenes and specific compounds containing sulphur or nitrogen. Hundreds of new natural substances are constantly isolated and identified, but data concerning their biological activities are limited. In certain plants one main constituent may predominate. In basil, for example, methyl chavicol represents 75% of the oil. In other species there is no single component which predominates. Instead there is a balance of various components, as for example in the oil of sweet marjoram where the individual chemicals are represented by 0.1-10% of total volume. The presence of trace components, even those as yet unidentified, can influence the odour, flavour and possibly also the biological activity significantly (Svoboda and Hampson, 1992).

3.2. Materials and methods

3.2.1. Thin layer chromatography (TLC)

One part of concentrated essential oil was diluted with seven parts of hexane and 2µl of the dilution was applied to silica plates (Alugram Sil G/UV_{254}). Toluene and ethyl acetate (9.3: 0.7) were used as the mobile phase. Two TLC plates were prepared, developed and thereafter sprayed separately with two different reagents i.e. anisaldehyde-sulphuric acid reagent which is used for the detection of terpenoids and propylpropanoids, and vanillin-sulphuric acid reagent which is used for the detection of components of essential oils e.g. terpenoids, lignanes and cucurbitacins. The first reagent was prepared by mixing 0.5ml anisaldehyde with 10ml glacial acetic acid, followed by 85ml methanol and 5ml concentrated sulphuric acid. The second reagent was prepared by making a 1% ethanolic vanillin solution and a 10% ethanolic sulphuric acid solution separately. The plate was first sprayed with the ethanolic vanillin solution, followed immediately by the ethanolic sulphuric acid solution. Both plates were thereafter immediately heated at 100°C for five minutes and then evaluated. TLC analysis was not performed on the essential oil of *Agathosma ovata* (hook-leaf) due to insufficient sample.

3.2.2. Gas chromatography-mass spectroscopy (GC-MS)

Analysis of essential oils by gas chromatography (GC) and mass spectroscopy (MS) was performed using a Hewlett Packerd (HP) 1800A GCD system operating under the following conditions; column: HP-Innowax (60m x 0.25mm id., 0.25µm 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). Compound identification was done using the Başer, Adams and Wiley libraries search of

retention indices in comparison with literature values. The mass spectra obtained were matched to those present in the abovementioned libraries. Quantitative data (percentage composition) was determined from the GC peak areas. GC-MS analysis was not performed on the essential oil of *Agathosma ovata* (hook-leaf) due to insufficient sample.

3.2.3. Cluster analysis

The percentage composition of the essential oil samples was used to determine the relationship between the different *Agathosma* species by cluster analysis using the NTSYS software developed by Rohlf (1992). Correlation was selected as a measure of similarity, and the unweighted pair-group method with arithmetic average (UPGMA) was used for cluster definition.

3.3. Results and Discussion

3.3.1. Essential oil yield

There was considerable variation in the percentage oil yield based on the dry weight of the samples (Figure 3.1). *Agathosma hirsuta* produced the highest yield (1.15%) whilst *A. ovalifolia* produced the lowest yield (0.16%). All the essential oils all had a pale yellow colour. *Agathosma capensis* (Besemfontein) had a greater oil yield (0.86%) then *Agathosma capensis* (Gamka) (0.68%) which may be attributed to different localities and hence the plants growing under different conditions (e.g. temperature, soil type and climate). Lower temperatures in one area would cause a higher proportion of stems to occur, hence less leaves which contain the pellucid oil glands, thus producing a lower oil yield.

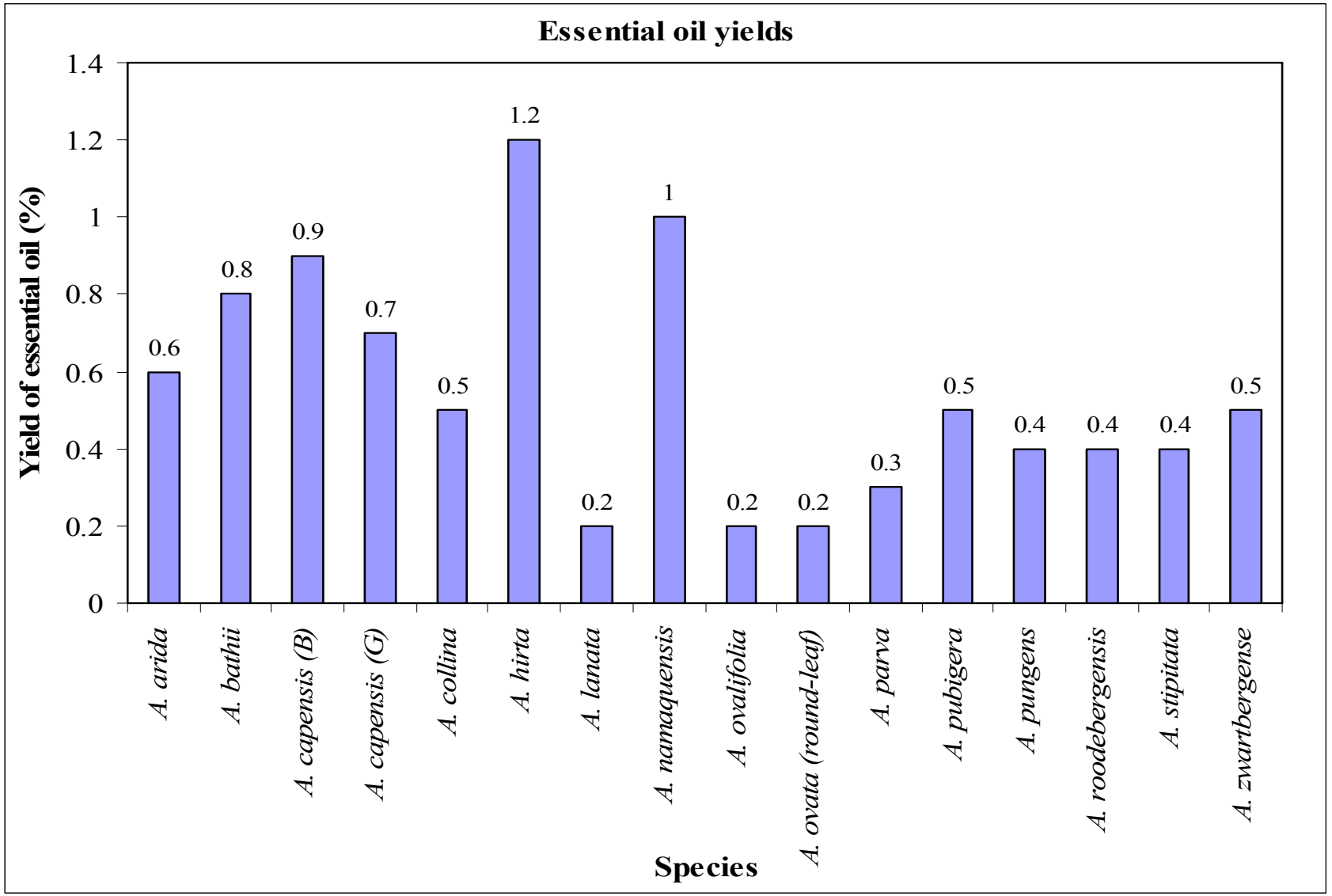
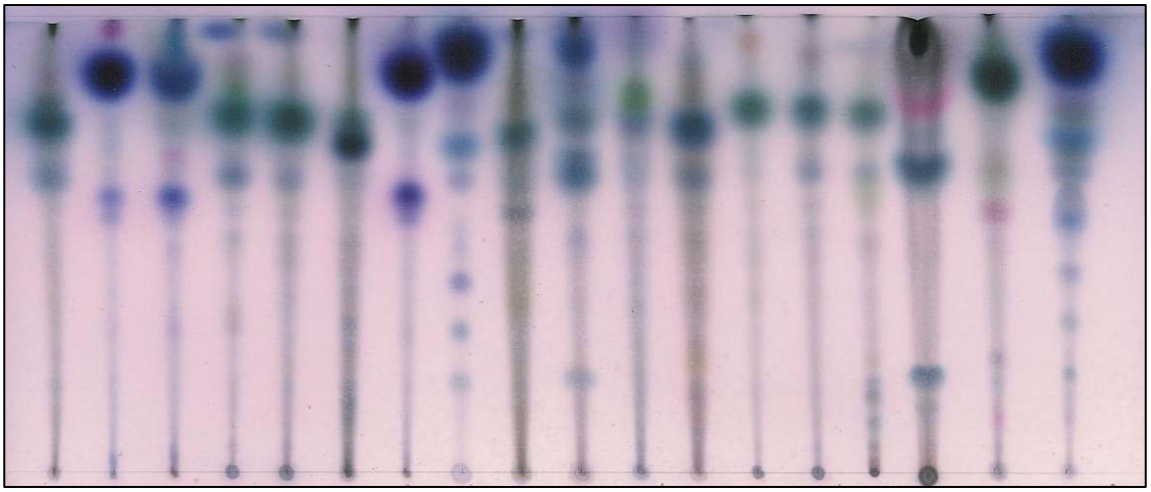


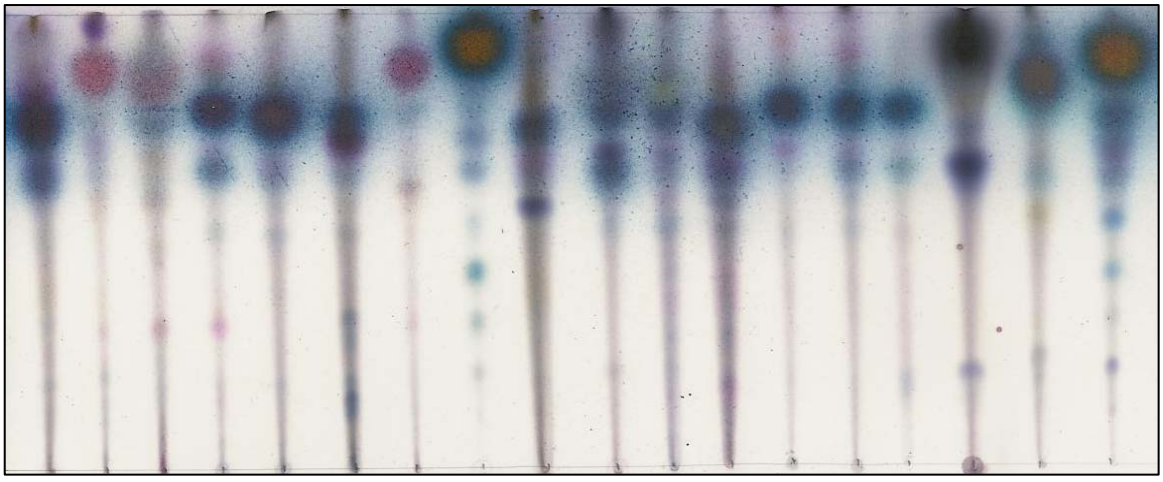
Figure 3.1: Bar graph comparing the percentage essential oil yields of indigenous *Agathosma* species.

3.3.2. Thin layer chromatography



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 3.2: TLC plate of the essential oils of *Agathosma* species sprayed with anisaldehyde-sulphuric acid reagent.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 3.3: TLC plate of the essential oils of *Agathosma* species sprayed with vanillin-sulphuric acid reagent.

Key to samples:

1. *A. arida*
2. *A. bathii*
3. *A. betulina*
4. *A. capensis* (Besemfontein)
5. *A. capensis* (Gamka)
6. *A. collina*
7. *A. crenulata*
8. *A. hirsuta*
9. *A. lanata*
10. *A. namaquensis*
11. *A. ovalifolia*
12. *A. ovata* (round-leaf)
13. *A. parva*
14. *A. pubigera*
15. *A. pungens*
16. *A. roodebergensis*
17. *A. stipitata*
18. *A. zwartbergense*

The results of the TLC analysis are depicted in Figures 3.2 and 3.3. Vanillin produced prominent spots with a khaki to dark green colour (Figure 3.3) while anisaldehyde resulted in bright purple spots (Figure 3.2). The TLC plates of the essential oils indicate an immense chemical variation amongst the species. *Agathosma hirsuta* and *A. zwartbergense* revealed a similar chromatographic profile in terms of their major compound (Figures 3.2 and 3.3, tracks 8 and 18), which GC-MS data revealed to be citronellal (Figures 3.4 and 3.5). The essential oils of *Agathosma capensis* (Besemfontein) and *A. capensis* (Gamka) portrayed an almost identical chemical constitution (Figures 3.2 and 3.3, tracks 4 and 5), whilst those of *Agathosma arida*, *A. parva*, *A. pubigera* and *A. pungens* proved to be similar to one another and to both the samples of *A. capensis*. This indicated that there is little variation in the essential oil composition of these species. In addition to many of the common components revealed

by GC-MS, the essential oils of these six samples have in common a major compound with an R_f value of 0.77. GC-MS data has revealed that linalool is a major compound in each of these species.

The essential oils of *Agathosma bathii*, *A. betulina* and *A. crenulata* also have similar constituents. *Agathosma roodebergensis* is anomalous to the others in terms of some of its constituents (Figures 3.2 and 3.3, track 16). GC-MS data supports this by revealing that geijerene and dictamnol are two of its major compounds which are also present in other species but not in such high quantities. *Agathosma collina*, *A. lanata*, *A. ovalifolia* and *A. stipitata* are chemically unique. Essential oil analysis has revealed that neral and geranial are characteristic of *Agathosma stipitata*. The results obtained from the TLC screening are also confirmed by GC-MS analysis.

Because of the chemical variation portrayed amongst the samples, the variation had to be further investigated using gas chromatography and gas chromatography coupled to mass spectroscopy.

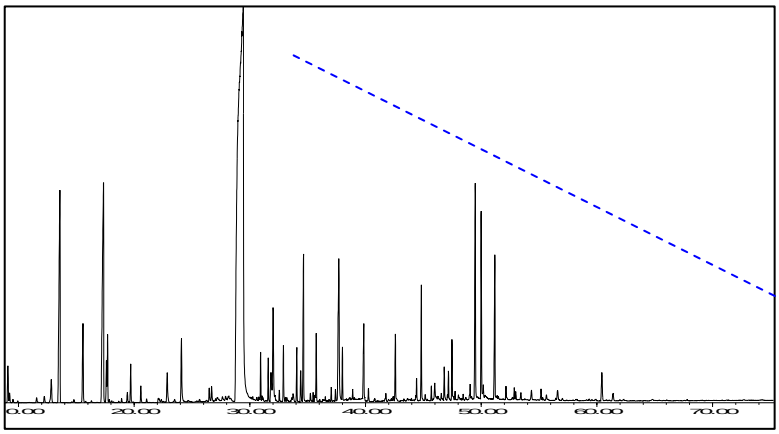
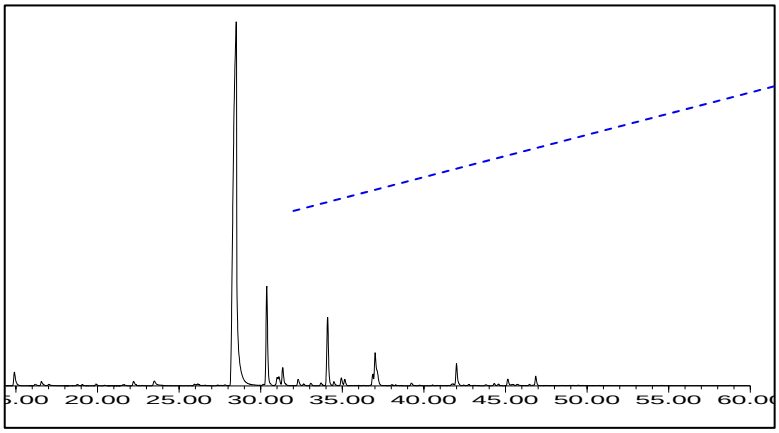
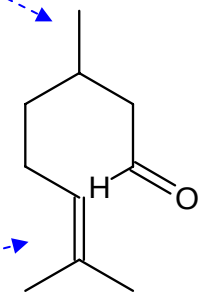


Figure 3.4: GC-MS chromatogram of *Agathosma hirsuta*.





citronellal

Figure 3.5: GC-MS chromatogram of *Agathosma zwartbergense.*

3.3.3. Gas chromatography-mass spectroscopy and cluster analysis

The GC-MS chromatograms, the compounds identified in each of the species, their percentages and relative retention indices are presented in the monographs (Appendix I).

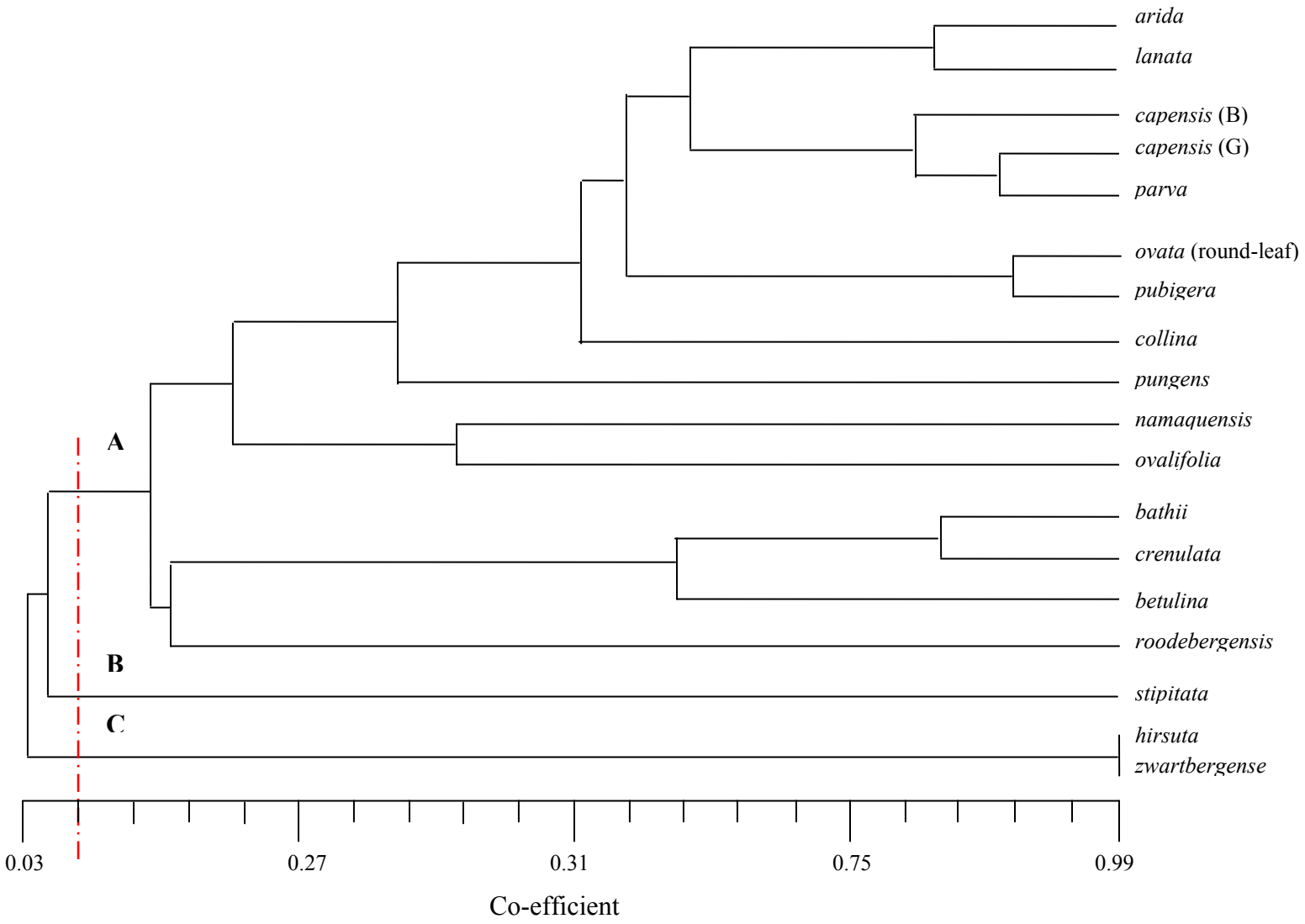


Figure 3.6: Dendrogram constructed from the volatile constituents of selected indigenous *Agathosma* species.

The composition of the essential oils of 17 indigenous *Agathosma* species (18 samples) was analyzed by GC-MS, leading us to compare the relative retention times and the mass spectra of oil components with those of authentic samples and mass spectra from data libraries. GC-MS analysis resulted in the identification of 333 compounds in 18 of the samples. To evaluate the chemical similarities and differences, cluster analysis was used to assess the essential oil composition of the 18 samples. A dendrogram was obtained and an arbitrary phenoline drawn at a co-efficient > 0.03, revealed three main distinct clusters (A, B and C) (Figure 3.6). The results showed qualitative and quantitative differences amongst the taxa.

Agathosma arida and *A. lanata* are united in a single cluster due to the compounds β-pinene, linalool and spathulenol being major components in both species (Cluster A, Figure 3.6). Linalool, myrcene and limonene are the major constituents of both *Agathosma capensis* (Gamka) and *A. capensis* (Besemfontein). Both these species are very similar in terms of their compositions with the exception of some compounds which are only present in either one of the species (e.g. 2-methyl-3-buten-2-ol, α-terpinene, (*Z*)-3-hexenal, γ-terpinene, allo-ocimene and few other compounds). Although it cannot be certain, the qualitative and quantitative differences in the chemical compositions of the leaf oils of *Agathosma capensis* (Gamka) and *A. capensis* (Besemfontein) may be attributed to the plants growing in different localities and hence under different conditions (e.g. temperature, soil type and climate) . The small differences could also be ascribed to either a genetic or phenetic divergence, or to environmental differences. Hence they are closely related to each other in the dendrogram but not tightly clustered

(Cluster A, Figure 3.6). A study performed by Campbell and Williamson (1991) found α-pinene (8.0%), myrcene (12.3%), limonene (16.7%), linalool (21.1%), estragole (= methyl chavicol) (18.1%) and *trans*-anethole (10.5%) to be the major components in the essential oil of *Agathosma capensis*. GC-MS data obtained from this study also revealed large quantities of myrcene, limonene and linalool in both of the samples, however lower quantities of α-pinene were detected. The compound methyl chavicol was only detected in *Agathosma capensis* (Besemfontein) (2.8%) whilst the compound *trans*-anethole was not detected in either of the species. The presence of myrcene, limonene and linalool in high percentages in both of the samples makes the oil strongly odourous (Campbell and Williamson, 1991).

The essential oils of *Agathosma ovata* (44.4%) and *A. pubigera* (24.3%) have sabinene as their major compound, hence they are tightly clustered in the dendrogram (Cluster A, Figure 3.6). The presence of 1,8-cineole in large quantities in both *Agathosma namaquensis* (23.5%) and *A. ovalifolia* (9.7%), unites them in a single cluster (Cluster A, Figure 3.6). *Agathosma bathii*, *A. crenulata* and *A. betulina* are tightly clustered (Cluster A, Figure 3.6) due to the high yields of limonene and pulegone. In addition menthone and isomenthone are also major constituents of both *Agathosma betulina* and *A. crenulata*. Dictamnol (14.2%) and traginone (5.2%) are two constituents of *Agathosma roodebergensis* which differentiates it from the rest of the species (Figure 3.6). *Agathosma stipitata* is mainly composed of neral (39.9%), geranial (18.4%) and α-pinene (10.1%). Its chemical composition indicates that it is different from the rest of the species and hence it is removed from the greater cluster A (Cluster B, Figure 3.6). The essential

oils of *Agathosma hirsuta* (72.5%) and *A. zwartbergense* (64.7%) are particularly rich in citronellal (Figures 3.4 and 3.5) hence they are tightly clustered in the dendrogram (Cluster C, Figure 3.6).

The compounds α-pinene, β-pinene, *p*-cymene, linalool and terpinen-4-ol are present in all of the species in various percentages. α-Pinene ranges from 0.1% (*Agathosma pungens*) to 10.4% (*A. parva*), β-pinene ranges from trace quantities (*A. pungens*) to 16.9% (*A. lanata*), *p*-cymene ranges from 0.1% (*A. hirsuta* and *A. pungens*) to 9.6% (*A. ovalifolia*), linalool ranges from 0.1% (*A. bathii*) to 33.3% (*A. capensis* [Gamka]), while terpinen-4-ol ranges from 0.3% (*A. hirsuta*) to 8.3% (*A. ovalifolia*).

α-Thujene, camphene, sabinene, myrcene, limonene, β-phellandrene, (*E*)-β-ocimene, perillene, *trans*-linalool oxide (furanoid), *cis*-1,2-limonene epoxide, cryptone, α-terpineol, carvone, citronellol, *trans*-carveol, *p*-cymen-8-ol, methyl eugenol and spathulenol are some components common to many of the species. Many components are present in selected species only; *p*-menthane-1,2,8-triol in *Agathosma pungens*, menthofurolactone in *A. arida*, geranic acid in *A. stipitata* and many more.

S-prenyl thioesters discovered previously in the essential oils of some *Agathosma* species were not discovered in any of these species (Campbell *et al*., 1980; Campbell and Williamson, 1991). *S*-prenyl-thioisobutyrate was detected in the essential oils of *Agathosma apiculata* and *A. puberula* (Rivett, 1974; Moran *et al*., 1975).

The compounds α-pinene, β-pinene, myrcene, limonene, ocimene, linalool and terpineol detected in *Agathosma ovata* by Moran *et al*. (1975) were also found to occur in the same species in this study. GC-MS data revealed that the compounds detected in *Agathosma apiculata*, *A. clavisepala* and *A. puberula* by Moran *et al*. (1975) also occurred in many of the species in this study.

In a study performed by Campbell and George (1982) two phenolic ethers, *trans*-1,2-methylenedioxy-4-(3'-methoxy-1'-propenyl)benzene and *trans*-1,2-dimethoxy-4-(3'-methoxy-1'-propenyl)benzene, were isolated from the essential oils of *Agathosma ciliaris*, *A. imbricata* and *A. scaberula*. These were not detected in any of the species in this study.

Owing to 'buchus' characteristic minty-fruity odour, the steam distilled oils of *Agathosma betulina* and *A. crenulata* are frequently used as valuable flavour ingredients of fruit aromas and fragrance compositions (Köpke *et al*., 1992). In previous studies, one of the major compounds was found to be pulegone (Köpke *et al*., 1992) and this was confirmed in the study where GC-MS data revealed that *Agathosma betulina* contained 8.4% pulegone, whilst *A. crenulata* contained 34.9%. Limonene, menthone and isomenthone were also found to be amongst the major compounds, but not the impact flavour compounds. The constituents responsible for the typical black currant odour are the diastereomeric isomers of 8-mercapto-*p*-menthan-3-one and its thiolacetates, 8-acetylthio-*p*-menthan-3-one and 8-methylthio-*p*-menthan-3-one (Köpke *et al*., 1992; Kaiser *et al*., 1975). GC-MS data revealed the presence of 8-(3-oxo-2-*p*-menthanylthio)-

3-*p*-menthanone-I and 8-(3-oxo-2-*p*-menthanylthio)-3-*p*-menthanone-II, as trace components of *Agathosma crenulata*. The compound *trans*-*p*-menthon-8-thioacetate was also found to occur in both species.

Collins and Graven (1996) performed a chemotaxonomic study of commercial 'buchu' species. The essential oils of *Agathosma betulina*, *A. crenulata* and their hybrids were analyzed to determine whether the taxa could be distinguished by their monoterpene chemistry. *Agathosma betulina* was characterized by a high percentage of limonene, menthone, isomenthone, ψ-diosphenol, *cis*-8-mercapto-*p*-menthan-3-one, 4-hydroxydiosphenol and 1-hydroxydiosphenol. The composition would account for the warmer, rounded, minty-type odour with the catty note emanating from the 8-mercapto-*p*-menthan-3-one. *Agathosma crenulata* had a sharper, minty note that emanated from the very high percentages of pulegone and isopulegone isomers that characterize the oil. Pulegone was found to be the key component for the identification of the oils. *Agathosma betulina* was identified by a pulegone content of 2.4% to 4.5% and *A. crenulata* by 31.6% to 73.2%. This study revealed that *Agathosma betulina* and *A. crenulata* contained 8.4% and 34.9% pulegone respectively. The authors found that *Agathosma betulina* had a higher content of 8-mercapto-*p*-menthan-3-one than 8-acetylthio-*p*-menthan-3-one, while the reverse was true for *A. crenulata*. They also found that *cis*-8-mercapto-*p*-menthan-3-one content is higher than that of the *trans*- isomer for *Agathosma betulina* but the reverse is true for *A. crenulata*. GC-MS data revealed that both species had the same quantities of *trans*-8-mercapto-*p*-menthan-3-one (0.1%), whilst A. *betulina* had a greater quantity of *cis*-8-mercapto-*p*-menthan-3-one. Two *Agathosma betulina* chemotypes were

identified by Collins and Graven (1996). The diosphenol chemotype is characterized by high ψ-diosphenol (>10%) and diosphenol (>12%), and low isomenthone (<28%) concentrations. The isomenthone chemotype is characterized by high isomenthone (>31%), and low ψ-disophenol (<0.16%) and diosphenol (<0.14%) concentrations. No chemotypes were found for *Agathosma crenulata*. From the results obtained in this study, *Agathosma betulina* would fall into the isomenthone chemotype since it is characterized by a high isomenthone (14.2%), and low ψ-diosphenol (2.9%) and diosphenol (2.5%) content.

In the same year, Posthumus and van Beek (1996) performed a study to determine the chemical composition of the essential oils of *Agathosma betulina*, *A. crenulata* and an *A. betulina* x *A. crenulata* hybrid. *Agathosma betulina* was characterized by 31% (iso)menthone, 41% (ψ)-diosphenol and 3% of the olfactory *cis*- and *trans*-8-mercapto-*p*-menthan-3-ones. The essential oil of *Agathosma crenulata* contained very high quantities of pulegone (54%) and considerable quantities of *trans*-acetylthio-*p*-menthan-3-one (7%). This study has revealed that both *Agathosma betulina* (14.2%) and *A. crenulata* (7.3%) contained isomenthone. (ψ)-Diosphenol and the olfactory compounds were also present in both species, but in smaller quantities. One of the few compounds that was not found in this study was 8-hydroxy-4-menthen-3-one. This compound as well as 8-hydroxy menthone, found in both *Agathosma betulina* and *A. crenulata*, are suspected to be decomposition products of pulegone on the basis of the fact that their concentration in a sample of *A. crenulata* essential oil doubled over a period of three years with a corresponding decrease in the amount of pulegone (Posthumus and van Beek, 1996).

Chemically this is not impossible, as for instance 8-hydroxy-4-menthen-3-one can be synthesized by photo-oxygenation of pulegone (Posthumus and van Beek, 1996).

Taxonomically both species are mainly distinguished by their leaf form (Collins and Graven, 1996). *Agathosma betulina* ('round-leaf buchu') is characterized by a smaller, round leaf with a recurved apex whereas *Agathosma crenulata* ('oval-leaf buchu') is characterized by a larger, oval-shaped leaf without the recurved apex (Collins and Graven, 1996). With the cultivation of both species outside their natural distribution, hybridization has occurred which has led to confusion in identification (Collins and Graven, 1996). It has been shown that the two taxa can be distinguished by their monoterpene chemistry. The key characters are the pulegone and 8-mercapto-*p*-menthan-3-one isomer ratios. As may be expected, the essential oils of these two taxa showed remarkable qualitative similarities in their chemical composition. Most of the components were common to both species, although some had varying percentages.

Generally there was a good correspondence, both qualitatively and quantitatively, between the results obtained from the study and those of previous studies regarding the chemical compositions of these two species. The study confirms the data obtained from previous phytochemical studies regarding the chemical compositions of *Agathosma betulina* and *A. crenulata* (Fluck *et al*., 1961; Kaiser *et al*., 1975). In most of the studies a similar chemical profile as to this study was obtained.

The essential oil compositions of many of the above species have not been previously reported and therefore the results obtained from the study can be evaluated as the first on the essential oil compositions of some of these species.

CHAPTER 4: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS

4.1. Introduction

Previous studies have revealed that the coumarin and flavonoid components of *Agathosma* species are responsible for their biological activities. In the continuing task aimed at evaluating the biological activities of these species and the compounds responsible for their activities, a study was embarked on to evaluate the non-volatile fractions of *Agathosma* species using high performance liquid chromatography (HPLC). The study also aimed at identifying any phenolic patterns if they were present amongst the species.

4.1.1. Chromatography

'Chromatography' is the general term for a variety of physico-chemical separation techniques, all of which have in common the distribution of a component between a mobile phase and a stationary phase. The stationary phase is fixed in the system and the mobile phase which is a fluid, streams through the chromatographic system. The various chromatographic techniques are sub-divided according to the physical state of these two phases. The molecules of the analytes are distributed between the mobile and the stationary phases. When present in the stationary phase, they are retained and do not move through the system but when present in the mobile phase, they migrate with a velocity equivalent to that of the mobile phase. Due to the different distributions of the analytes, their mean residence time in the stationary phase differs, resulting in a different

net migration velocity. This is the principle of the chromatographic system (Scott R.P.W., 1995).

4.1.2. The high performance liquid chromatography system

HPLC instrumentation includes a pump, injector, column, detector and recorder or data system. The heart of the system is the column where separation occurs. Since the stationary phase is composed of micrometer size porous particles, a high pressure pump is required to move the mobile phase through the column. The components (or analytes) are first dissolved in a solvent, and then forced to flow through a chromatographic column under a high pressure. The chromatographic process begins by injecting the solute onto the top of the column where the mixture is resolved into its components. The amount of resolution is important and is dependant upon the extent of the interaction between the solute components and the stationary phase. The stationary phase is defined as the immobile packing material in the column. Separation of components occurs as the analytes and mobile phase are pumped through the column. Eventually each component elutes from the column as a narrow band (or peak) on the recorder (Scott R.P.W., 1995). The interaction of the solute with the mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems and it has the ability to easily separate a wide variety of chemical mixtures (Scott R.P.W., 1995).

Modern HPLC uses a non-polar solid phase, like C-18, and a polar liquid phase, generally a mixture of water and another solvent. High pressures up to 400 bars are required to elute the analytes through the column before they pass through a diode-array detector. The detector measures the absorption spectra of the analytes to aid in their identification. HPLC is useful for compounds that cannot be vaporized or that decompose under high temperatures (Scott R.P.W., 1995).

Initially, pressure was selected as the principle criterion of modern liquid chromatography and thus the name was 'high pressure liquid chromatography'. This was, however, an unfortunate term because it seems to indicate that the improved performance is primarily due to the high pressure. This is not true. In fact, high performance is the result of many factors: very small particles of narrow distribution range and uniform pore size and distribution, high pressure column slurry packing techniques, accurate low volume sample injectors, sensitive low volume detectors and good pumping systems (Scott R.P.W., 1995).

4.1.3. Photodiode-array ultra-violet detection

The photodiode-array detector is based on a dynamic system using reversed optics. Light is projected through the flow cell, dispersed via a holographic grating onto a number of photosensitive diodes, resulting in voltage, which is then converted to digital signals for further electronic processing. Diode-array detection enables continuous spectral determination during elution and allows rapid simultaneous access to data of the total preprogrammed wavelength range. Widely different compounds can be detected

simultaneously (Barendse, 1987). HPLC coupled with an ultra-violet photodiode-array detector and mass spectrometer provides more structural information on the compounds, thus enabling us to identify peaks more reliably (He *et al*., 1997).

4.1.4. Advantages of using high performance liquid chromatography

There is a growing interest in the chemical composition of plants and the use of HPLC has several advantages over other methods. The technique is sensitive, rapid and does not require the preparation of derivatives before separation. A further advantage is that ultraviolet absorption detectors set in the aromatic region of the spectrum (e.g. 275 nm) may also be used. Several reports have also been published of the application of HPLC to various phenolic acids and aldehydes that occur in plants (Hartley, 1987).

4.1.5. Flavonoids

Flavonoids are a group of C-15 phenolic compounds which comprise one of the most abundant groups, as well as the most structurally and functionally diverse group, of secondary metabolites in plants. Flavonoids serve a wide variety of roles: UV protectants in leaves, an important cue in pollen development and pollen germination, phytoalexins giving resistance to pathogenic microbial attack, inducing *nod* genes of rhizobia for nodule formation of roots and as defense agents against predation and pathogens. It has also been well established that some flavonoids, such as anthocyanins, are significant factors in floral colouration and can serve as pollinator cues. Biosynthesis of the C-15 flavonoid skeleton has been well studied and reviewed. Commonly flavonoids are further modified by the addition of substituent groups such as methyl groups, aromatic acyl

groups, and/or sugar moieties. For example, naringenin, a bitter tasting flavanone diglycoside, accounts for up to 40-70% of the dry weight of very young green fruit and leaves of grapefruit plants. In comparison, very little of the latter flavonoids, such as dihydroflavonols and flavonols, are produced in these tissues. Alternatively the flavanone aglycone can serve as an intermediate in reactions that form other types of flavonoids such as dihydroflavonols, isoflavones or flavones (Figure 4.1). In plants such as *Petunia*, flavonoid accumulation in wild type plants favours flavonols and anthocyanin glycoside accumulation as well as some dihydroflavonol glycosides (Pelt *et al*., 2003).

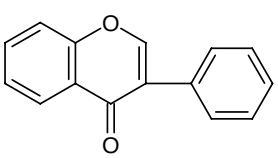
The flavonoids are all structurally derived from the parent substance, the flavone. Ten classes of flavonoids are recognized (Table 4.1). They are mainly water-soluble phenolic compounds that change colour when treated with base or with ammonia; thus they are easily detected on chromatograms or in solution (Harborne, 1973). They contain conjugated aromatic systems and thus show intense absorption bands in the UV and visible regions of the spectrum. Flavonoids are generally present in plants bound to sugar as glycosides and any flavonoid aglycone may occur in a single plant in several glycosidic combinations. For this reason, when analyzing flavonoids, it is usually better to examine the aglycones present in hydrolyzed plant extracts before considering the complexity of glycosides that may be present in the original extract (Harborne, 1973). Flavonoids are present in all vascular plants but some classes are more widely distributed than others; while flavones and flavonols are universal, isoflavones and biflavonyls are found in only a few plant families. Flavonoids are present in plants as mixtures and it is very rare to find only a single flavonoid component in a plant tissue. In addition, there are

often mixtures of different flavonoid classes. The coloured anthocyanins in flower petals are almost invariably accompanied by colourless flavones or flavonols and research has established that the flavones are important co-pigments, being essential for the full expression of anthocyanin colour in floral tissues (Harborne, 1973).

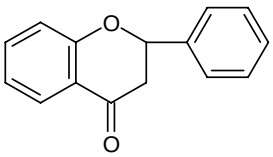
Flavonols are widely distributed in plants, both as co-pigments to anthocyanins in petals and also in leaves of higher plants (Harborne, 1973). They occur most frequently in glycosidic combination. Although there are over a hundred flavonol aglycones known, only three are at all common: kaempferol (corresponding in hydroxylation pattern to the anthocyanidin pelargonidin); quercetin (*cf*. cyanidin) and myricetin (*cf*. delphinidin). The other known flavonols are mostly simple structural variants of the common flavonols and are of limited natural occurrence. Flavones only differ from flavonols in lacking a 3-hydroxyl substitution; this affects their UV absorption, chromatographic mobility and colour reactions and simple flavones can be distinguished from flavonols on these bases. There are only two common flavones apigenin and luteolin, corresponding in hydroxylation pattern to kaempferol and quercetin. Flavones occur as glycosides but the range of different glycosides is less than in the case of the flavonols. Almost every higher plant contains a characteristic pattern of flavone and flavonol glycosides in the leaf or flower and thus these substances are ideal taxonomic markers for use in studying problems of plant classification, hybridization or phytogeography.

Table: 4.1: Properties of different flavonoid classes (Harborne, 1973).

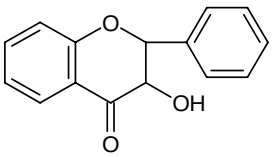
Flavonoid class	Distribution	Characteristic properties
Anthocyanins	Scarlet, red, mauve and blue flower pigments; also in leaf and other tissues.	Water soluble, visible max. 515 to 545 nm.
Leucoanthocyanidins	Mainly colourless, in heartwoods and in leaves of woody plants.	Yield anthocyanidins (colour extractable into amyl alcohol) when tissue is heated for 0.5 h in 2M HCl.
Flavonols	Mainly colourless co-pigments in both cyanic and acyanic flowers; widespread in leaves.	After acid hydrolysis, bright yellow spots in UV light on Forestal chromatograms; spectral max. 350 to 386 nm.
Flavones	As flavonols.	After acid hydrolysis, dull absorbing brown spots on Forestal chromatograms; spectral max. 330 to 350 nm.
Glycoflavones	As flavonols.	Contain C-C linked sugar; mobile in water unlike normal flavones.
Biflavonyls	Colourless, almost entirely confined to the gymnosperms.	Dull absorbing spots with very high R_f value.
Chalcones and aurones	Yellow flower pigments; occasionally present in other tissues.	Give red colours with ammonia (colour change can be observed *in situ*) visible max. 370 to 410 nm.
Flavanones	Colourless; in leaf and fruit (especially in *Citrus*).	Give intense red colours with Mg/HCl; occasionally an intense bitter taste.
Isoflavones	Colourless; often in root; only common in one family, the Leguminosae.	Mobile on paper in water; no specific colour tests available.



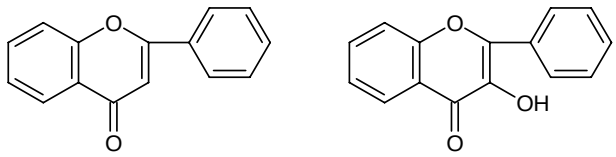
isoflavone



flavanone



dihydroflavonol



flavone flavonol

Figure 4.1: Structures of the various structural classes of flavonoids.

4.1.5.1. Value of flavonoids

Flavonoids play a role in disease prevention in humans. They help provide a defense system against UV radiation, fungi, bacteria and insects. These health benefits may be due, in part, to the anti-oxidant properties of flavonoids. Anti-oxidants are substances that neutralize damaging chemicals in the body such as free radicals, which are thought to contribute to a number of diseases, including atherosclerosis, cancer, degenerative brain diseases like Alzheimer's disease as well as premature ageing. Flavonoids have also been found to help the body fight viruses and decrease allergic reactions. There is abundant evidence to support the role of flavonoids in disease prevention: a high dietary intake of quercetin is related to a reduced risk of heart disease. Rutin has been found to help prevent stomach ulcers by protecting the stomach lining and it also protects blood vessels. Flavonoids have also been found to reduce the 'stickiness' of blood platelets and thus reduce the formation of blood clots, which reduce the risks of certain cardiovascular diseases. The isoflavones in soy products may help to lower blood cholesterol levels and

reduce the side effects of menopause by acting like estrogen-like substances (Peterson and Dwyer, 1998).

While they are not considered essential nutrients, some flavonoids support health by strengthening capillaries and other connective tissue, and some function as anti-inflammatory, antihistaminic and antiviral agents. Quercetin has also been reported to block the 'sorbitol pathway' that is linked to many problems associated with diabetes. It also protects LDL cholesterol from oxidative damage (So *et al*., 1996). Others, such as anthocyanidins from bilberry, purple cabbage, and grapes, may help protect the lens of the eye from cataracts. Animal research suggests that naringenin, found in grapefruit, may have anticancer activity. Soy isoflavones are also currently being studied to determine if they can help fight cancer (So *et al*., 1996).

In a small preliminary trial, rutoside (500mg twice daily), a derivative of the flavonoid, rutin, combined with vitamin C (500mg twice daily) produced a marked improvement in three women with progressive pigmented purpura (PPP), a mild skin condition (Reinhold *et al*., 1999).

4.1.6. Coumarins

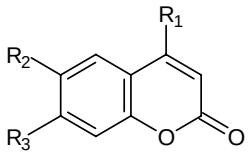
Coumarins owe their class name to 'coumarou', the vernacular name of the tonka bean (*Dipteryx odorata* Willd., Fabaceae), from which coumarin itself was isolated in 1820 (Bruneton, 1999). Coumarins belong to a group of compounds known as the benzopyrones, all of which consist of a benzene ring joined to a pyrone (Figure 4.2).

Coumarins may also be found in nature in combination with sugars, as glycosides. Coumarins can be roughly categorized as follows:

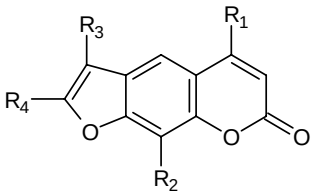
- simple - these are hydroxylated, alkoxylated and alkylated derivatives of the parent compound, coumarins along with their glycosides
- furanocoumarins - these compounds consist of a five-membered furan ring attached to the coumarin nucleus, divided into linear and angular types with substituents at one or both of the remaining benzenoid positions
- pyranocoumarins - members of this group are analogous to the furanocoumarins, but contain a six-membered ring
- coumarins substituted in the pyrone ring.

Like other phenylpropanoids, they arise from the metabolism of phenylalanine via *p*-coumaric acid which is a cinnamic acid (Bruneton, 1999). The specificity of the process resides in the 2'-hydroxylation, followed by the photocatalysed isomerisation of the double bond followed by spontaneous lactonisation. In some rare cases, glucosylation of cinnamic acid occurs, precluding lactonisation. In such cases, coumarin only arises after tissue injury and enzymatic hydrolysis. The formation of di- and trihydroxycoumarins and of their ethers involves the hydroxylation of umbelliferone rather than the lactonisation of the corresponding cinnamic acids. Prenylation of the benzene ring by dimethylallyl pyrophosphate in C-6 of a 7-hydroxycoumarin yields the so called linear furano- and pyranocoumarins and in C-8 it affords the angular homologues.

The primary site of synthesis of coumarins is suggested to be the young, actively growing leaves, with stems and roots playing a comparatively minor role.



Compound	R_1	R_2	R_3
coumarin	H	H	H
herniarin	H	H	OCH_3
methyl-umbelliferone	CH_3	H	OH
scopoletin	H	OCH_3	OH
umbelliferone	H	H	OH



Compound	R_1	R_2	R_3	R_4
bergapten	OCH_3	H	H	H
isopimpinellin	OCH_3	OCH_3	H	H
peucedanin	H	H	OCH_3	$CH(CH_3)_2$
psoralen	H	H	H	H
xanthotoxin	H	OCH_3	H	H

Figure 4.2: Structures of the classes of coumarins.

4.1.6.1. Value of coumarins

Coumarins have a variety of bioactivities including anticoagulant, estrogenic, dermal, photosensitizing, antimicrobial, vasodilator, molluscidal, antihelmintic, sedative, hypnotic, analgesic and hypothermic activity (Soine, 1964). Various coumarins have been

reported to possess anti-inflammatory activity as shown in carrageenan-induced inflammation and cotton pellet granuloma tests (Lino *et al*., 1997; Hoult *et al*., 1994, Härmälä *et al*., 1992; Atta and Alkofahi, 1998; Al-Said, 1990). There have been reports on efficacies of pure coumarins as well as extracts containing them; against Gram-positive and Gram-negative bacteria as well as fungi (Bisignano *et al*., 2000; Kayser and Kolodziej, 1997). The free OH group at C-6 in the coumarins nucleus has been found to be important for antifungal activity, while the free hydroxyl group at C-7 is important for antibacterial activity (Sardari *et al*., 1999). The linear furanocoumarin xanthotoxin, is capable of inactivating human P450 2A6, the major coumarin 7-hydroxylase present in human liver, at physiologically relevant concentrations (Koenigs *et al*., 1997) and bergapten (Figure 4.2) against intestinal CYP3A4 (Ho *et al*., 2000), and therefore these compounds carry the potential of causing a serious drug-drug interaction with any drug, compound or toxin whose clearance is largely dependant on these enzymes. Psoralen, xanthotoxin (Figure 4.2) and sphondin proved to be inhibitors of coumarins 7-hydroxylase activity in both mice and human liver microsomes (Mäenpää *et al*., 1993). Woo and co-workers (1983) investigated the effects of coumarins from *Angelica koreana* on the drug metabolizing enzymes and found them to retard drug metabolism both *in vitro* and *in vivo*.

The bioactivities of phototoxic psoralens and dicoumaroul derivatives are well known and several of these compounds are used in antipsoriatic and anticoagulant therapy (Hönigsmann *et al*., 1989). Other than psoriasis, skin disease like cutaneous T-cell lymphoma, atopic dermatitis, urticaria pigmentosa and lichen planus (Goodman and

Gilman, 1996) are treated with the photochemotherapy of linear furanocoumarins (psoralens). The most widely used compound is xanthotoxin (Conconi *et al*., 1998). Bergapten (Figure 4.2) is considered a valuable alternative for chemotherapy of psoriasis, since its clinical efficacy is comparable to that of xanthotoxin, although bergapten requires higher cumulative UVA doses (Figure 4.2).

Coumarin is the parent molecule of warfarin, which acts as a vitamin K antagonist. Warfarin is a clinically useful anticoagulant and widely employed rodenticide. The inherent fluorescent properties of many coumarins are a key factor in many applications. Areas where coumarins are widely used include estimation of enzymatic activity (Egan *et al*., 1990), labeling of proteins, antibodies, DNA and lipids, derivatising agents in chromatography, etc.

Coumarins have a wide variety of uses in industry, mainly due to their strong fragrant odour (Egan *et al*., 1990). Uses include that of a sweetener and fixative of perfumes (e.g. 3,4-dihydrocoumarin), an enhancer of natural oils such as lavender, a food additive in combination with vanillin, a flavour/odour stabilizer in tobaccos and an odour masker in paints and rubbers. 6-Methylcoumarin is mainly used as a flavour enhancer, and 7-hydroxycoumarin is mainly used in sunscreens.

4.2. Materials and methods

The dichloromethane and methanol (1:1) extracts were diluted with methanol to obtain a concentration of 50mg/ml. The samples were then qualitatively analyzed by using a

Walters 2690 HPLC system (Phenomenon Aqua C18 column, 250mm x 2.1mm) equipped with a 996 photodiode-array detector (PDA). The injection of the sample (10µl) was done under the following conditions: the flow rate was 0.2ml/min, the gas flow in the nebuliser: 30l/h, the nebuliser temperature: 80ºC, the expansion region: 90ºC, and the source of temperature: 225ºC. The mobile phase started off with 10% acetonitrile and 90% water (containing 100mM formic acid). The solvent ratio was changed through a linear gradient to 90% acetonitrile and 10% water (containing 100mM formic acid) at 40 min. This ratio was maintained for 10 min and thereafter the solvent ratio was changed back to the initial starting conditions.

4.3. Results

The HPLC chromatograms of selected extracts are displayed in Figure 4.3. The retention times and percentage areas obtained for peaks detected in each of the species are presented in the monographs (Appendix I).

Using Mabry *et al*. (1970) for identification purposes, all species were found to be rich in flavonoids. A compound with a retention time of 4.02 min and maximum absorbance at 203.8 nm occurred in all of the species. Another compound with a retention time of approximately 12.88 min and maximum absorbance at 266.3 nm was common to all species, ranging in integration value from 2.43% to 16.80%. A third compound with a retention time of 12.33 min and a maximum absorbance at 258.0 nm occurred in all of the species (ranging in integration value from 0.52% to 14.06%) with the exception of *Agathosma collina* and *A. ovalifolia*.

A coumarin isolated by Cassim and Noorgat (2005) from *Agathosma ovata* (round-leaf) was analyzed by HPLC for comparative purposes. The coumarin identified as puberulin, had a retention time of 24.33 min and its absorbance maxima at 229.7 nm, 293.4 nm and 343.2 nm (Figure 4.4). It was detected in the extract of *Agathosma namaquensis* (Figure 4.5).

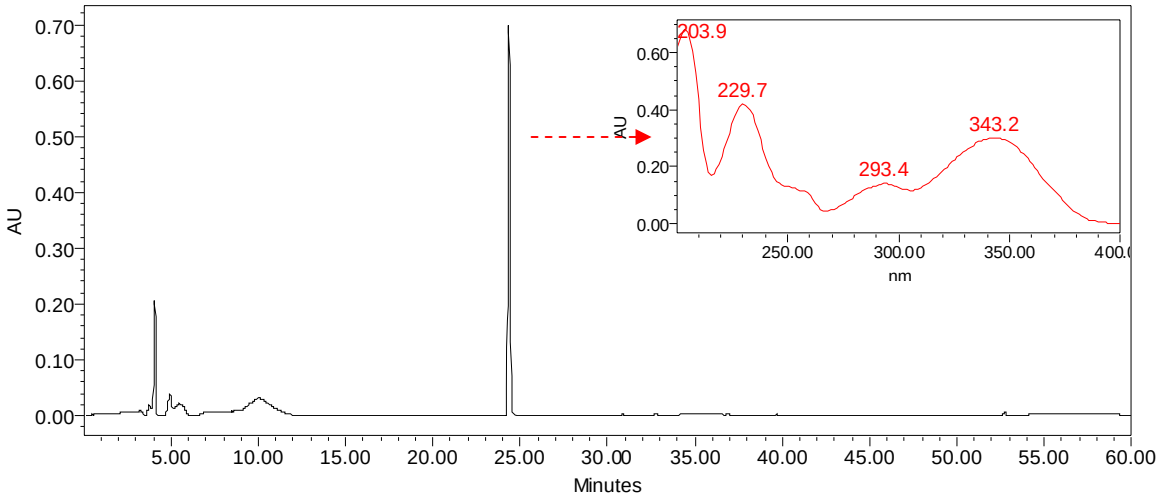


Figure 4.4: HPLC chromatogram and UV spectrum of puberulin, isolated by Cassim and Noorgat (2005) from the diethyl ether extract of *Agathosma ovata* (round-leaf).

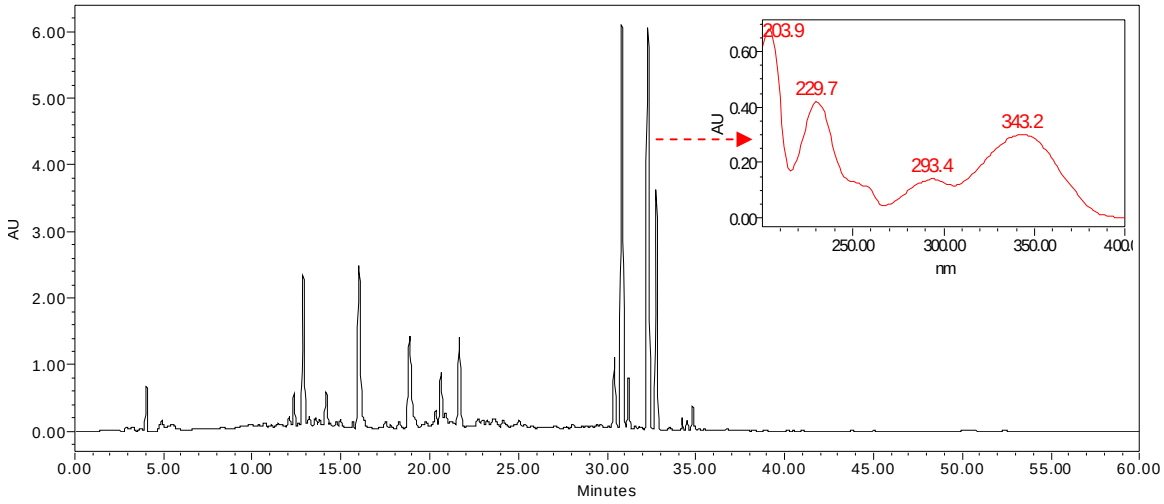


Figure 4.5: HPLC chromatogram of the dichloromethane and methanol (1:1) extract of *Agathosma namaquensis* and UV spectrum of puberulin.

4.4. Discussion

The HPLC chromatograms revealed the presence of numerous flavonoids in all species according to Mabry *et al*. (1970). The authors report that the spectra of flavones and flavonols exhibit two major absorption peaks in the region 240 – 400 nm. These two peaks are commonly referred to as Band I (usually 300 – 380 nm), and Band II (usually 240 – 280 nm). Band I is considered to be associated with absorption due to the B-ring cinnamoyl system, and Band II with absorption involving the A-ring benzoyl system. The position of Band I provides information about the type of flavonoid as well as its oxidation pattern. It distinguishes between flavones and 3-hydroxyflavones (flavonols). Band I of flavones occurs in the range 304 – 350 nm whereas Band I of 3-hydroxyflavones appears at a longer wavelength (352 – 385 nm). According to Mabry *et al*. (1970), isoflavones and flavanones give similar UV spectra as a result of having little or no conjugation between the A- and B- rings. They are all readily distinguished from flavones and flavonols by their UV spectra, which typically exhibit an intense Band II absorption with only one shoulder or low intensity peak representing Band I. The Band II absorption of isoflavones usually occurs in the region 245 – 270 nm. Flavanones have a major absorption peak (Band II) in the range 270 – 295 nm and are therefore clearly distinguished from the spectra of isoflavones.

Analysis has revealed that similar compounds are present in many of the species. Sixteen species had a compound with a retention time of approximately 9.57 min and a maximum absorbance at 208.5 nm. A flavonol with a retention time of 18.93 min and absorbance maxima at 255.7 nm and 355.6 nm occurred in *Agathosma arida*, *A. capensis*

(Besemfontein), *A. capensis* (Gamka), *A. namaquensis*, *A. ovalifolia*, *A. parva*, *A. pubigera* and *A. pungens*. A flavanone with a retention time of approximately 21.67 min and absorbance maxima at 284.1 nm and 328.1 nm was detected in 13 of the species. *Agathosma bathii*, *A. crenulata*, *A. lanata*, *A. ovata* (hook-leaf), *A. ovata* (round-leaf) and *A. zwartbergense* were the only species found to contain a compound with a retention time of 1.81 min and absorbance maxima at 291.2 nm and 396.2 nm. *Agathosma ovalifolia* contained a compound with a retention time of approximately 2.04 min and a maximum absorbance at 291.2 nm. Sixteen of the species contained a flavanone with a retention time of 21.63 min and absorbance maxima at 284.2 nm and 326.9 nm. A flavanone with a retention time of 26.11 min and absorbance maxima at 284.1 nm and 329.3 nm occurred in *Agathosma bathii*, *A. betulina*, *A. parva*, *A. pubigera*, *A. pungens* and *A. roodebergensis*. A flavonol with a retention time of 20.27 min and absorbance maxima at 255.7 nm and 353.2 nm was common to *Agathosma arida* and *A. capensis* (Besemfontein). Another flavonol with the same absorbance maxima and retention time of 20.67 min occurred *Agathosma arida*, *A. capensis* (Besemfontein), *A. capensis* (Gamka), *A. namaquensis*, *A. parva* and *A. pungens*.

The major compounds present in *Agathosma arida*, *A. capensis* (Besemfontein), *A. capensis* (Gamka), *A. collina*, *A. hirsuta*, *A. parva*, *A. pubigera*, *A. pungens*, *A. roodebergensis*, *A. stipitata* and *A. zwartbergense* appear to be flavonoids. *Agathosma bathii* (29.46%) and *A. lanata* (40.86%) have a major compound with absorbance maxima at approximately 206.2 nm and 370.0 nm and a retention time of 9.64 min. The major compounds in *Agathosma betulina* (14.27%), *A. ovalifolia* (16.84%), *A. ovata*

(hook-leaf) (34.54%) and *A. ovata* (round-leaf) (30.05%) have a retention time of 9.62 min and a maximum absorbance at 208.5 nm. *Agathosma crenulata* has a compound (23.03%) with absorbance maxima at 206.2 nm, 373.3 nm and 396.2 nm and a retention time of 7.62 min. *Agathosma namaquensis* has a compound (20.24%) with absorbance maxima at 207.3 nm, 235.6 nm, 287.6 nm and 333.7 nm and a retention time of 30.83 min.

A reference compound of the coumarin isolated by Cassim and Noorgat (2005), from *Agathosma ovata* (round-leaf) was made available to us and analyzed by HPLC. The results obtained (Figure 4.4) revealed a spectrum similar to that of a prenyloxy-coumarin, puberulin (6,8-dimethoxy-7-prenyloxycoumarin) (Figure 4.6), isolated by Finkelstein and Rivett (1976), from an Eastern Cape species of *Agathosma puberula*. The compound isolated was found to have its absorbance maxima at wavelengths similar to those obtained by Finkelstein and Rivett (1976). It was detected in *Agathosma namaquensis* (19.64%) at a retention time of 32.28 min (Figure 4.5) and appeared to be a major compound in this species.

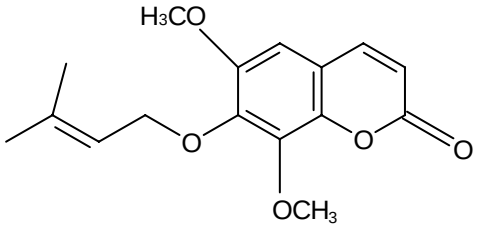


Figure 4.6: Structure of puberulin as proposed by Finkelstein and Rivett (1976).

The Diosmeae, one of the five tribes of a sub-family of the Rutaceae, the Rutoideae, consists of ten genera and approximately 260 shrubby species, more than half belonging to the genus *Agathosma* (Campbell *et al*., 1986). Relatively little is known of the chemistry of the Diosmeae. Simple coumarins have been isolated from a few species of the genera *Agathosma*, *Coleonema*, *Diosma* and *Phyllosma* (Campbell *et al*., 1986). Coumarins having prenyl, geranyl and farnesyl substituents have been found chiefly amongst members of the Rutaceae and Umbelliferae.

Campbell *et al*. (1986) investigated 24 species from the genera *Agathosma*, *Diosma* and *Empleurum* (tribe Diosmeae) for the presence of coumarins. Nine coumarins were identified in total amongst the species namely: 7-(3',3'-dimethylallyloxy)-coumarin, 6,7-dimethoxycoumarin, 6-methoxy-7-(3',3'-dimethylallyloxy)-coumarin, 7-(3',3'-dimethylallyloxy)-8-methoxycoumarin, 6,7,8-trimethoxycoumarin, 6-methoxy-7-(3',3'-dimethylallyloxy)-8-hydroxycoumarin, 6,8-dimethoxy-7-(3',3'-dimethylallyloxy)-coumarin, 6-methoxy-7,8-methylenedioxycoumarin and 5,6,7-trimethoxycoumarin. These results confirmed previous findings (Finkelstein and Rivett, 1976; Campbell and Cragg, 1979; Campbell *et al*., 1982; Gray, 1981; Khalid and Waterman, 1983), namely that the Diosmeae would appear to elaborate the 'simple' coumarins and not the furano- and pyranocoumarins. *Agathosma martiana*, *A. mucronulata*, *A. puberula*, *A. recurvifolia* and *A. spinosa* have been the only taxa investigated so far which contain the 6,7,8-tri-oxygenated coumarins with an *O*-prenyl group at C-7. These five species are found in the South Eastern Cape and have a close affinity with many other similar morphological characters (Campbell *et al*., 1986). *Agathosma affinis* was found to contain 6,7-

dimethoxycoumarin (scoparon) and 5,6,7-trimethoxycoumarin; *Agathosma capensis* was found to contain 6,7-dimethoxycoumarin and *Agathosma collina* contained 6,7-dimethoxycoumarin and 6-methoxy-7-(3',3'-dimethylallyloxy)-coumarin. HPLC data obtained in this study did not reveal either *Agathosma capensis* or *A. collina* to contain any of the compounds reported previously. This may be due in part, to the species being obtained from different localities.

Campbell *et al*. (1982) investigated the aerial parts of two collections of *Diosma acmaeophylla* (Rutaceae-Diosmeae), and revealed the presence of seven simple coumarins, three of which were common to both. Two were identified as herniarin and scoparone by direct comparison with authentic samples and a further two, 7-(3',3'-dimethylallyloxy)-coumarin and 7,8-methylenedioxycoumarin, by comparison with physico-chemical data published previously. Another was identified as 6-methoxy-7-(3',3'-dimethylallyloxy)-coumarin. Compared to other tribes of the Rutaceae, little is known of the distribution of coumarins in the Diosmeae (Gray and Waterman, 1978). Herniarin has also been detected in the leaves of *Prunus* (Rosaceae) species; whilst the barks have been found to contain scopolin (Santamour and Riedel, 1994). Simple coumarins but not furano- or pyranocoumarins have now been recorded in four genera: *Agathosma* (Finkelstein and Rivett, 1976), *Coleonema* (Gray, 1981), *Diosma* and *Phyllosma* (Campbell and Cragg, 1979). In *Agathosma*, *Diosma* and *Phyllosma* isolated coumarins are characterized by oxygenation patterns involving C-6, C-7 and C-8.

In another study performed by Campbell *et al*. (1987), the aerial parts of 42 taxa of the genera *Agathosma*, *Coleonema*, *Diosma*, *Empleurum* and *Phyllosma* (tribe Diosmeae) were screened for alkaloids. Positive results were obtained for five *Agathosma* species and the alkaloids halfordamine (trimethoxy-2[1H]-quinolinone) and skimmianine (trimethoxyfuro[2,3b]quinoline) were found to occur in *Agathosma bisulca* and *A. capensis*. Skimmianine was also found to occur in *Agathosma peglerae*, *A. thymifolia* and *A. virgata*. Of all the *Agathosma* species screened, the following gave negative results for the presence of alkaloids: *Agathosma abrupta*, *A. affinis*, *A. barosmaefolia*, *A. cedrimontana*, *A. ciliaris*, *A. collina*, *A. dielsiana*, *A. eriantha*, *A. geniculata*, *A. glandulosa*, *A. hispida*, *A. lanceolata*, *A. latipetala*, *A. marifolia*, *A. martiana*, *A. mucronulata*, *A. mundtii*, *A. ovata*, *A. puberula*, *A. pungens*, *A. recurvifolia*, *A. roodebergensis*, *A. rosmarinifolia*, *A. salina*, *A. scaberula*, *A. serpyllacea*, *A. spinosa* and *A. unicarpellata*. In a study performed by Campbell *et al*. (1990), the aerial parts of a new species of the genus *Agathosma* yielded skimmianine and two new alkaloids which were identified by means of spectral data and synthesis as 4,6-dimethoxy-1-methyl-2(1H)-quinolinone and 2,6-dihydro-9-methoxy-2,2,6-trimethyl-5H-pyrano[3,2*c*]quinolin-5-one. Quinoline alkaloids were also detected in *Agathosma barosmaefolia* (Campbell and Bean, 1996). The alkaloids detected previously in *Agathosma* species were not detected in any of the species in this study.

In a study performed by Kinoshita and Firman (1996), *Murraya paniculata* (Rutaceae), a shrub widely distributed in tropical and sub-tropical Asia and used traditionally for various medicinal reasons, was found to contain eight highly oxygenated flavones. Seven

of them were identified as 5-hydroxy-6,7,8,3',4',5'-hexamethoxyflavone (gardenin A), 5,3'-dihydroxy-6,7,8,4',5'-pentamethoxyflavone (gardenin C), 6,7,8,4'-tetramethoxy-5,3',5'-trihydroxyflavone (gardenin E), 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone (5-*O*-desmethylnobileton), 6,7,8,3',4',5'-hexamethoxyflavone, 5-hydroxy-6,7,3',4',5'-pentamethoxyflavone (umhengerin) and 5,3'-dihydroxy-6,7,4',5'-tetramethoxyflavone, either by direct comparison with authentic samples or by comparing their melting points and spectroscopic data with those in reported literature. In an investigation of the peel and pulp of the fresh ripe fruits by Ferracin *et al*. (1998) nine flavonoids were again identified. In a study performed by Cambie *et al*. (1996) the bark of *Melicope simplex* was found to contain the flavones melisimplin, melisimplexin, and ternatin, and the dimethylchromenes evodionol and alloevodionol 7-methyl ether, while the bark of *Melicope ternata* was found to contain the flavones meliternatin, meliternin, ternatin and wharangin, as well as the dimethylchromene xanthoxyletin.

Casimiroa tetrameria (Rutaceae), commonly known as Yuy, is used for treating gastrointestinal problems, especially diarrhea, dysentery and gastrointestinal cramps (Heneka *et al*., 2005). 'Buchu' leaves, belonging to the same family, are used to treat the same conditions. The phytochemistry of the plant was investigated in a study performed by Heneka *et al*. (2005). Eight flavonoids and a furanocoumarin were islolated and characterized as 5,6,2',3',5',6'-hexamethoxyflavone, 5,6,2',3',6'-pentamethoxyflavone and 5-methoxy-8-(3"-hydroxymethyl-but-2"-enyloxy)-psoralen.

A number of *citrus* species have been recorded in the Chinese Pharmacopoeia as appropriate for medicinal use. *Zhi Shi* (sour orange, also known as bitter orange) is the dried, immature fruit of *Citrus aurantium* L. As a traditional Chinese remedy, it has been used to activate vital energy and circulation, eliminate phlegm and disperse stagnation (He *et al*., 1997). Due to its sour and bitter taste, it has not been used as an edible fruit. The primary active biological constituents of sour orange are flavonoids, of which it has a high content and a sympathomimetic amine, synephrine (He *et al*., 1997). *Citrus* flavonoids, which occur principally in the peel, have been studied for a century. More than 60% flavonoids have been isolated and structurally determined. Three types of flavonoids have been found to occur in *citrus* species: flavanones, flavones and flavonols (He *et al*., 1997). The flavanones predominate amongst the *citrus* flavonoids, with the flavonols present in considerably smaller amounts. HPLC data confirms this by revealing that flavanones are more common in *Agathosma* species than flavonols (Appendix I). The flavonoids in *citrus* usually occur as glycosides, the permethoxylated flavones are an exception: they occur as free aglycones (He *et al*., 1997). Many potentially health promoting effects have been ascribed to the *citrus* flavonoids. Hesperitin and naringenin are effective at inhibiting the *in vitro* proliferation of human breast cancer cells. Tangeritin and nobiletin are the most active antimutagens of the flavonoids tested so far, and may have chemopreventive potential (He *et al*., 1997). The analysis of *citrus* flavonoids has become increasingly requisite. Several papers have been published on the HPLC analysis of *citrus* flavonoids (Rouseff and Ting, 1979; Ishii *et al*., 1996). However, each has paid attention only to an individual flavone. Another paper reported the analysis

of 25 standard *citrus* flavonoids using HPLC with a UV photodiode-array detector (Nogata *et al*., 1994).

Previous studies revealed that flavonoids are common in the Rutaceae, hence confirming the results obtained in the study. The anti-oxidant properties of flavonoids and the antimicrobial, analgesic and anti-inflammatory properties of coumarins justify the use of *Agathosma* species traditionally.

CHAPTER 5: ANTIMICROBIAL ACTIVITY

5.1. Introduction

'Buchu' has been used traditionally as a cough remedy, as well as for the treatment of colds and flu, kidney and urinary tract infections, for the treatment of cholera and other stomach ailments. The San have used these aromatic plants lubricated with fat and smeared all over their bodies as an antibacterial and antifungal agent (antibiotic protectant). A mixture of 'buchu' and vinegar is still used today to clean wounds (van Wyk *et al*., 1997). Based on the vast traditional use of these plants in treating infections and the lack of scientific evidence, a study was embarked on to determine the antimicrobial activity of these species.

5.1.1. Plants as antimicrobials

Infectious diseases account for approximately one-half of all deaths in tropical countries (Iwu *et al*., 1999). In industrialized nations, despite the progress made in the understanding of microbiology and their control, incidents of epidemics due to drug resistant micro-organisms and the emergence of hitherto unknown disease-causing microbes, pose enormous public health concerns. Historically plants have provided a good source of anti-infective agents; emetine, quinine and berberine remain highly effective instruments in the fight against microbial infections. Phytomedicines derived from plants have shown great promise in the treatment of intractable infectious diseases including opportunistic AIDS infections. Plants containing protoberberines and related alkaloids, picralima-type indole alkaloids and *Garcinia* biflavanones used in the

traditional African system of medicine, have been found to be active against a wide variety of micro-organisms. The profile of known drugs like *Hydrastis canadensis* (goldenseal), *Garcinia kola* (bitter kola), *Polygonum* sp. and *Aframomum melegueta* (grains of paradise) have illustrated the enormous potential of anti-infective agents from higher plants (Iwu *et al*., 1999).

Antibiotics are potent antimicrobial agents with high specificities; however, we face a worldwide rapid increase in multi-resistant micro-organisms. This alarming situation has its origin in the excessive and often inappropriate use of antibiotics in human and animal health care for treatment and prevention of infections. Since the development of the first commercially available antibiotic penicillin in the 1940's, the high expectations by man in the healing power of these drugs has not been entirely fulfilled, as resistance is a vital part of the survival strategy of bacteria. Alternatives hence need to be investigated, leading man to analyze plant extracts and essential oils.

5.1.2. Microbiological activity of essential oils

Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives (Cowan, 1999). Most are secondary metabolites, of which at least 12,000 have been isolated (Cowan, 1999). In many cases these substances serve as plant defense mechanisms against predation by micro-organisms, insects and herbivores. Some, such as terpenoids, give plants their odours; others (quinines and tannins) are responsible for plant pigment. Many compounds are responsible for plant flavour (eg. the terpenoid capsaicin from chili peppers), and some of

the same herbs and spices are used by humans to season food yield useful medicinal compounds (Cowan, 1999).

Aromatic plant volatile oils have been known since antiquity to possess biological activities, notably antibacterial, antifungal and anti-oxidant properties (Deans and Svoboda, 1990). The use of natural antimicrobial compounds is important not only in the preservation of food but also in the control of human and plant diseases of microbial origin (Baratta *et al*., 1998). Bacterial and fungal infections pose a greater threat to health, most notable in immunocompromised subjects, hence the need to find cheap and effective antimicrobial agents (Baratta *et al*., 1998).

Essential oils contain terpenes which are active against bacteria, fungi, viruses and protozoa (Cowan, 1999). In 1977, it was reported that 60% of essential oil derivatives examined to date were inhibitory to fungi while 30% inhibited bacteria (Cowan, 1999). The mechanism of action of terpenes involves membrane disruption by the lipophilic compounds (Cowan, 1999).

Many essential oils have strong to medium antimicrobial activities (Lis-Balchin *et al*., 1998). The activity could act as a chemical defense against plant pathogenic diseases. Pathogens can readily penetrate at wound sites, for example, by herbivores. Wounding of leaves which are covered with volatile oil glands result in the rupture of glands causing the oil to flow over the wound. The existence, therefore, of antimicrobial activity in the oil would be of considerable benefit to the plant. A good majority of aromatic and

medicinal plants do not succumb to many of the most common diseases. It is also suggested that a complex oil presents a greater barrier to pathogen adaptation than would a more simple mixture of monoterpenes. This theory is well documented in the detailed study of *Myrca gale* volatile oil and its inhibitory properties against a broad spectrum of fungal species (Carlton *et al*., 1992). The complicated mixtures of monoterpenes and sesquiterpenes in the whole oil represented the strongest barrier to fungal infection. Deans and Ritchie (1987) examined 50 plant volatiles for their antibacterial properties against 25 genera of bacteria, using an agar diffusion technique. The volatile oils exhibited various reductions in growth of micro-organisms depending on the oil concentration and chemical composition. The activity of essential oils could also be explored for their utilization as food preservatives, as they are widely used in food products as flavour additives (Araújo, 2002).

Testing and evaluating of the antimicrobial activity of essential oils is difficult because of their volatility, water insolubility, and complexity. Factors that are important when evaluating essential oils include: the assay technique, the growth medium and the micro-organism (Janssen *et al*., 1986).

5.2. Materials and methods

A number of different testing methods are used in order to investigate the antimicrobial properties of essential oils, extracts and their constituents. However, the results obtained with different testing methods are often limited, because they are strongly dependant on the applied experimental conditions (Pauli and Kubeczka, 1996). The antimicrobial

activity of the essential oils and extracts of indigenous *Agathosma* species was evaluated using the disc diffusion and minimum inhibitory concentration (MIC) assays. The reference stock cultures were obtained from the National Health Laboratory Services (NHLS), Johannesburg, and were maintained in the Department of Pharmacy and Pharmacology, University of the Witwatersrand.

5.2.1. Disc diffusion assay

5.2.1.1. Principle

When a filter paper disc impregnated with a sample is placed on agar (Figure 5.1), the compounds present within the sample will diffuse from the disc into the agar. This diffusion will cause the compounds to diffuse into the agar around the disc. The solubility of the compounds and their molecular size will determine the size of the area of the sample infiltration around the disc. If an organism is placed in the agar it will not grow in the area around the disc if it is susceptible to the test substance. This area of no growth around the disc is known as a 'zone of inhibition' (Figure 5.2).

Many conditions can affect the disc diffusion susceptibility test. When performing these tests certain conditions should be maintained constant. Conditions that must be constant from test to test include: the type of agar used, inoculum size, the concentration of the sample and the incubation conditions (time, temperature and atmosphere). For the controls, the antibiotic concentrations are predetermined and commercially available. Each test method has a prescribed media to be used and incubation is to be at 35-37°C in ambient air for 18-24 h.

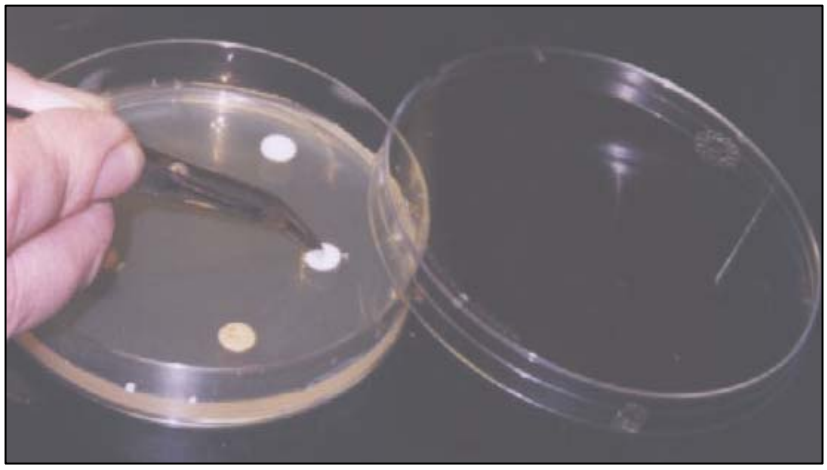


Figure 5.1: An illustration of the disc diffusion method:

Demonstration of aseptic introduction of disc on to agar.

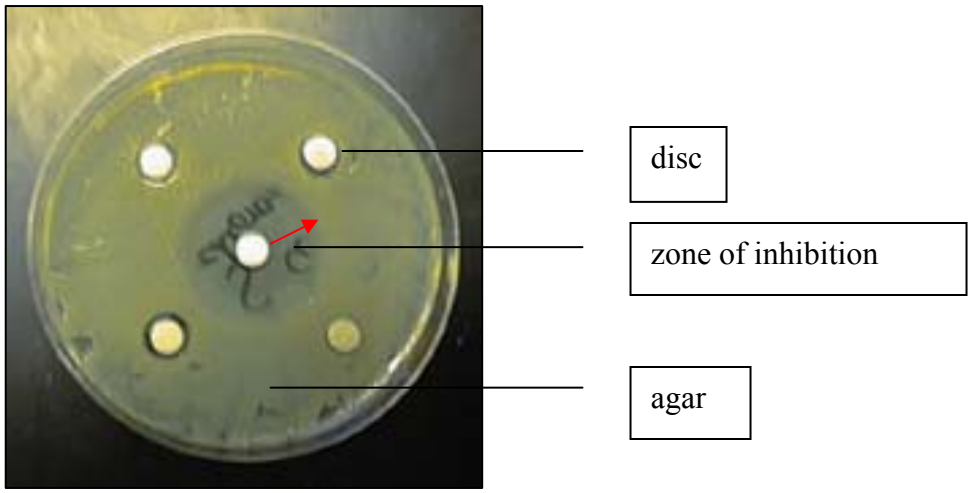


Figure 5.2: An illustration of the disc diffusion method:

Agar plate showing the clear zone of inhibition.

5.2.1.2. Method

The preliminary screening of indigenous *Agathosma* species for antimicrobial activity was performed using the disc diffusion assay. This broad screen was performed once, on ten extracts using three pathogens, to enable us to determine the activities of these species so that further analysis may be performed utilizing more pathogens and evaluating the essential oils. Two Gram-positive bacteria, *Staphylococcus aureus* (ATCC 12600) and *Bacillus cereus* (ATCC 11778); and a Gram-negative bacterium, *Klebsiella pneumoniae* (NCTC 9633) were used to evaluate the antimicrobial activity. The antibiotic, Neomycin (30µg, Oxoid) was used as a positive control.

The culture medium (agar) was prepared by dissolving 30g of Tryptone Soya (Oxoid) agar in 750ml of distilled water. The solution was autoclaved for 15 min at 121°C to sterilize the medium. To prepare the inoculum, 150µl of the bacterial spore suspension yielding an inoculum size of approximately 1 x10 ^{6} colony forming units (CFU)/ml was mixed with 15ml of agar. This was placed onto a base agar layer (15ml), which was left to solidify. After solidification, paper discs impregnated with extract (50mg/ml) were aseptically introduced, using a sterile needle, onto the agar containing microbial culture. The petri dishes were kept in the fridge for one hour to allow the extracts to diffuse into the culture medium. They were incubated overnight at 37°C. The compounds diffused into the culture medium and after incubation, the antimicrobial activity was determined by measuring the zones of inhibition from the disc edge to the edge where growth begins.

5.2.2. Minimum inhibitory concentration assay

5.2.2.1. Principle

A current definition of the minimum inhibitory concentration, (MIC), is 'the lowest concentration which results in maintenance or reduction of inoculum viability' (Carson *et al*., 1995). The determination of the MIC involves a semi-quantitative test procedure which gives an approximation to the least concentration of an antimicrobial needed to prevent microbial growth (Lambert and Pearson, 2000). In the recent past, the method used tubes of growth broth containing a test level of antimicrobial, into which a microbial inoculum was added. The end result of the test was the minimum concentration of antimicrobial which gave no visual growth (Davidson and Parish, 1989). The sensitivity of this assay is increased compared to the disc diffusion method. It is inexpensive, gives reproducible results, is more sensitive then most other methods used in literature, requires a small quantity of sample and can be used for a large number of samples (Eloff, 1998).

5.2.2.2. Method

Based on the preliminary screening, further quantitative analysis of the antimicrobial activity of the extracts and essential oils was performed using the MIC assay. Analysis was not performed on the essential oil of *Agathosma ovata* (hook-leaf) due to insufficient sample. Two Gram-positive bacteria *Staphylococcus aureus* (ATCC 12600) and *Bacillus cereus* (ATCC 11778), a Gram-negative bacterium, *Klebsiella pneumoniae* (NCTC 9633) and a yeast *Candida albicans* (ATCC 10231) were used to evaluate the antimicrobial activity. Two antibiotics, ciprofloxacin (at a starting concentration of 0.01 mg/ml, Oxoid)

and amphotericin B (at a starting concentration of 0.01 mg/ml, Oxoid) were used as controls for the bacteria and yeast, respectively.

The inoculum was prepared by mixing 1ml of a 24 h broth suspension with 100ml of Tryptone Soya (Oxoid) broth. Stock solutions of 128mg/ml for the essential oils and 64mg/ml for the extracts were prepared using acetone. The stock solution of amphotericin B (0.01mg/ml) was prepared using dimethyl sulphoxide (Saarchem) while the stock solution of ciprofloxacin (0.01mg/ml) and serial dilutions for both controls were prepared using sterile water. Sterile water (100µl) was also added to all wells. The test solution (100µl) was added aseptically to the first row and 1:1 serial dilutions were performed longitudinally in the microtitre plate. Inoculum (100µl) was added to each well to achieve a final sample concentration ranging from 32mg/ml in row A to 0.25mg/ml in row H for the essential oils. The final sample concentration for the extracts ranged from 16mg/ml in row A to 0.125mg/ml in row H (Figure 5.3), whilst the final concentration for the antibiotic controls ranged from 2.5×10^{-3} mg/ml in row A to 1.95×10^{-5} mg/ml in row H. The essential oil plates were sealed with a film to prevent the samples from evaporating and all plates were incubated overnight for bacteria or 48 h for the yeast. *p*-Iodonitrotetrazolium (INT) chloride (Sigma) was prepared by dissolving 40mg in 100ml sterile water. Tetrazolium salts are frequently used to indicate biological activity because the colourless compound acts as an electron acceptor and is reduced to a coloured product by biologically active organisms. The INT solution had to be placed in a water bath in order to allow the powder to dissolve. Once dissolution had occurred, the solution was stored in the refrigerator at 4°C until use. After incubation, 50µl of a 0.04% (w/v) INT

was added to each well and the plates were kept at ambient temperature for 6 h for the bacteria or 24 h for the yeast. Thereafter the MIC value was visually determined and the value corresponds to the lowest concentration which inhibits the bacterial growth and is seen as the last clear well as opposed to the first red well which indicates growth (Figure 5.3).



Figure 5.3 An illustration of a microtitre plate showing serial dilutions and the corresponding MIC values of the extracts.

5.3. Results

5.3.1. Disc diffusion assay

The extracts screened for antimicrobial activity using the disc diffusion assay displayed poor activity (Table 5.1). The zones ranged from 1mm to 4mm in diameter from the disc edge to where growth begins. The maximum antibacterial effect was demonstrated for *Agathosma parva* against *Staphylococcus aureus*, having a zone of inhibition of 4mm. *Agathosma namaquensis*, *A. parva*, *A. pubigera* and *A. recurvifolia* showed very poor

activity against *Klebsiella pneumoniae* each having a zone of inhibition of 1mm. Overall, *Staphylococcus aureus* and *Bacillus cereus* seemed to be more sensitive to the extracts than *Klebsiella pneumoniae*, with the exception of *Agathosma hirsuta*.

Table 5.1: Preliminary disc diffusion results of *in vitro* antimicrobial activity of extracts of indigenous *Agathosma* species (measured in mm from disc edge to zone of growth).

Species	***B. cereus*** **ATCC 11778**	***K. pneumoniae*** **NCTC 9633**	***S. aureus*** **ATCC 12600**
A. capensis (Besemfontein)	2	2	2
A. hirsute	1	2	1
A. mundtii	2	2	2
A. namaquensis	2	1	2
A. ovata (round-leaf)	2	2	2
A. parva	2	1	4
A. pubigera	2	1	2
A. recurvifolia	2	1	2
A. stipitata	2	2	2
Neomycin	6.2	5	5.4

number of experimental runs (n) = 1

5.3.2. Minimum inhibitory concentration assay

The results from the MIC assay for the extracts and essential oils are depicted in Tables 5.2 and 5.3. The data shows that all of the extracts were active against the four pathogens tested with the exception of *Agathosma bathii* which showed very poor activity against *Klebsiella pneumoniae* (MIC value of 32mg/ml). The extracts exhibited stronger activity against the pathogens as compared to the essential oils. The MIC values of the extracts

ranged between 0.125mg/ml and 32mg/ml, whereas those of the essential oils ranged between 3mg/ml and 32mg/ml. Both the essential oils and extracts exhibited greater activity towards the Gram-positive bacteria than the Gram-negative bacterium with the extract of *Agathosma ovata* (round-leaf) displaying the greatest activity against *Staphylococcus aureus* and *Bacillus cereus* (MIC values of 0.156mg/ml and 0.125mg/ml).

The extract of *Agathosma parva* displayed the greatest activity against *Candida albicans* and *Klebsiella pneumoniae* having an MIC value of 1.5mg/ml for both pathogens. Amongst the essential oils (Table 5.3), *Agathosma pungens* proved to be the most active against the Gram-positive pathogen, *Bacillus cereus*, having an MIC value of 3mg/ml. *Agathosma collina* was the most active against *Candida albicans*, also having an MIC value of 3mg/ml. The essential oil of *Agathosma zwartbergense* showed the least activity against most of the tested bacteria, having MIC values ranging between 8mg/ml and 32mg/ml.

Table 5.2: MIC results (mg/ml) of antimicrobial activity of crude extracts of indigenous *Agathosma* species.

Species	***B. cereus*** **ATCC 11778**	***S. aureus*** **ATCC 12600**	***K. pneum-oniae*** **NCTC 9633**	***C. albicans*** **ATCC 10231**
A. arida	3	0.75	12	0.375
A. bathii	4	4	32	4
A. betulina	4	4	4	2
A. capensis (Besemfontein)	3	3	3	4
A. capensis (Gamka)	2	6.5	4	2
A. collina	4	8	2	16
A. crenulata	2	2	4	2
A. hirsute	0.75	0.25	2	3
A. lanata	4	1.5	6	4
A. namaquensis	1.25	0.5	2.5	3
A. ovalifolia	0.5	3	4	8
A. ovata (hook-leaf)	1.06	1	7	6
A. ovata (round-leaf)	0.125	0.156	8	4
A. parva	2	1	1.5	1.5
A. pubigera	2	0.8	2.5	3
A. pungens	1	0.75	4	6
A. roodebergensis	0.5	1	12	4
A. stipitata	2	2	3	3
A. zwartbergense	4	1.5	4	3
Controls	3.1×10^{-3}	3.1×10^{-3}	6.3×10^{-3}	3.1×10^{-3}

n = 3

Table 5.3: MIC results (mg/ml) of antimicrobial activity of essential oils of indigenous *Agathosma* species.

Species	***B. cereus*** **ATCC 11778**	***S. aureus*** **ATCC 12600**	***K. pneum-oniae*** **NCTC 9633**	***C. albicans*** **ATCC 10231**
A. arida	4	4	16	6
A. bathii	8	8	16	8
A. betulina	4	8	8	32
A. capensis (Besemfontein)	4	8	8	8
A. capensis (Gamka)	8	4	12	6
A. crenulata	3	4	8	24
A. collina	4	12	8	3
A. hirsute	4	6	8	8
A. lanata	4	4	12	4
A. namaquensis	8	4	8	8
A. ovalifolia	8	4	16	4
A. ovata (round-leaf)	6	4	16	6
A. parva	8	8	8	8
A. pubigera	8	8	8	16
A. pungens	3	4	12	4
A. roodebergensis	12	6	12	24
A. stipitata	8	4	8	8
A. zwartbergense	32	8	24	12
Controls	3.2×10^{-3}	2.5×10^{-3}	6.3×10^{-3}	3.1×10^{-3}

n = 3

The bar graphs below depict the antimicrobial activity of the essential oils and their corresponding extracts for each of the pathogens (Figures 5.4, 5.5, 5.6 and 5.7).

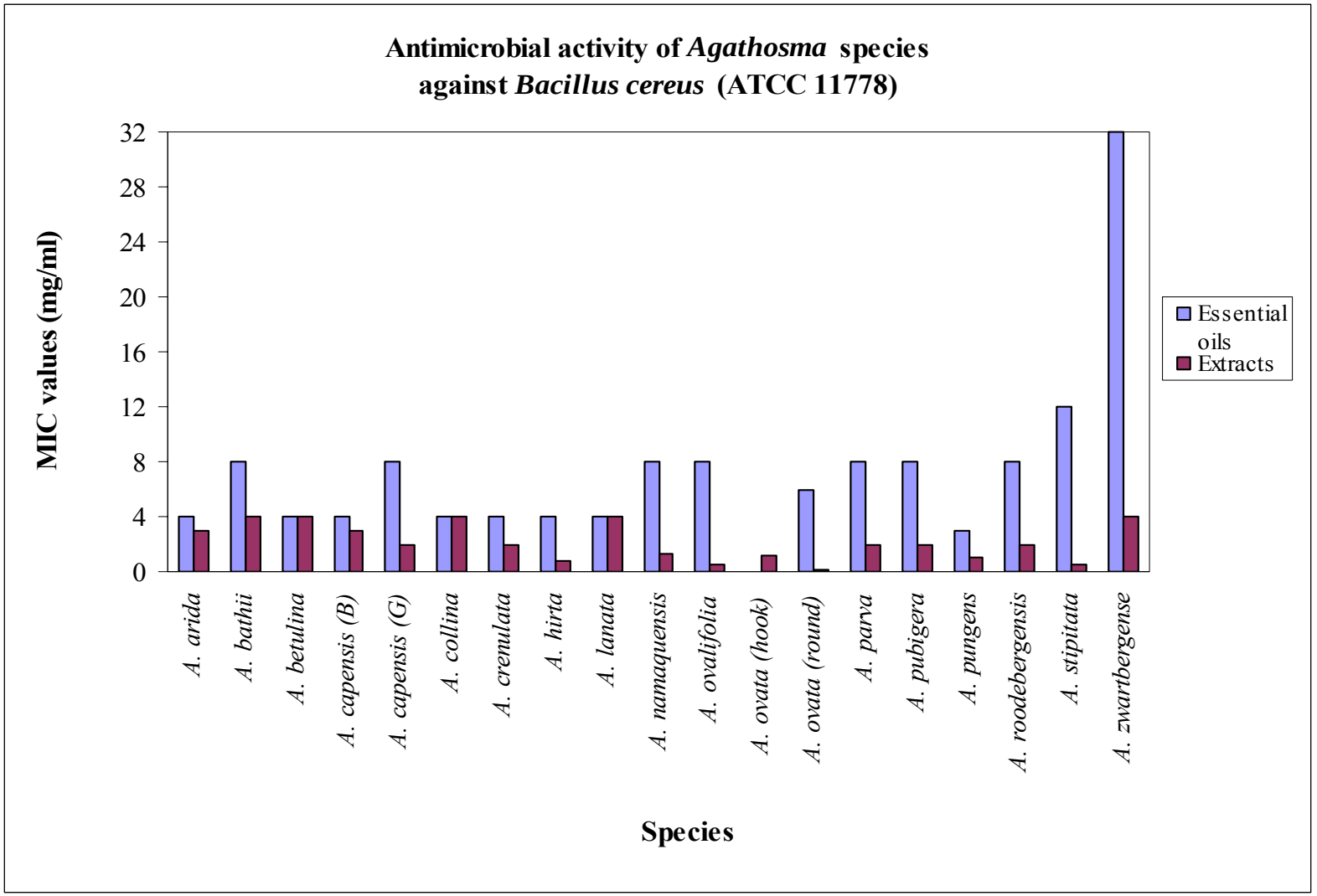


Figure 5.4: Bar graph depicting the antimicrobial activity (MIC) of the essential oils and corresponding extracts of indigenous *Agathosma* species against *Bacillus cereus* (ATCC 11778).

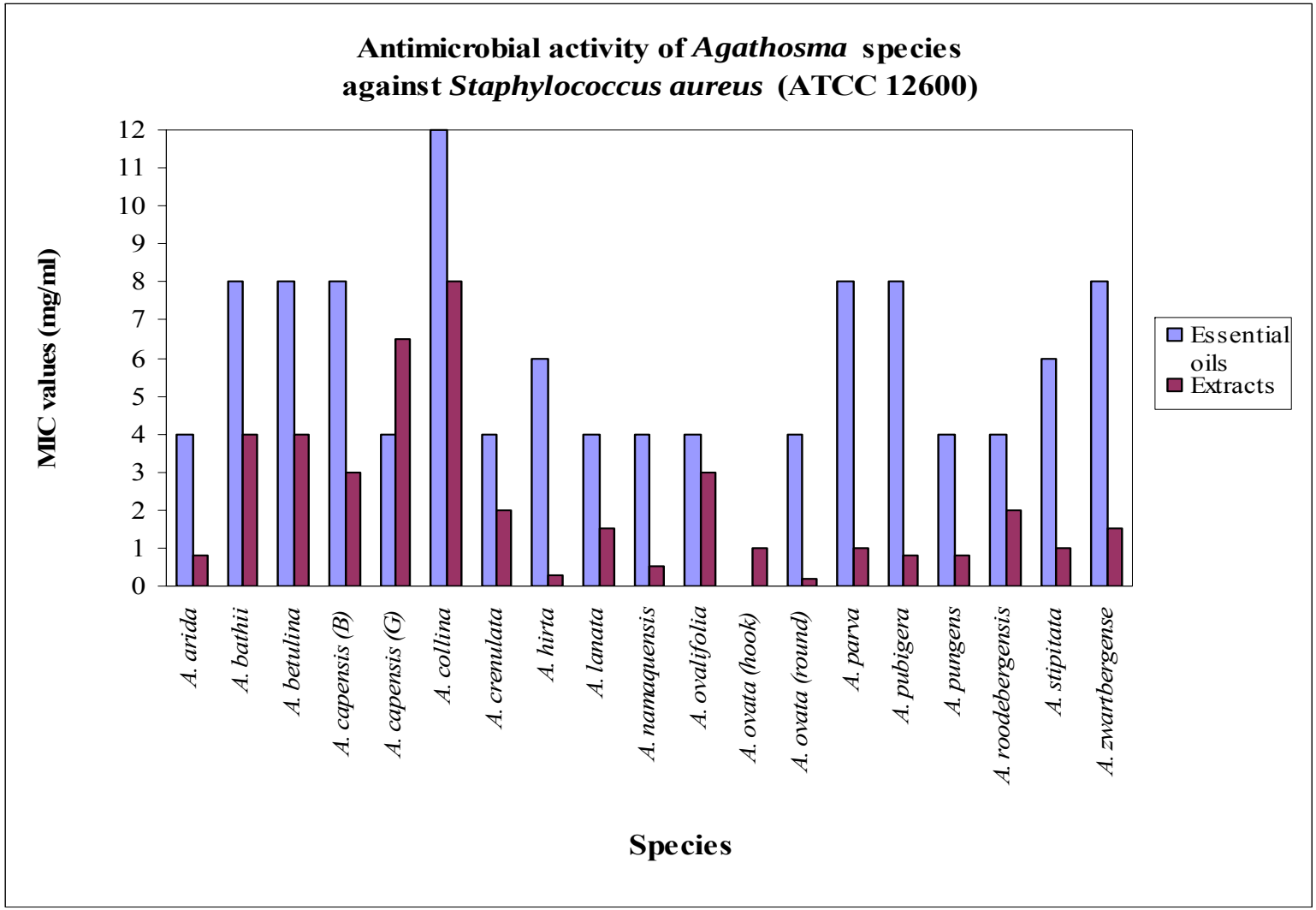


Figure 5.5: Bar graph depicting the antimicrobial activity (MIC) of the essential oils and corresponding extracts of indigenous *Agathosma* species against *Staphylococcus aureus* (ATCC 12600).

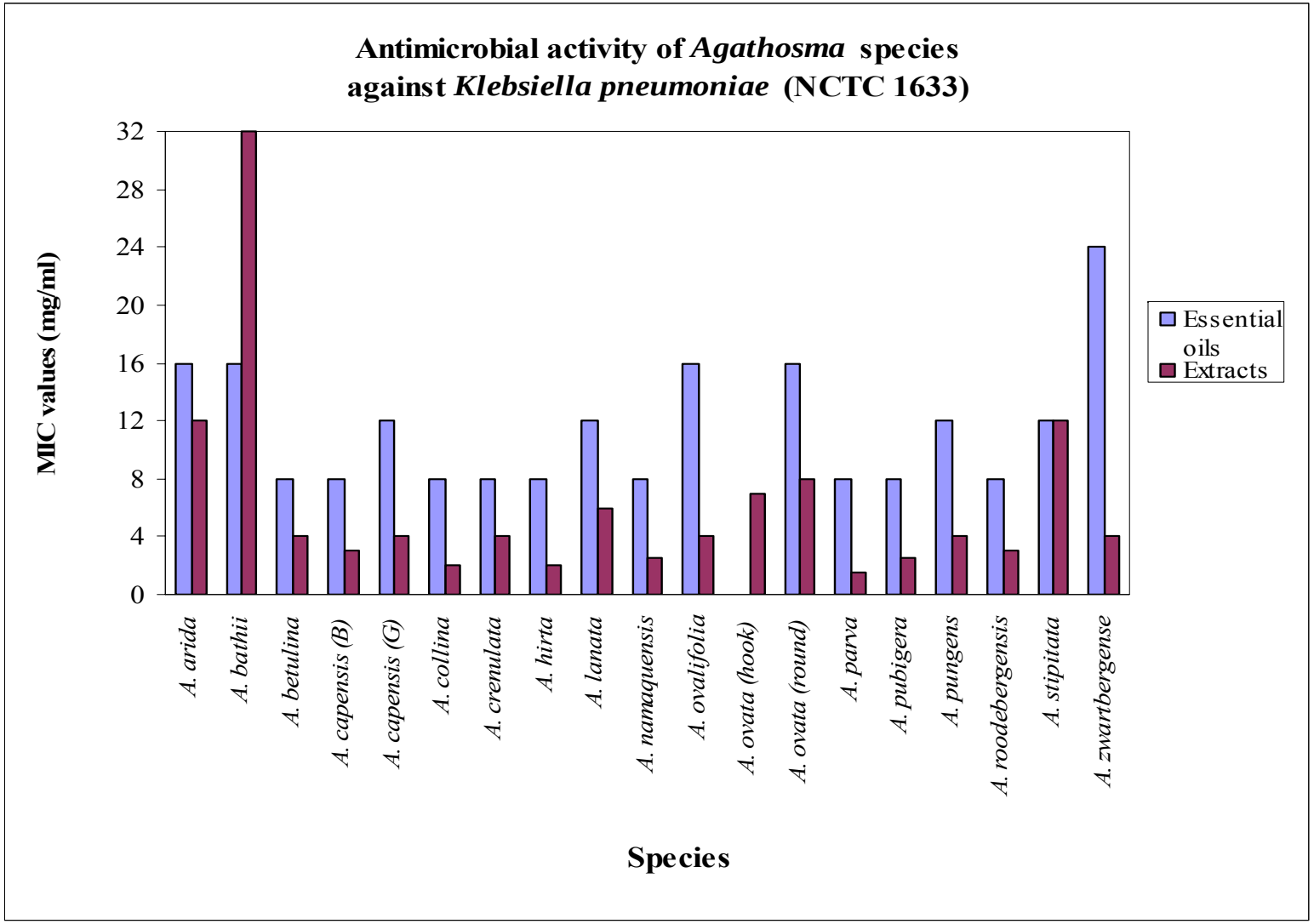


Figure 5.6: Bar graph depicting the antimicrobial activity (MIC) of the essential oils and corresponding extracts of indigenous *Agathosma* species against *Klebsiella pneumoniae* (NCTC 1633).

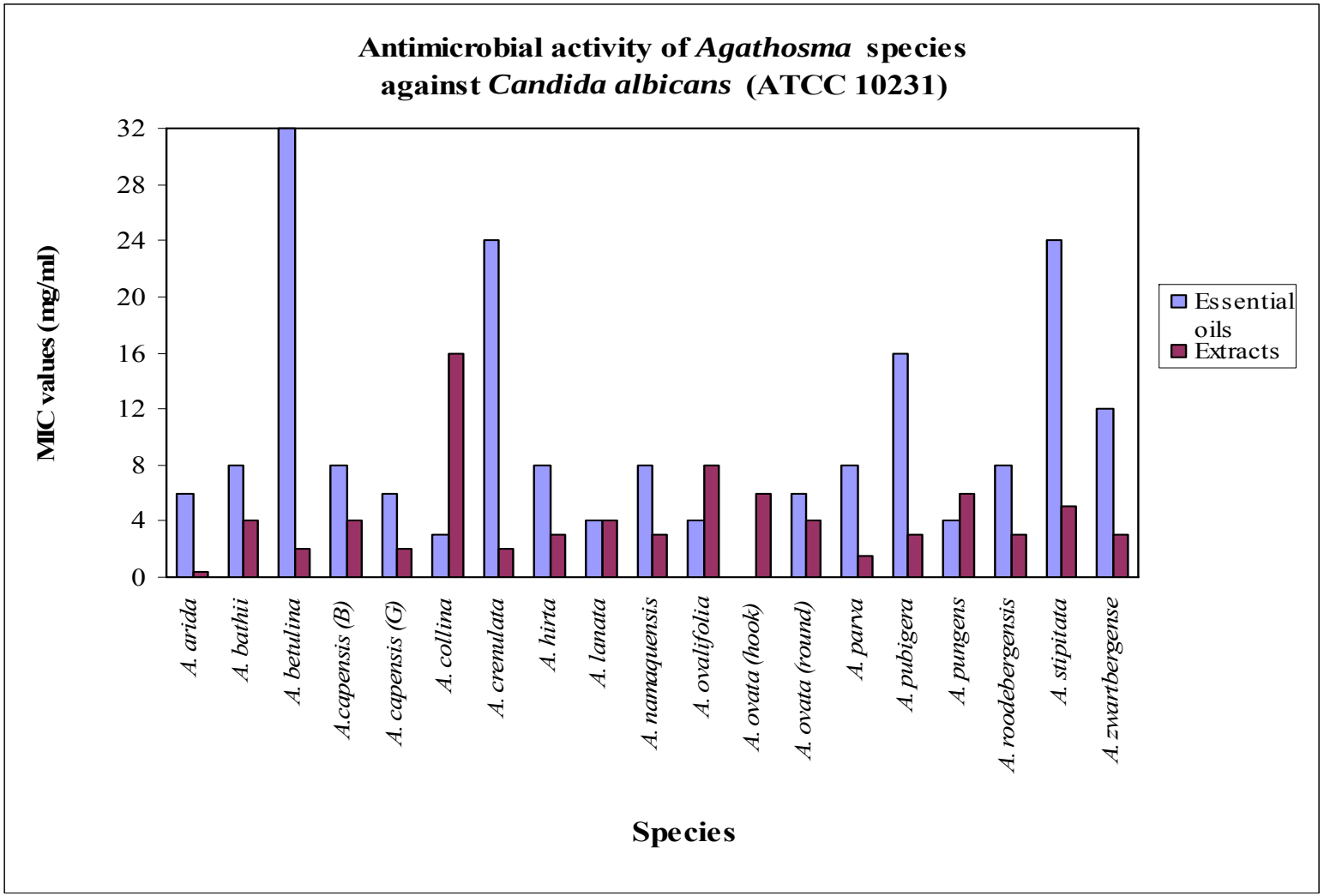


Figure 5.7: Bar graph depicting the antimicrobial activity (MIC) of the essential oils and corresponding extracts of indigenous *Agathosma* species against *Candida albicans* (ATCC 10231).

5.4. Discussion

5.4.1. Disc diffusion assay

A comparison of the diameters of the inhibition zones produced revealed that most of the species displayed similar activities. It may be speculated that the poor activity in the disc diffusion assay could be due to the hydrophobic nature and the low water solubility of the extract constituents which limit their diffusion through the water-based medium (Knobloch *et al*., 1988). Other factors that could have influenced the results are: the amount of sample applied, the agar composition, pH and the type of pathogens used (Pauli and Kubecza, 1996). The disc size and volume of agar used was constant, hence these could have not influenced the results.

5.4.2. Minimum inhibitory concentration assay

The extracts displayed better activity than the essential oils against the pathogens tested. Fabry *et al*. (1998) reported that MIC values ≥ 8mg/ml indicate very low antibacterial activity for extracts. Based on this it is concluded that most of the extracts exhibited good antimicrobial activity against the pathogens tested. HPLC analysis has revealed that these species are rich in flavonoids (Chapter four), which may be a reason for the extracts displaying better activity than the essential oils. Since flavonoids are noted for being synthesized by plants in response to microbial infection, it is not surprising that they have been found *in vitro* to be effective antimicrobial substances. Their activities are due to their ability to complex with bacterial cell walls. The more lipophilic flavonoids may also disrupt microbial membranes (Cowan, 1999).

In 1976, Finkelstein and Rivett discovered a new prenyloxy-coumarin, puberulin. A further study performed by Campbell *et al*. (1986) discovered the presence of coumarins in *Agathosma* species. Coumarins are phenolic substances made of fused benzene and α-pyrone rings and several have been found to have antimicrobial properties (Cowan, 1999). A coumarin was also found *in vitro*, to inhibit *Candida albicans* (Cowan, 1999). As a group, they have been found to stimulate macrophages, which could have an indirect negative effect on infections. Data on specific antimicrobial properties of coumarins are scarce, although many reports give reason to believe that some utility may reside in these phytochemicals (Cowan, 1999). Column and thin layer chromatographic analysis of *Agathosma ovata* (round-leaf) resulted in the detection of coumarins, visible as blue fluorescent bands under UV_{366nm} (Cassim and Noorgat, 2005). HPLC analysis confirmed that puberulin is present in *Agathosma namaquensis* (Chapter four). Coumarins may be another contributing factor to the antimicrobial properties of these species.

The aerial parts of *Agathosma* species were screened in a study by Campbell *et al*. (1986) for alkaloids, which are heterocyclic nitrogen compounds. Positive results were obtained for five species and the compounds halfordamine and skimmianine were identified. Quinoline alkaloids were also detected in *Agathosma barosmaefolia* (Campbell and Bean, 1996). Diterpenoid alkaloids, commonly isolated from the plants of the Ranunculaceae or buttercup family are commonly found to have antimicrobial properties (Cowan, 1999). Berberine is an important representative of the alkaloid group. It is potentially active against trypanosomes and plasmodia (Cowan, 1999). The mechanism of action of highly aromatic planar quaternary alkaloids such as berberine and harmane are attributed to their ability to intercalate with DNA (Cowan,

1999). Alkaloids in general, have been found to have microbiocidal effects and although determining the presence of alkaloids in these species, isolating them and evaluating their antimicrobial activities was not part of the research study, the possibility of alkaloids being present and contributing to the antimicrobial effect is probable.

Some of the active essential oils against *Staphylococcus aureus* having MIC values of 4mg/ml include: *Agathosma arida*, *A. capensis* (Gamka), *A. crenulata*, *A. lanata*, *A. namaquensis*, *A. ovalifolia*, *A. ovata* (round-leaf) and *A. pungens* (Table 5.3). Linalool and myrcene are components present in large quantities and common to all of the above species, which may contribute to their overall activities (Carson and Riley, 1996). The presence of terpinen-4-ol may also contribute to their activities since it has been found to exhibit strong antimicrobial properties (Carson and Riley, 1996). The antimicrobial activity of *Agathosma* species may be attributed to more then one component. The two most active essential oils against *Bacillus cereus* were those of *Agathosma crenulata* and *A. pungens* having MIC values of 3mg/ml. *Agathosma collina* was the most active essential oil against *Candida albicans* (MIC value of 3mg/ml). Borneol is a constituent of *Agathosma bathii* (0.1%), *A. betulina* (0.1%), *A. capensis* (Gamka) (0.1%), *A. crenulata* (0.1%), *A. ovata* (round-leaf) (0.1%) and *A. pungens* (0.2%); 1,8-cineole is a constituent of many species including *A. namaquensis* (22.1%) and carvacrol is a constituent of *Agathosma bathii* (0.1%), *A. capensis* (Besemfontein) (trace), *A. namaquensis* (0.2%) and *A. ovata* (round-leaf) (0.2%). A previous study has shown that borneol, 1,8-cineole and carvacrol are well known antimicrobial compounds isolated from different plant species (Uzel *et al*., 2004), hence these compounds may contribute to the activity of these essential oils.

Due to their low concentrations, activity is not very good but neither is it as poor as those species that lack the compounds. A study performed by Lambert *et al*. (2001) found that mixing carvacrol and thymol in specific ratios exerted inhibition on *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Such inhibition is due to damage in membrane integrity, which affects pH homeostasis and equilibrium of inorganic ions (Lambert *et al*., 2001). The antimicrobial activity of *Agathosma namaquensis* could then be due to the combination of carvacrol (0.2%) and thymol (0.1%) which have an additive effect.

The *in vitro* antimicrobial activity assay indicated that most of the essential oils exhibited a wide range of activity (MIC values ranging between 3mg/ml and 32mg/ml). The antibacterial activity of the essential oils may be attributed to the compound 1,8-cineole as the activity of the essential oil increases proportionally to the respective yield of 1,8-cineole, which is known to possess some antimicrobial activity (Viljoen *et al*., 2003). The presence of linalool, which is known to possess good antimicrobial activity (Knobloch, 1988) may contribute to the antibacterial effect of all species. Lis-Balchin *et al*. (1998) reported that samples of essential oils containing linalool showed no correlation with antifungal activity but did correlate with antibacterial activity.

Dorman and Deans (2000) found that the antibacterial constituents of black pepper, clove, geranium and oregano involved thymol, carvacrol, α-terpineol, eugenol, linalool, nerol and α-pinene. These results suggest that the presence of these compounds in most of the species have contributed to their overall antimicrobial effect. The major compound citronellal, present in *Agathosma hirsuta* (72.5%) and *A.*

zwartbergense (64.7%) may contribute to their antimicrobial activities. In a study performed by Lis-Balchin *et al*. (1998), it was found that *E. citriodora* with a high citronellal content had anti-*Listeria* activity. The compounds methyl chavicol, geraniol and citronellol present in small amounts in most of the species are not conducive to strong antimicrobial activity (Lis-Balchin *et al*., 1998). Those plants that contain monoterpenes and generally essential oils with high monoterpene hydrocarbon levels are very active against bacteria although not against fungi (Lis-Balchin *et al*., 1998). Compounds like limonene, *p*-cymene and α-pinene have been shown to exhibit low antibacterial activity (Cimanga *et al*., 2002). The excellent activity of *Agathosma stipitata* against all pathogens can be attributed to a high content of geranial (16.1%) and neral (34.8%), both of which have antibacterial properties (Cimanga *et al*., 2002). However, compounds present in the greatest proportions are not necessarily responsible for the greatest share of the total activity. The involvement of the less abundant constituents should also be considered (Cimanga *et al*., 2002). The activity could then be attributed to the presence of minor components such as nerol, borneol, linalool, cinnamaldehyde, carvacrol, geraniol, myrtenal and eugenol known already to exhibit an antibacterial effect (Cimanga *et al*., 2002) or a synergistic effect between different components. It has been suggested that the antibacterial activity of some essential oils are related to the presence of some favourable classes of compounds such as alcohols, aldehydes, alkenes, esters and ethers (Cimanga *et al*., 2002). Species containing favourable compounds could exhibit poor activity due to the presence of unfavourable compounds in high amounts.

Both the essential oils and extracts exhibited greater activity towards the Gram-positive bacteria than the Gram-negative bacterium. This is consistent with results that

have emerged from other *in vitro* studies in which it has been found that plant extracts and essential oils inhibit more Gram-positive rather than Gram-negative bacteria (Voravuthikunchai *et al*., 2004). Various authors reported that these differential susceptibilities are related to the morphological structure and chemical composition of the membrane of the bacteria. Nikaido and Nakae (1979) reported that Gram-negative bacteria have an outer membrane composed mainly of lipopolysaccharides, which is rather impermeable to lipophilic molecules and hydrophobic dyes and acts as a selective barrier to hydrophilic molecules. Gram-positive bacteria possess a much thicker peptidoglycan layer, which is not an effective permeability barrier to hydrophilic solutes.

The possible poor activity of the essential oils as compared to the extracts could be due to medium constituents reacting with essential oil components and inactivating them, for example some aldehydes can react with the sulfhydryl groups in proteins (Janssen *et al*., 1987). Another reason could be due to the essential oil insolubility in the growth medium (Janssen *et al*., 1987). The pH of the medium could influence the activity of the essential oil. For phenols and carboxylic acids, it is known that only the uncharged form is able to penetrate the microbial cell and exhibit activity. For Gram-positive non-lactic bacteria, the activities of eugenol, *l*-carvone, *d*-carvone and menthol are larger at pH 6 than pH 8 (Janssen *et al*., 1987). Another reason for the low activity may be the decomposition of the oil constituents which may occur during the test period or the oil may evaporate while plating out (Janssen *et al*., 1987).

'Buchu' has been an important part of the San and Khoi healing culture in the Cape and is still used throughout South Africa. It has been used as a cough remedy, for the

treatment of colds and flu, kidney and urinary tract infections, for the treatment of cholera and other stomach ailments and as an antibiotic protectant. This scientific evidence provides a basis and justifies the use of *Agathosma* species in treating infections.

CHAPTER 6: A SEASONAL VARIATION STUDY OF *AGATHOSMA OVATA*

6.1. Introduction

Due to the availability and accessibility of *Agathosma ovata*, a seasonal variation study was performed on the chemical composition of the essential oils and how this may impact on the antimicrobial activity. Furthermore, this species has recently been earmarked for commercial development by the flavour and fragrance industry and information on variability is required to establish the harvesting protocol.

6.2. Materials and methods

6.2.1. Collection of plant material

Agathosma ovata (round-leaf) was harvested monthly from May 2003 to April 2004 during the full flowering and non-flowering stages by Mr E.M. Goosen in Bredasdorp. The plants were harvested from the same site but were not harvested during the months of August 2003 and January 2004 due to insufficient plant material for hydrodistillation. A voucher specimen was made for identification purposes and duplicate specimens have been maintained.

6.2.2. Preparation of plant material

Refer to the preparation of essential oils in 2.3.1.

6.2.3. Essential oil analysis

6.2.3.1. Gas chromatography-mass spectroscopy

Refer to method in 3.2.2.

6.2.4. Antimicrobial activity

6.2.4.1. Minimum inhibitory concentration assay

Refer to method in 5.2.2.

6.3. Results and discussion

6.3.1. Essential oil yields

The pattern of variation in oil yield throughout the year was visualized by plotting the oil yield (%) against the harvest month (Figure 6.1).

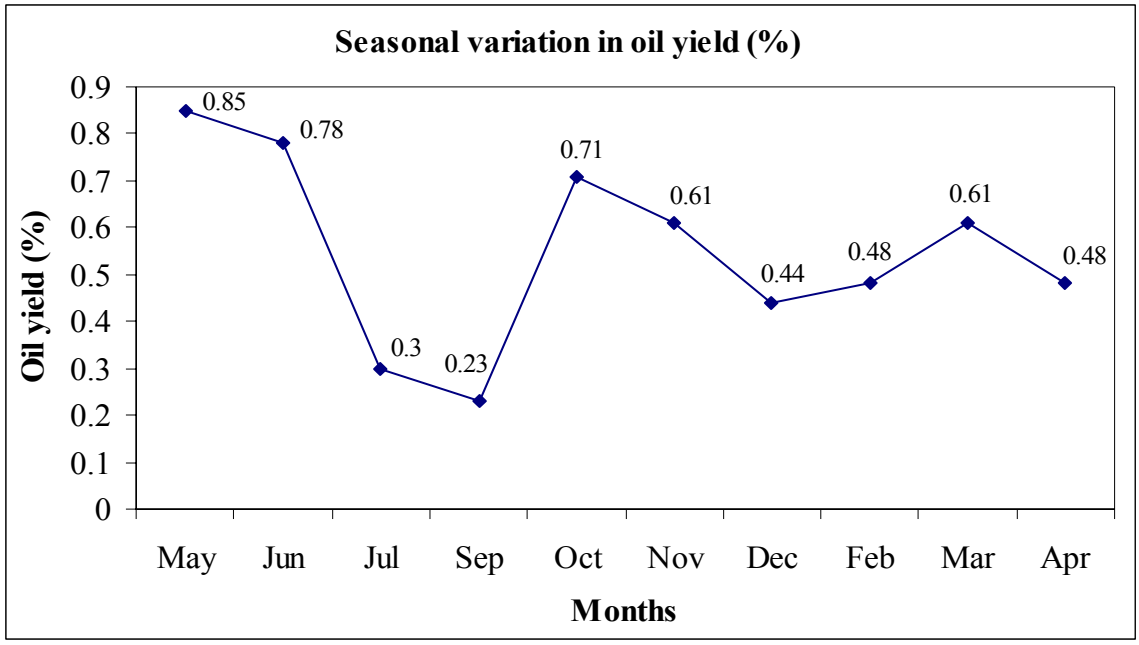


Figure 6.1: Graph displaying the seasonal variation in oil yield (%).

There was a substantial variation in the oil yield throughout the year, ranging from 0.23% in early Spring to 0.85% in late Autumn. The flowering season for *Agathosma ovata* is mid Autumn to early Spring (Gould, 1990). A higher yield was observed during the flowering season as compared to the non-flowering season. The Rutaceae family are characterized by the presence of oil glands sometimes on the petals (Gould,

1990). This may contribute to the higher yield during the flowering season (Gould, 1990). A study performed by Perry *et al*., (1999) found the flowering parts of *Salvia officinalis* to have a higher oil content then the non-flowering parts. A seasonal variation study on the essential oil of *Salvia officinalis* found the yield and composition of the oil to fluctuate from month to month (Pitarević *et al*., 1984).

Oil yields were low during Summer (0.44%-0.48%). This could be due to the low oil content in stems and higher proportion of stems after flowering (Perry *et al*., 1999). The proportion of oil-rich green leaves also decreased markedly, hence affecting the yield. Oil yields were dependant on the amount of leaf and flower harvested.

The lowest yields (0.30% and 0.23%) were recorded during the mid Winter and early Spring seasons. However, since the yield presents the higher values in the months of flowering, it is possible that the plant increases the amount of essential oil in order to favour the pollination (Palá-Paúl *et al*., 2001). Several terpenoids have been previously reported as pollination vectors (Palá-Paúl *et al*., 2001).

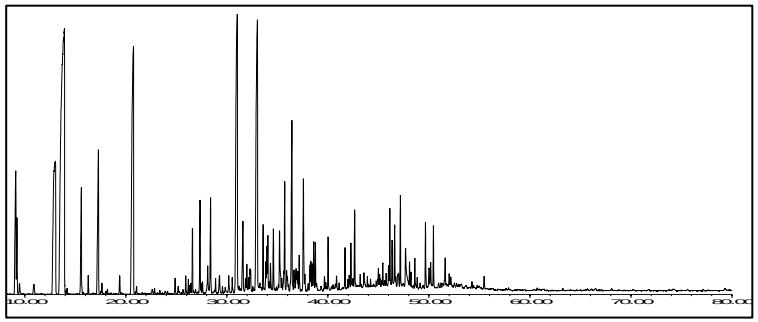
Overall the yields were dependant on the season harvested and proportion of plant parts distilled. Understanding this factor can make a difference between a good yield of a high quality oil and a poor yield of an undesirable oil.

6.3.2. Gas chromatography-mass spectroscopy

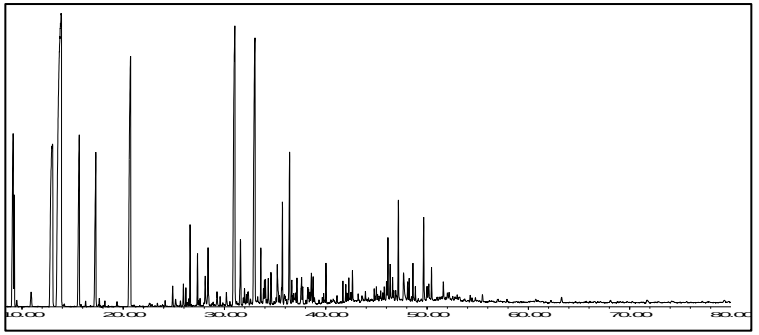
The GC-MS chromatograms are depicted in Figure 6.2 below. The compounds identified in each of the samples, their percentages and relative retention indices are listed in order of their elution in Table 6.1.

For the 10 samples analyzed by GC-MS, 145 compounds were identified. The patterns of variation in the essential oil composition of *Agathosma ovata* was visualized by plotting the percentages of each of the major components against the month of harvest (Figures 6.4 and 6.5). All samples contained a large number of common monoterpenes. GC-MS data revealed that they have very similar compositions, with mainly the percentages of the compounds fluctuating. Some components common to all samples include: sabinene, *p*-cymene, β-pinene, α-pinene, α-thujene, myrcene, limonene, linalool and terpinen-4-ol (Figure 6.3). Sabinene (Figure 6.3) was found to be the most dominant component in all samples, ranging between 25.6% and 44.4% (Figure 6.4). The level dropped in December, from 44.4% to 25.6%, and thereafter began to increase in March. This could be due to the approaching of the flowering season. During the flowering season, its level remained between 30.7% and 38.2%.

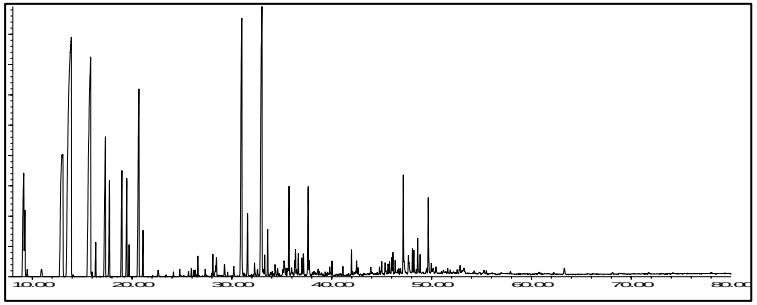
Myrcene levels dropped sharply between the beginning of Spring and end of Summer, from 14.9% to 1.0% (Figure 6.4). Peak levels occurred in September (14.9%) and March (13.9%). β-pinene followed a similar trend, peaking during Spring and decreasing during the Summer months (Figure 6.4). Some of the changes can be explained by different compositions of the essential oils during the flowering and non-flowering stages. In particular, *p*-cymene levels were lowest in early Spring (4.1%) and increased gradually until late Summer (14.1%), after which it decreased again (Figure 6.4). Limonene and terpinen-4-ol did not vary considerably throughout the year (Figure 6.4). Linalool levels peaked in late Summer (11.7%) and early Winter (10.7%) (Figure 6.4).



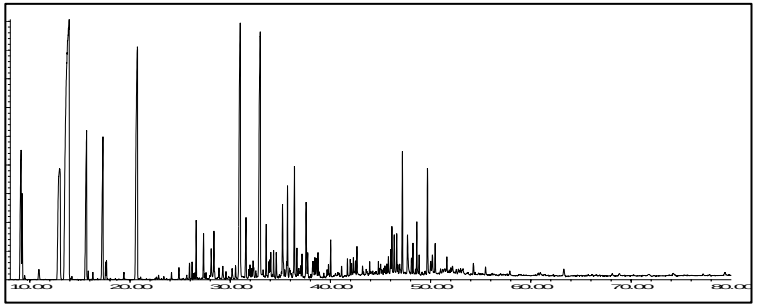
May



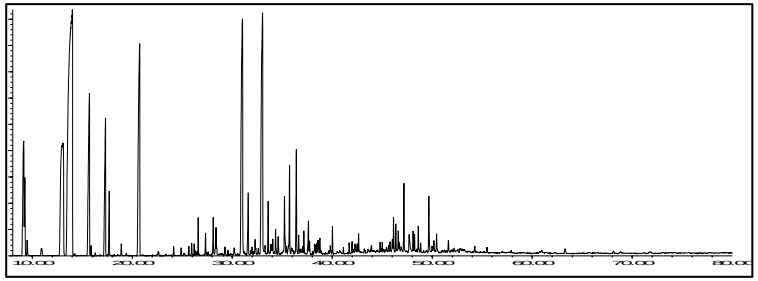
June



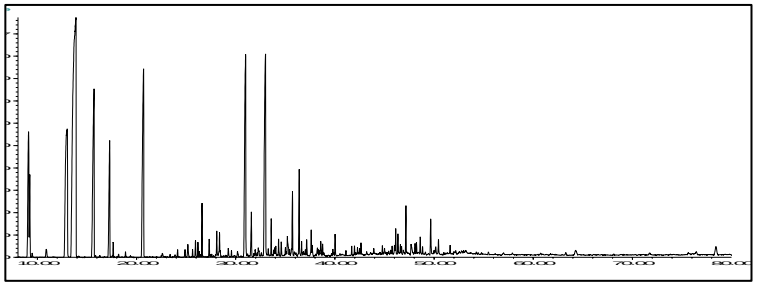
July



September



October



November

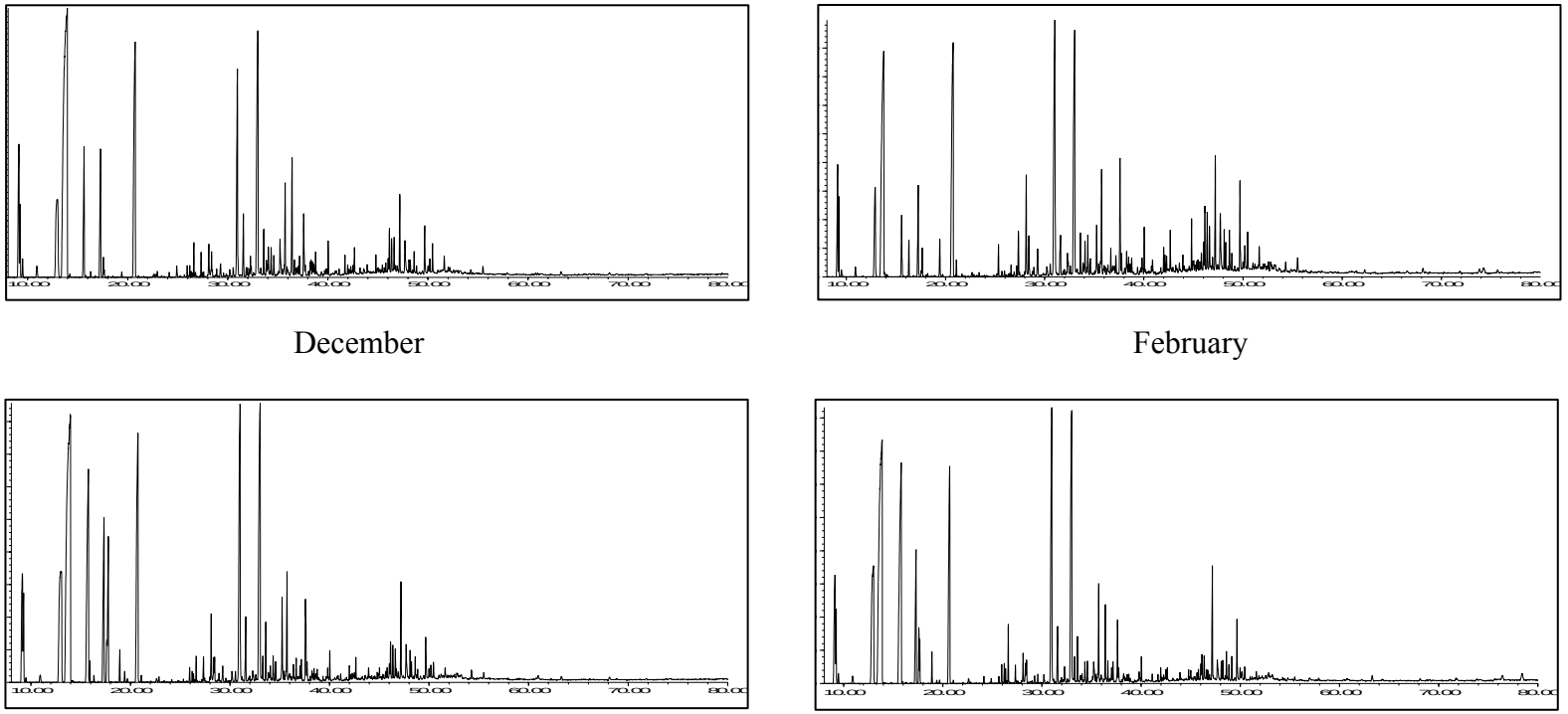


Figure 6.2: GC-MS chromatograms of the seasonal samples of the essential oils of *A. ovata*.

Table 6.1: Compounds identified in the seasonal samples of the essential oils of *A. ovata*.

RRI	Compound	May	Jun	Jul	Sep	Oct	Nov	Dec	Feb	Mar	Apr
1032	α-pinene	2.6	3.7	3.8	3.7	3.0	3.2	3.5	4.2	5.1	3.4
1035	α-thujene	1.0	1.2	1.3	1.3	1.5	1.2	1.1	1.3	tr	1.4
1048	2-methyl-3-buten-2-ol	0.2	0.1	0.1	0.2	0.1	0.3	0.4	0.2	0.2	0.1
1076	camphene	0.2	0.2	0.2	0.2	0.19	0.2	0.3	0.3	0.2	0.2
1118	β-pinene	7.0	7.4	6.0	8.6	7.9	8.1	4.2	3.9	7.3	6.2
1132	sabinene	38.2	30.7	35.5	31.3	34.2	39.6	44.4	25.6	32.2	36.4
1141	thuja-2,4(10)-diene								tr	tr	
1174	myrcene	1.4	3.6	3.5	14.9	7.2	4.6	2.6	1.0	13.9	8.2
1176	α-phellandrene								0.1		
1183	pseudolimonene			0.1			tr	tr	tr	tr	0.1
1187	1,4-cineole							tr		tr	
1188	α-terpinene		tr	tr				tr	0.1	tr	0.1
1195	dehydro-1,8-cineole							tr	tr	tr	
1203	limonene	2.3	2.6	2.8	2.9	2.6	2.7	2.4	1.9	2.8	3.2
1205	3-hexanol									tr	
1213	1,8-cineole			0.1						1.0	tr
1218	β-phellandrene	0.1	0.1	0.3	1.3	0.2	0.6	0.3	0.3	tr	2.5
1220	*cis*-anhydrolinalool oxide									tr	
1224	*o*-mentha-1(7),5,8-triene			tr					tr		
1225	(*Z*)-3-hexenal		0.1			tr			tr		
1246	(*Z*)-β-ocimene	tr		tr	1.6	0.1			tr	0.3	0.3

RRI	Compound	May	Jun	Jul	Sep	Oct	Nov	Dec	Feb	Mar	Apr
1255	γ-terpinene	0.1	tr	tr	1.2	tr		tr	0.2	tr	0.1
1266	(*E*)-β-ocimene	tr		tr	0.3	tr			tr	tr	tr
1280	*p*-cymene	9.1	8.1	8.3	4.1	7.5	7.1	9.4	14.1	6.3	7.8
1290	terpinolene	tr			0.4		tr	tr	0.1	tr	0.1
1296	octanal								tr		
1319	3-octen-1-one								tr	tr	
1327	3-methyl-2-butenol	0.1	0.1		0.1	0.1	0.1	0.1		0.1	0.1
1337	geijerene									tr	
	thiol derivative									tr	
1348	6-methyl-5-hepten-2-one								0.1		
1382	allo-ocimene				0.1						
1384	α-pinene oxide		0.2	0.1		0.1	0.1	tr		tr	
1386	1-hexanol					0.1					
1391	(*Z*)-3-hexen-1-ol		0.1			0.3	tr		0.2		
1398	(*E*)-4,8-dimethyl-1,3,7-nonatriene (tentative identification)	0.1	0.2	0.1	tr	0.2	tr		tr	0.2	0.1
1400	nonanal								tr		
1409	1,3,8-*p*-menthatriene								tr		
1415	(*E*)-4-hexen-1-ol										
1424	*o*-methylanisole	0.1		0.1	0.1	0.1	tr	0.1	tr	0.1	0.1
1429	perillene	0.5	0.7	0.5	0.1	0.6	0.3	0.3	0.1	0.5	0.2
1430	α-thujone								tr	tr	
1435	*p*-menthatriene isomer								0.1		

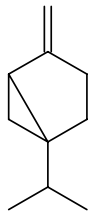
RRI	Compound	May	Jun	Jul	Sep	Oct	Nov	Dec	Feb	Mar	Apr
1450	*trans*-linalool oxide (furanoid)	0.7	0.4	0.3	tr	0.1	0.1	tr	0.4	tr	0.1
1451	β-thujone		0.1	tr				0.1	0.1	0.1	
1458	*cis*-1,2-limonene epoxide		0.1	0.1			tr	tr		tr	
1466	α-cubebene			0.1	tr				tr	tr	tr
1474	*trans*-sabinene hydrate	0.3	0.4	0.4	0.2	0.3	0.3	0.4	1.5	0.3	0.6
1476	4,8-epoxyterpinolene				tr			0.1			
1476	(*Z*)-β-ocimene epoxide				0.2		tr			0.3	0.3
1478	*cis*-linalool oxide (furanoid)	0.7	0.5	0.4	tr	0.3	0.2	0.2	0.1		tr
1487	citronellal										0.1
1497	α-copaene	0.2	0.1	0.1	0.1	0.1			0.3	0.1	0.1
1498	(*E*)-β-ocimene epoxide									tr	
1505	dihydroedulane II						tr	tr			
1532	camphor	0.2	0.2		0.1	0.1	tr	0.1	0.2	0.1	0.1
1553	linalool	10.0	10.7	8.0	6.9	8.7	8.8	4.3	11.7	7.6	7.2
1562	isopinocamphone	tr			0.1				tr	0.1	
1571	*trans-p*-menth-2-en-1-ol	0.7	0.8	0.1	0.6	0.7	0.7	0.7	0.6	0.6	0.6
1586	pinocarvone		0.1			0.1	0.1	0.1	tr	0.1	0.1
1597	bornyl acetate	0.2	tr	0.1	0.1	0.1	0.1	0.1	0.1	0.1	tr
1600	β-elemene	tr	0.1	0.1	0.1				0.1		
1602	6-methyl-3,5-heptadien-2-one					tr				tr	
1611	terpinen-4-ol	7.8	8.9	7.5	8.9	9.7	9.3	9.0	11.3	8.3	8.44
1626	2-methyl-6-methylene-3,7-octadien-2-ol	0.1			0.3	0.1	0.1	0.4		0.2	

RRI	Compound	May	Jun	Jul	Sep	Oct	Nov	Dec	Feb	Mar	Apr
1638	*cis*-*p*-menth-2-en-1-ol	0.5	0.5	0.4	tr	0.4	0.4	0.2	0.4	0.4	
1639	*trans*-*p*-mentha-2,8-dien-1-ol				0.2						
1642	thuj-3-en-10-al		0.1	0.1	tr	0.1	0.1		0.3	0.1	
1648	myrtenal		0.2	0.2	0.1	0.1	tr		tr	0.1	0.1
1651	sabinaketone	0.4	0.2	0.3	0.1	0.2	0.1	0.3	0.3	0.2	
1657	umbellulone		tr					tr		tr	
1664	*trans*-pinocarveol	0.6	0.3	0.3	0.1	0.2		tr	tr		
1668	citronellyl acetate						0.2			tr	0.2
1678	*cis*-*p*-menth-2,8-dien-1-ol							tr		0.1	
1685	ethyl benzoate					0.1					
1687	α-humulene				tr						
1689	*trans*-piperitol	0.6			0.2			0.1			
1690	cryptone		0.5	0.8		0.4	0.6	0.4	0.5	0.3	0.7
1700	limonen-4-ol	0.1	0.1	0.1	0.1	0.1	tr	0.1	0.1	0.1	0.1
1704	myrtenyl acetate				0.1					tr	
1704	γ-muurolene		0.1	0.1	tr	tr	0.1		0.1		0.1
1706	α-terpineol	0.7	0.9	0.7	0.7	0.8	0.7	0.9	1.2	0.8	0.8
1719	borneol				0.1				0.1	0.1	0.1
1719	germacrene D				tr						
1720	*trans*-sabinol		0.1	0.1		0.1	0.1	0.1			
1729	*cis*-1,2-epoxyterpinen-4-ol		1.9	1.1	0.2	1.4	1.0	1.4	0.2	0.7	0.2
1729	thujol			0.1							
1740	α-muurolene	0.1	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.2	0.1

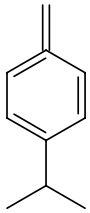
RRI	Compound	May	Jun	Jul	Sep	Oct	Nov	Dec	Feb	Mar	Apr
1748	piperitone		0.1	0.1		0.1	0.1	0.1	0.2	tr	
1751	carvone		0.2	0.1		0.1	tr	0.3	tr	0.2	
1755	bicyclogermacrene	0.3			0.2						
1756	2-oxo-1,4-cineole			0.9				0.8	1.6		1.1
1758	*cis*-piperitol	0.4	0.3	0.3	0.2	0.3	0.2		0.3	tr	0.2
1763	naphthalene								tr		
1772	citronellol		0.5			0.5					
1773	δ-cadinene				0.9		0.3			0.7	tr
1773	γ-cadinene	0.1			0.1	tr	0.1	tr	0.1	tr	0.3
1776	γ-calacorene		0.1	0.1			tr	0.1	0.1		
1786	*cis*-sabinol		tr	tr		tr	0.1	0.1	tr	tr	
1793	guai-3,7-diene									0.1	
1797	*p*-methylacetophenone		0.2	0.2		0.2	0.1	0.3	0.2	0.1	0.1
1797	*p*-mentha-1,5-dien-7-ol	0.5	0.3	0.2	tr	0.2	tr	0.3	tr	tr	tr
1802	cuminaldehyde		0.1	0.2		0.1	0.2		0.2	tr	0.1
1804	myrtenol		0.4	0.3	tr	0.2	0.1	0.2	0.1	0.2	
1830	2,6-dimethyl-3(*E*),5(*E*),7-octatriene-2-ol									tr	
1845	*trans*-carveol			0.1			0.1	0.1	0.1	0.1	
1853	calamenene	tr	0.1	0.1	tr	0.1	tr	tr	0.1	tr	0.1
1857	geraniol									tr	
1864	*p*-cymen-8-ol		0.4	0.4	0.1	0.3	0.3	0.4	0.5	0.2	0.2
1868	(*E*)-geranylacetone								tr		

RRI	Compound	May	Jun	Jul	Sep	Oct	Nov	Dec	Feb	Mar	Apr
1900	epi-cubebol	tr	0.1	0.1	0.1	0.1	0.1	0.1	tr	0.1	tr
1941	α-calacorene		0.1	0.1			tr		tr	0.1	tr
1949	(*Z*)-3-hexenyl nonaoate		0.3	0.2		0.1	0.1	0.1	0.3	tr	0.1
1957	cubebol	tr	tr	0.2	0.1	0.1	0.1	0.1		0.1	tr
2008	caryophyllene oxide	0.1	0.1	0.1	tr	0.1		0.1	0.2	0.1	0.1
2037	salvial-4(14)-en-1-one								tr		
2050	(*E*)-nerolidol	tr	0.1	0.1	tr	0.1	tr	0.1	0.3	0.1	0.1
2069	germacrene-D-4-ol				0.1						
2071	humulene epoxide II							0.1	0.1	tr	tr
2073	*p*-mentha-1,4-dien-7-ol	0.2	0.1				0.1	0.1	0.1	0.1	0.1
2073	1,10-diepicubenol	0.1									
2080	cubenol		0.1	0.1	0.1		0.1	0.1		0.1	
2081	humulene epoxide III								0.1		
2088	1-epi-cubenol		0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.2	0.1
2098	globulol	0.2	0.2	0.2	0.1	0.1	0.1	0.4	0.2	0.2	0.1
2100	4-hydroxy-4-methylcyclohex-2-enone	0.3	0.2	0.3	0.2	0.3				0.2	0.2
2113	cumin alcohol	0.4	0.2	0.3		0.1		tr	0.3	0.1	0.2
2144	spathulenol	0.6	0.9	1.2	0.9	0.6	0.5	0.7	1.1	1.0	0.7
2184	*cis*-*p*-menth-3-ene-1,2-diol	0.1	0.1	0.1	0.1	0.1	0.2	0.3	0.3	0.3	0.3
2209	τ-muurolol	0.2	0.3	0.4		0.2	0.2	0.1	0.3	0.1	0.2
2235	α-muurolol	0.1	0.1	0.1		0.1	0.1	tr	0.1	tr	0.1
2239	carvacrol					tr		0.2	tr	0.2	

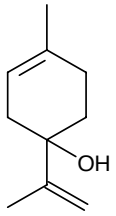
RRI	Compound	May	Jun	Jul	Sep	Oct	Nov	Dec	Feb	Mar	Apr
2239	τ-cadinol	0.1	0.2	0.2	0.1	0.1	0.1	0.1	0.2	0.1	0.2
2254	citronellic acid				0.1		tr		tr	0.1	
2255	α-cadinol	0.5	0.9	1.1	0.2	0.5	0.4	0.4	0.8	0.5	0.3
2256	4-hydroxycryptone	0.2	0.2	0.1		0.1	tr				0.1
2312	(*E*)-2,6-dimethyl-1,3,7-nonatriene									tr	0.1
2343	10-hydroxycalamenene		0.1	0.1		tr					
2392	caryophyllenol II				0.1					tr	
2713	tetradecanoic acid					tr					
Total		**91.3**	**93.7**	**92.6**	**95.9**	**95.3**	**95.0**	**94.1**	**91.5**	**96.5**	**95.7**



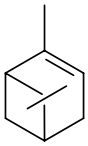
sabinene



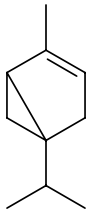
p-cymene



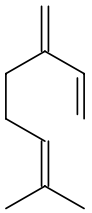
terpinen-4-ol



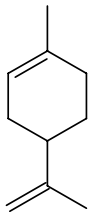
α-pinene



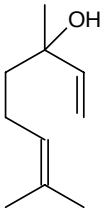
α-thujene



myrcene

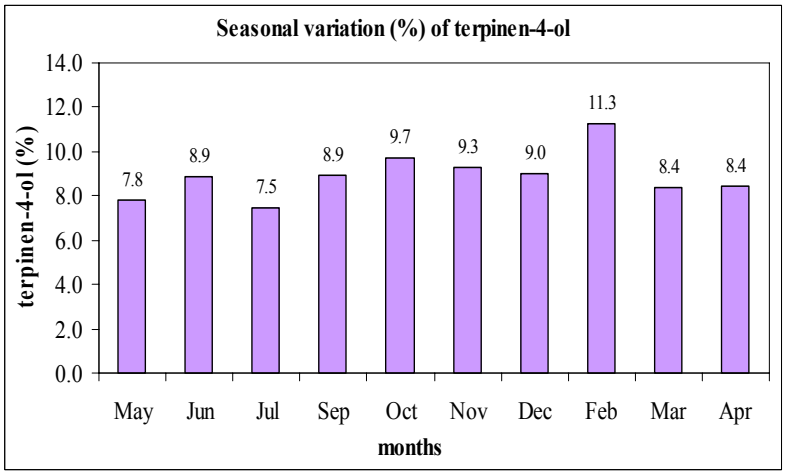


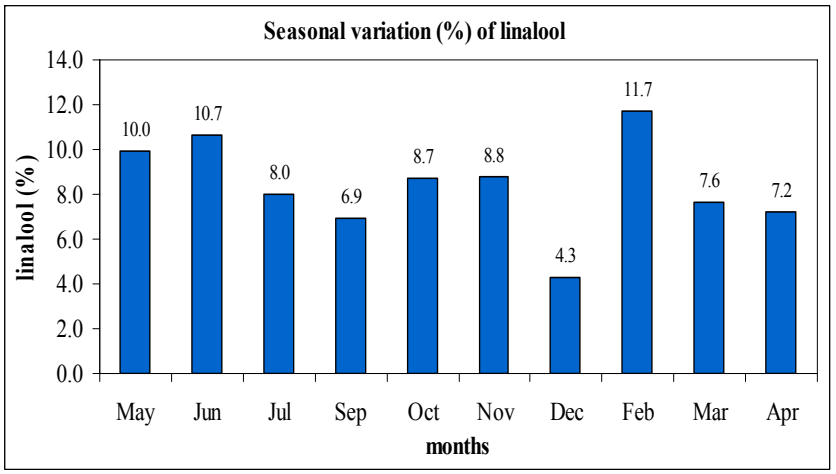
limonene

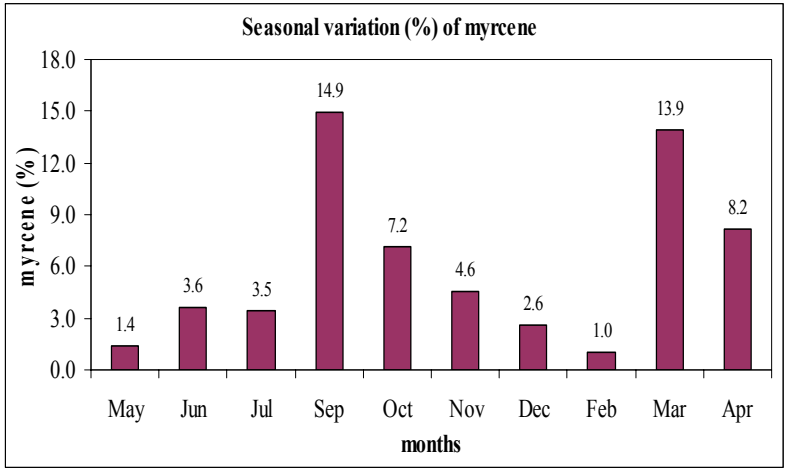


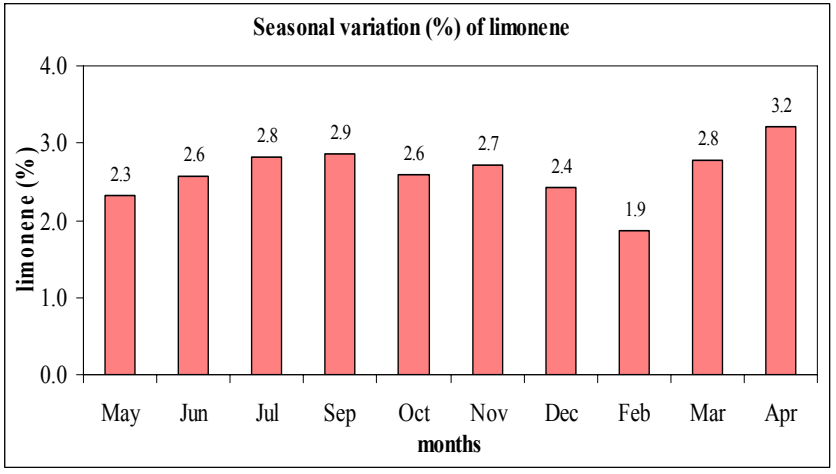
linalool

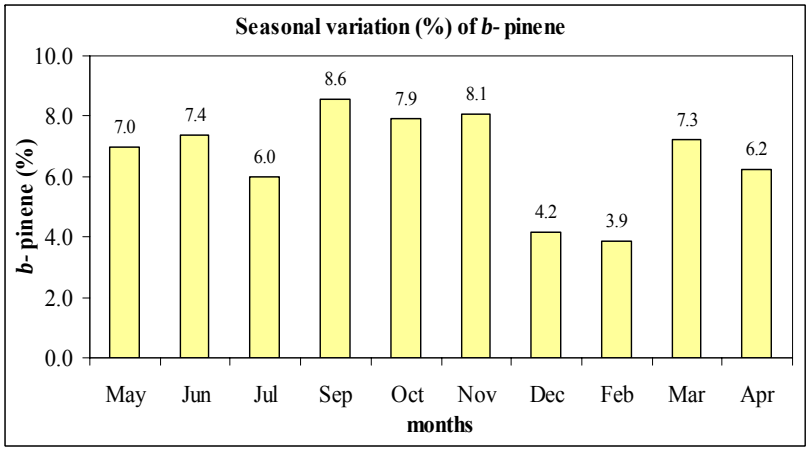
Figure 6.3: Structures of the major compounds present in the essential oils of *Agathosma ovata*.

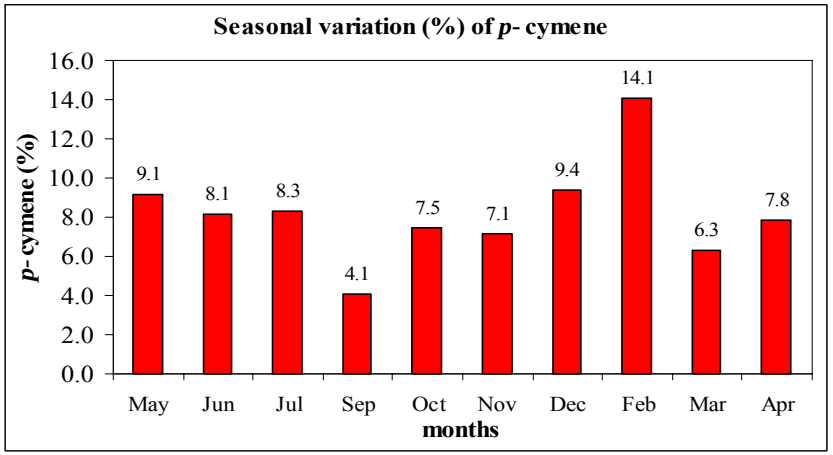

Seasonal variation (%) of terpinen-4-ol
terpinen-4-ol (%)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
7.8
8.9
7.5
8.9
9.7
9.3
9.0
11.3
8.4
8.4
May
Jun
Jul
Sep
Oct
Nov
Dec
Feb
Mar
Apr
months


Seasonal variation (%) of linalool
linalool (%)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
10.0
10.7
8.0
6.9
8.7
8.8
4.3
11.7
7.6
7.2
May
Jun
Jul
Sep
Oct
Nov
Dec
Feb
Mar
Apr
months


Seasonal variation (%) of myrcene
myrcene (%)
18.0
15.0
12.0
9.0
6.0
3.0
0.0
1.4
3.6
3.5
14.9
7.2
4.6
2.6
1.0
13.9
8.2
May
Jun
Jul
Sep
Oct
Nov
Dec
Feb
Mar
Apr
months


Seasonal variation (%) of limonene
limonene (%)
4.0
3.0
2.0
1.0
0.0
2.3
2.6
2.8
2.9
2.6
2.7
2.4
1.9
2.8
3.2
May
Jun
Jul
Sep
Oct
Nov
Dec
Feb
Mar
Apr
months





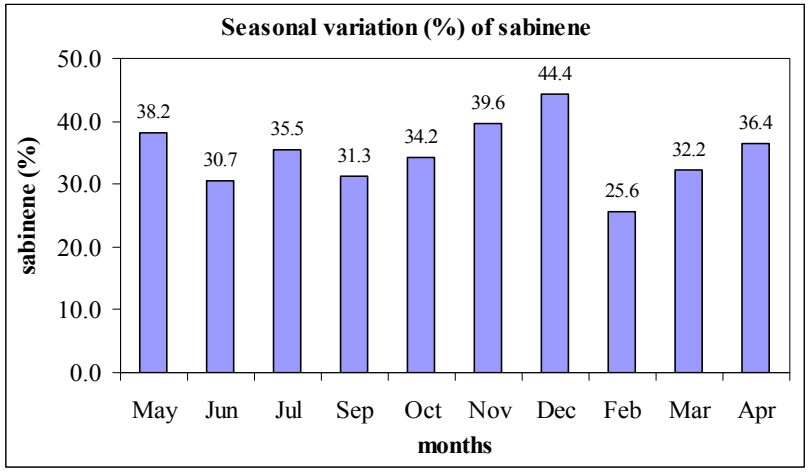


Figure 6.4: Graphs displaying the seasonal variation in the composition (%) of the major constituents of *Agathosma ovata*.

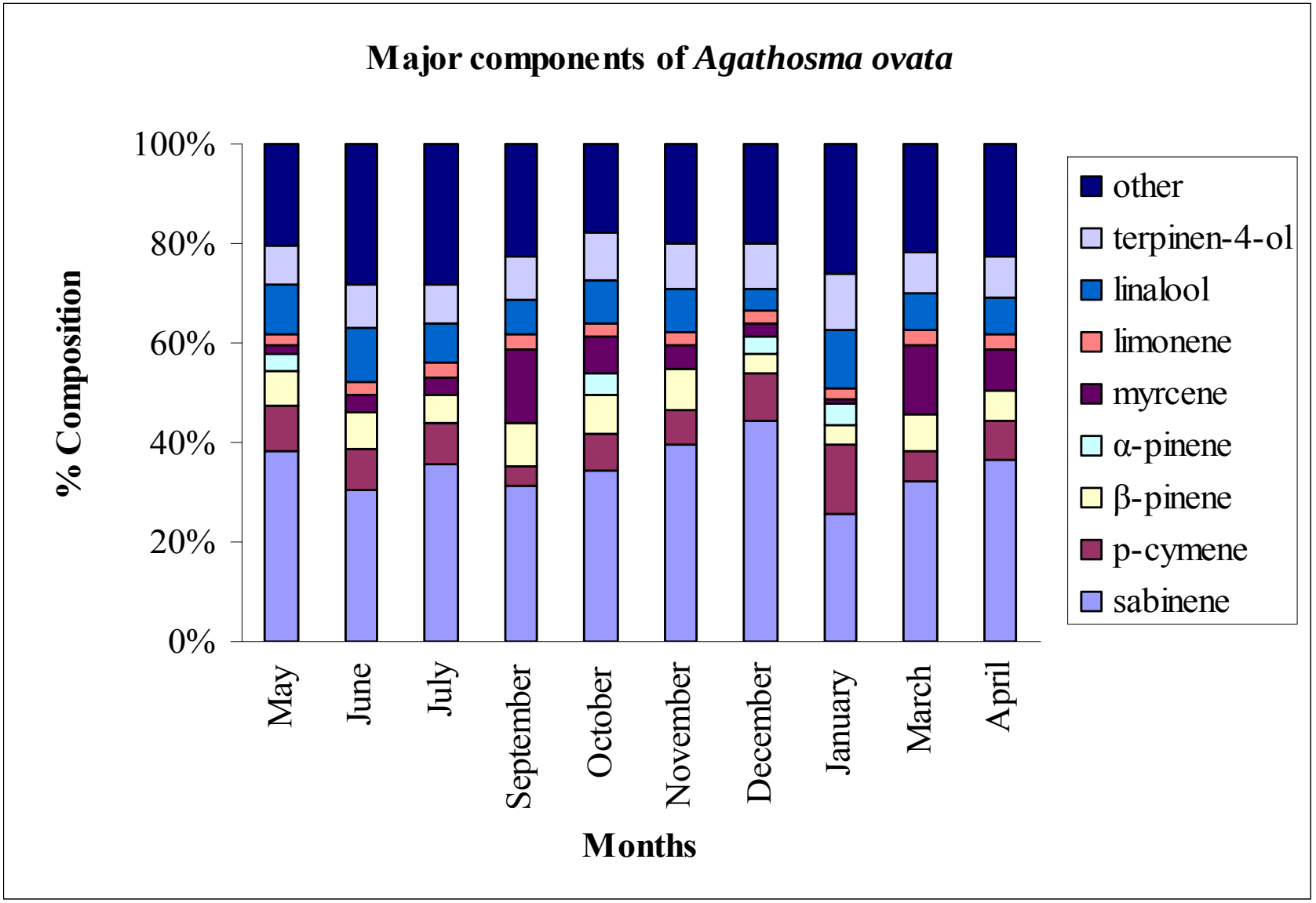


Figure 6.5: Bar graph displaying the major components in the essential oils of *Agathosma ovata*.

In a study performed by Perry *et al*., (1999), the flowering parts of *Salvia officinalis* were found to have higher levels of β-pinene and lower thujone levels of than the non-flowering parts. The same trend was observed in this study. The highest β-pinene level occurred during the flowering season (September, 8.6%). α-Thujone was not detected during the flowering season, while β-thujone levels were lower during this season (Table 6.1).

With regards to α-pinene, a decrease occurred during the Autumn months (5.1% to 2.6%). A similar effect was observed in the leaf oil of *Juniperus oxycedrus* ssp. *badia* in a study performed by Salido *et al*. (2002). The authors report that components such as β-pinene, myrcene, limonene, α-humulene and δ-cadinene increased from Winter to Autumn. A comparable effect was observed for limonene. Myrcene levels peaked in September (14.9%) and March (13.9%), while β-pinene levels peaked in September (8.6%). α-Humulene was only detected in September (tr), whilst δ-cadinene levels peaked in September (0.9%) and March (0.7%). Germacrene D was found to significantly increase in Autumn in the essential oil of *Juniperus oxycedrus* ssp. *badia*, while in this study it was only detected in Spring (tr).

The composition of the essential oil of sage has been found to change under the influence of temperature (Avato *et al*., 2005). Monoterpenes like limonene, 1,8-cineole and β-phellandrene have shown a negative correlation with temperature (Palá-Paúl *et al*., 2001). Similarly the lowest levels of linalool (4.3%), myrcene (1.0%), β-pinene (3.9%), limonene (1.9%) and sabinene (25.6%), occurred during the Summer months when the

temperatures were high. This is evidence that temperature affected the chemical composition of the essential oils and a negative correlation was observed.

According to Palá-Paúl *et al*. (2001), the compounds found in essential oils can be classified into one of the three following categories, according to the correlation between their concentration values and the temperature: 1) constituents whose variations during the whole year seem to be random and are not influenced by the climatic conditions, 2) constituents that present percent concentration that is more or less constant, such as α-thujene, camphene and 2-methyl-3-buten-2-ol (Table 6.1), and 3) constituents influenced by temperature, such as limonene. These results can be explained by supposing that *Agathosma ovata* varies its secondary metabolite production according to the climatic conditions.

A study performed by Kim *et al*. (2005), involved determining the seasonal variation of monoterpene emission from coniferous species. It was found that the total emission rates and the components of monoterpene varied significantly with species, age and season. Higher monoterpene emission rates were found in Spring and Summer as compared to Autumn and Winter emissions. Monoterpene emissions from coniferous plants are reported to be mainly temperature dependant and in the study they were found to increase with temperature (Kim *et al*., 2005). The composition ratios of all monoterpene components from the plants varied with season. In another study, total emission rates were investigated for different seasons, ages and leaf moistures; the highest emission rates occurred during the Spring months (Kim, 2001). Kim (2001) suggested bud

elongation during Springtime and terpene pool variations as possible explanations for these findings.

A study performed by Adams (1970) on the seasonal variation of terpenoid constituents in natural populations of *Juniperus pinchotti* Sudw. revealed that significant differences occurred from Summer to Winter in the relative composition of the terpenoids. The Summer collections were more variable than the Winter collections which indicated the desirability of Winter sampling when practical.

Flowering may not be the only reason for the seasonal changes in the composition of the essential oil of *Agathosma ovata*. There is a Springtime increase in the levels of β-pinene, terpinen-4-ol, linalool, sabinene, limonene and *p*-cymene in the non-flowering *Agathosma ovata*. These changes could be due to the higher proportion of young leaves during Spring, and young leaves may have oil compositions slightly different to those of mature leaves (Langer *et al*., 1993).

The Febuary sample contains various compounds that are present in small quantities and that are unique to this sample (Table 6.1). Some of these include: octanal (tr), 3-octen-1-one (tr), 6-methyl-5-hepten-2-one (0.1%), nonanal (tr), 1,3,8-*p*-menthatriene (tr), naphthalene (tr), *p*-menthatriene isomer (0.1%), salvial-4(14)-en-1-one (tr) and α-phellandrene (0.1%). The compound allo-ocimene (0.1%) was only detected in the September sample and 1-hexanol was confined to the October sample. The compounds thuja-2,4(10)-diene and 1,4-cineole were only detected in two samples during the non-

flowering season. Some compounds that were absent during the flowering season include: dehydro-1,8-cineole, 1,4-cineole, (*E*)-β-ocimene epoxide and carvacrol. The samples of the flowering season are very similar in composition and the same applies to the samples of the non-flowering season.

The April sample has the largest quantity of limonene (3.2%) and β-phellandrene (2.5%). The November and December samples are unique in that they are the only samples that contain the compound dihydroedulane II (tr) (Table 6.1).

A rare thiol derivative (tr) that could not be identified was detected in the March sample, in lower quantities than other sulphur containing compounds that have been detected in various *Agathosma* species (Table 6.1). The remainder of the essential oil components did not show any noticeable trend.

Many of the changes in the study were associated with flowering. They can be explained by the different compositions of the flower and leaf oils. However, flowering is not the only reason for seasonal changes in the composition of *Agathosma ovata*. The changes could also be due to the higher proportions of young leaves in Spring, which may have oil compositions different to those of adult leaves.

There are no data available in literature on the relationship of the seasonal profile documented for any morphological differentiation through the year in *Agathosma ovata*, nor any other related *Agathosma* species, and this can be regarded as the first. Moreover

there are no available data concerning the phytogeographical patterns in seasonal profile of any *Agathosma* species. The results obtained reveal that the chemical composition of the essential oil of *Agathosma ovata* is subject to seasonal variation.

6.3.3. Minimum inhibitory concentration assay

The results obtained from the antimicrobial assay are depicted in Table 6.2 below. Most of the samples had MIC values of 8mg/ml. The MIC values varied between 4mg/ml and 8mg/ml for *Bacillus cereus*. Although the essential oils were active against all of the pathogens tested, they were most active against the Gram-positive organism *Staphylococcus aureus*, with the June, July and October samples having MIC values of 1.5mg/ml, 2mg/ml and 3mg/ml.

The December and March samples displayed poor activity against *Staphylococcus aureus* (MIC values of 12mg/ml and 14mg/ml). Overall, the MIC values for *Staphylococcus aureus* varied largely between 1.5mg/ml and 14mg/ml. The July sample was most active against the Gram-negative pathogen, *Klebsiella pneumoniae* (MIC value of 3mg/ml), while the December sample was least active (MIC value of 16mg/ml). The July and October samples were the only two having MIC values of 12mg/ml against the yeast *Candida albicans*.

Table 6.2: MIC results (mg/ml) of the antimicrobial activity of the essential oils of *Agathosma ovata*.

Month	***B. cereus*** **ATCC 11778**	***S. aureus*** **ATCC 12600**	***K. pneum-oniae*** **NCTC 9633**	***C. albicans*** **ATCC 10231**
May	8	8	8	8
June	4	1.5	4	8
July	8	2	3	12
September	8	8	8	8
October	8	3	4	12
November	8	8	8	8
December	8	12	16	8
February	8	8	8	8
March	4	14	8	8
April	8	8	8	8
Controls	6.25×10^{-4}	3.125×10^{-4}	6.25×10^{-4}	3.125×10^{-4}

Controls = ciprofloxacin for the bacteria and amphotericin B for the yeast

(at a starting concentration of 0.01mg/ml).

n = 3

The study demonstrates differences in the potency of antimicrobial activity of the essential oils distilled each month. The Winter samples were more active against the Gram-positive bacteria *Bacillus cereus*, *Staphylococcus aureus* and the Gram-negative bacterium *Klebsiella pneumoniae* as compared to the other samples. Activity in mid Spring also seemed to be greater against *Staphyloccocus aureus* and *Klebsiella pneumoniae*, whilst activity decreased in Summer (Table 6.2). *Bacillus cereus* and *Candida albicans* were not drastically affected.

Earlier studies have shown that plants are known to display variation in the concentration of bioactive phytochemicals depending on intrinsic factors like the age of the plant, its parts used and extrinsic factors like the geographical climate, circadian rhythm, the nature of the soil, season and processing (Jagetia and Baliga, 2005). The concentration of vitamin C, tocopherols, and tocotrienols have been reported to exhibit changes with seasons in *Hippophae rhamnoides* (Kallio *et al*., 2002). The flavonoid and phenolic contents in spinach have also been reported to vary with season (Howard *et al*., 2002). The isoflavonoids and astragalosides have been reported to vary according to season and age of the plant in *Astragalus membranaceus* var. *mongholicus* (Ma *et al*., 2002). Seasonal variation in the quantity of the phytochemicals in plants with anticancer activity like *Crinum macowanii*, *Taxus baccata*, *T. wallichiana* and *T. brevifolia* have also been reported (Wheeler *et al*., 1992; Vance *et al*., 1994; Glowniak *et al*., 1999, Mukherjee *et al*., 2002).

Overall the antimicrobial activity of the essential oil of *Agathosma ovata* is subject to seasonal variation (Table 6.2). The results reveal that there is a correlation between the concentrations of the active compounds and the antimicrobial activity. From the study, it is clear that the antimicrobial activity of the essential oil of *Agathosma ovata* may not depend on the level of one component but rather the ratio of several components.

CHAPTER 7: ANTI-OXIDANT ACTIVITY

7.1. Introduction

'Buchu' has been an important part of the San and Khoi culture in the Cape and is still used as a general tonic and medicine throughout South Africa. Tonics generally have a high anti-oxidant content in order to promote the overall well-being of the user. Due to the vast traditional use of buchu and the lack of scientific evidence the anti-oxidant activity of selected *Agathosma* species was investigated.

7.1.1. Free radicals, their formation and mechanism of action

Biological reduction of molecular oxygen in cells is accompanied by the production of dangerously reactive free radical and non-radical oxygen species (Figure 7.1). Because of the ubiquity of molecular oxygen and its ability to accept electrons, their production is readily associated with damage. The superoxide radical (O_2^-) is the first univalent reduction product of oxygen, which by dismutation via the enzyme superoxide dismutase (SOD) is transformed to hydrogen peroxide (H_2O_2), which can easily penetrate the membranes of surrounding cells, whereas O_2^- usually cannot. In the presence of ions of a suitable transition metal (usually iron), H_2O_2 can interact with the reduced form of the metal ion to form several highly oxidizing species, the most important of which is probably the hydroxyl radical ($OH^{\cdot}$). It is so reactive that it will combine with whatever molecules are present at or close to its site of formation. Reactive oxygen species when stimulated in the environment of critical biomolecules such as deoxyribonucleic acid (DNA), lipids, proteins and carbohydrates, promote oxidative damage (Dowling *et al*., 1990).

Free radicals are involved in several normal biological processes *in vivo*. Superoxide radicals and other active oxygen species are products of the action of oxidases such as xanthine oxidase, NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) oxidase, etc. and are generated by a variety of cells to perform useful functions in the body. For example, they are part of the cascade of events in the antimicrobial action of phagocytic cells via NADPH oxidase, in the arsenal of defense cells with which the human body is equipped (Rice-Evans, 1994). The reaction products of superoxide ions are believed to be partly responsible for the removal and destruction of bacteria and damaged cells (Rice-Evans, 1994).

Free radicals can act as regulatory molecules in biochemical processes; for example, lymphocytes and fibroblasts constantly generate small amounts of superoxide radicals as growth regulators. Nitric oxide from endothelial cells is involved in the regulation of vascular tone, including the relaxation of smooth muscle cells. Biological damage resulting from either non-oxy free radicals ($R^{\cdot}$), or (oxyl alternatively) radicals ($^{\cdot}OH$, $RO^{\cdot}$, $ROO^{\cdot}$, O_2^{-}), may be inhibited by suitable scavengers (Figure 7.1).

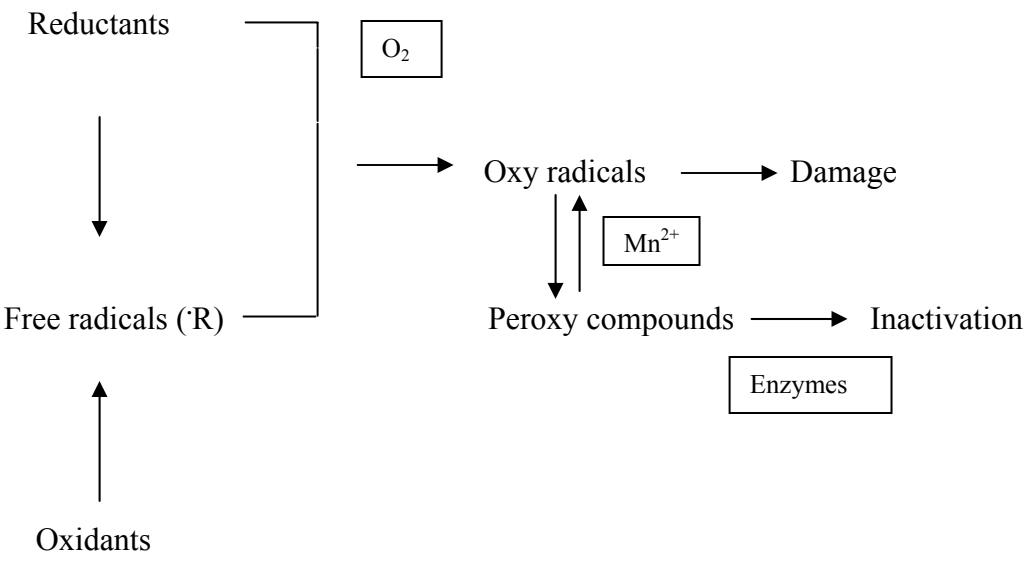


Figure 7.1: Generation of free radicals and their relationship to oxy (oxygen) radicals.

7.1.2. Free radicals and their role in the inflammatory response

Oxygen radicals have been implicated in a host of commonly occurring diseases which possess an inflammatory component, including rheumatoid arthritis, atherosclerosis, pulmonary emphysema, cancer, inflammatory bowel disease and periodontal disease. Radicals are also implicated in the normal course of ageing. A wide variety of oxidized biomolecules, known to be specific products of free radical reactions, have been detected in extracellular fluids from patients with these inflammatory conditions (Winyard *et al*., 1994).

In biological systems, oxygen is reduced to reactive oxygen intermediates (ROI) by a wide variety of both enzymatic and non-enzymatic pathways, as a result of normal metabolic pathways. It is also thought that in acute or chronic inflammation, one

pathogenic factor might cause the disruption of normal metabolic balance between production and removal of oxygen radicals, leading to cell damage (Winyard *et al*., 1994). Some pathways involving free radicals as second messengers in inflammation include: (1) Vasodilation: The free radical nitric oxide is an important factor in bioregulation. Its formation is essential for the cytotoxicity of activated macrophages against tumour cells and protozoa. (2) Fibrosis: An inflammatory response marked by infiltration of tissues by neutrophils, monocytes and macrophages is a prerequisite for fibrosis in major body organs and there is evidence that ROI released from such cells are vital factors in this process. Alveolar macrophages activated with agents known to produce fibrosis in man, i.e., silica, coal, dust or asbestos, are able to release ROI such as $\cdot O_2^-$, H_2O_2 and $\cdot OH$ (Winyard *et al*., 1994). Low concentrations of ROI, particularly H_2O_2, are known to increase replication rates in tissue fibroblasts. (3) Gene transcription: Recently it has been shown that oxidant stress can induce the expression and replication of human immunodeficiency virus-1 (HIV-1) in the human T cell line. The effect was shown to be mediated by a transcription factor, which was potently and rapidly activated by exposure of target cells to H_2O_2 (Winyard *et al*., 1994).

According to Dowling *et al*. (1990), the involvement of reactive oxygen species in promoting the inflammatory processes *in vivo* are evident by:

- the measurement of products by peroxidative decomposition of lipid (principally malondialdehyde as thiobarbituric acid (TBA) reactive material) at the site of injury, and also by the detection of ethane and pentane, a non-invasive indicator of *in vivo* lipid peroxidation in exhaled air

- the detection of modified protein, characteristic of radical damage
- the measurement of high levels of free radical activity, as intense luminal-amplified chemiluminescence (LAC) from the inflamed site.

7.1.3. Definitions of anti-oxidants

An anti-oxidant may be defined as any substance which can delay or prevent the oxidation of a substrate when it is present in small amounts relative to the amount of substrate. Halliwell *et al*. (1993) considered that anti-oxidants act at several different levels in the oxidative sequence, and that they may have multiple mechanisms of action. With respect to lipid peroxidation, they consider five different mechanisms of action:

- decreasing localized oxygen concentrations
- preventing chain initiation by scavenging initiating radicals
- binding catalysts such as metal ions to prevent initiating radical generation
- decomposing peroxides so that they cannot be reconverted to initiating radicals
- chain breaking to prevent continued hydrogen abstraction by active radicals.

7.1.4. Mechanisms of anti-oxidant action *in vivo*

Two large categories of substances can be distinguished that afford protection against oxidative attack by superoxide radical ions, hydrogen peroxide, hydroxyl radicals, singlet oxygen or its successor radicals, namely; enzymes and low molecular weight anti-oxidants (of which vitamins are an important subgroup). Anti-oxidant protection can be viewed as consisting of three sequential levels of defensive activity which are most clearly understood in the mechanism of lipid peroxidation. The first level of defense,

which is largely enzymatic, involves the activity of enzymes which depends principally on trace amounts of the minerals manganese, copper, zinc and selenium; it is concerned with the control of formation and proliferation of primary radical species derived from molecular oxygen (Diplock, 1996). The protective mechanism as a whole is thus dependant on the supply from dietary sources of certain specific minerals and nutrients and it is thus susceptible to being compromised by the failure to supply through the diet one or more of these essential substances which have been given the name 'anti-oxidant nutrients' (Diplock, 1996). The second, which involves the vitamins C and E, and probably the carotenoids, is concerned with the prevention of the proliferation of secondary radicals in chain reactions such as lipid peroxidation, initiated and driven by primary radicals. The third level is the enzymatic prevention of formation of secondary radicals from chain-terminated derivatives and enables removal of such molecules from an environment in which metal-catalyzed reactions might cause further oxidative damage (Diplock, 1996). The containment by enzymatic means of the initial process is thus a level of defense against free radical damage (Diplock, 1996). The enzymes involved include; superoxide dismutase, catalase and glutathione peroxidase.

7.1.5. Flavonoid containing plants as anti-oxidants

Traditional medicine all over the world is nowadays revalued by an extensive activity of research on different plant species and their therapeutic principles. As plants produce anti-oxidants to control the oxidative stress caused by sunbeams and oxygen, they can represent a source of new compounds with anti-oxidant activity (Scartezzini and Speroni, 2000).

Flavonoids are a group of polyphenolic compounds diverse in chemical structure and characteristics. They occur naturally in fruit, vegetables, nuts, seeds, flowers and bark and are an integral part of the human diet (Cook and Samman, 1996). The have been reported to exhibit a wide range of biological effects, including antibacterial, antiviral, anti-inflammatory, anti-allergic and vasodilatory actions. Over 4000 types of flavonoid compounds have been identified in vascular plants and these vary in type and quantity due to variations in plant growth, conditions and maturity. Only a small number of plant species have been examined systematically for their flavonoid content. Flavonoid aglycones, members of a ubiquitous class of phenols, have often been proposed to act as anti-oxidants. More recently this activity has been specifically attributed to their radical-scavenging capabilities (Wolf *et al*., 1990). Flavonoids inhibit lipid peroxidation *in vitro* at the initiation stage by acting as scavengers of superoxide ions and hydroxyl radicals. It has been proposed that flavonoids terminate chain radical reactions by donating hydrogen atoms to the peroxy radical forming a flavonoid radical. The flavonoid radical in turn reacts with free radicals thus terminating the propagating chain. In addition to their anti-oxidative properties, some flavonoids act as metal-chelating agents and inhibit the superoxide driven Fenton reaction, which is an important source of active oxygen radicals (Cook and Samman, 1996). It has been reported that flavonoid compounds have two to five fold greater anti-oxidant and free radical scavenging activities than vitamins C and E on an equimolar basis (Du Toit *et al*., 2001). Much evidence suggests that peroxidation of low density lipoproteins (LDL) is positively associated with atherogenesis (Cook and Samman, 1996). It has been reported that phenolic compounds (including flavonoids and non-flavonoid polyphenols) isolated from red wine inhibit

copper catalyzed oxidation of LDL *in vitro*. It is postulated that the anti-oxidant and free radical scavenging properties of phenolic compounds, present in red wine, may partly explain the anomaly observed in coronary heart disease rate between the French population who consume wine regularly and have rates of coronary heart disease lower than other populations despite similar fat intakes (Cook and Samman, 1996).

Several *in vitro* analytical tools can be used to characterize the anti-oxidant propensity of bioactive compounds in plants. For example, the oxygen radical absorbance capacity, ferric reducing anti-oxidant power, total oxidant scavenging capacity, the deoxyribose assay, assays involving oxidative DNA damage, assays involving reactive nitrogen intermediates (e.g. $ONOO^-$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalent anti-oxidant capacity, the 2, 2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay and the 2, 2-diphenyl-1-picrylhydrazyl assay (DPPH) assay (Aruoma, 2003).

7.2. Materials and methods

Two assays were utilized in order to evaluate the anti-oxidant activity of indigenous *Agathosma* species i.e. the DPPH assay and the ABTS assay.

7.2.1. Rationale for two assays

Due to the complexity of the oxidation-anti-oxidation processes, it is obvious that no single testing method is capable of providing a comprehensive picture of the anti-oxidant profile of a studied sample. Preliminary studies confirm that a multi-method approach is

necessary in the anti-oxidant activity assessment. A combination of rapid, sensitive and reproducible methods preferably requiring small sample amounts should be used. A rapid estimation of radical scavenging abilities by using the DPPH or superoxide inhibition method could save much laboratory work and provide preliminary information about screened samples, giving a basis for further isolation procedures. The DPPH reagent has also been reported to be more stable than the ABTS reagent (Du Toit *et al*., 2001). A number of assays have been introduced for the measurement of the total anti-oxidant activity of samples. Each method relates to the generation of a different radical, acting through a variety of mechanisms, and the measurement of a range of endpoints at a fixed time or over a range. Two approaches have been taken, namely, the inhibition assays in that the extent of the scavenging by hydrogen- or electron- donation of a pre-formed free radical is the marker of anti-oxidant activity, as well as assays involving the presence of anti-oxidant systems during the generation of the free radical.

7.2.2. The 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) assay

7.2.2.1. Principle

The method involves using a stable free radical DPPH with a dark violet colour, whereby anti-oxidants are allowed to react with the stable radical in methanol solution. Anti-oxidant compounds donate electrons to DPPH, resulting in decolourisation which is stoichiometric with respect to the number of electrons captured by DPPH (Figure 7.2). The reduction in the concentration of the DPPH radical is followed by monitoring the decrease in its absorbance at a characteristic wavelength during the reaction. In its radical form, DPPH absorbs at 515 nm, but upon reduction by an anti-oxidant or a radical

species, the absorption disappears. The anti-oxidant activities are determined using DPPH as a free radical and the antiradical activity is defined as the amount of anti-oxidant necessary to decrease the initial DPPH concentration by 50% (Aruoma, 2003). There is a need to agree governance on *in vitro* anti-oxidant methods based on the mechanisms involved. Generally a combination of these assays should be used in assessing these activities (Aruoma, 2003).

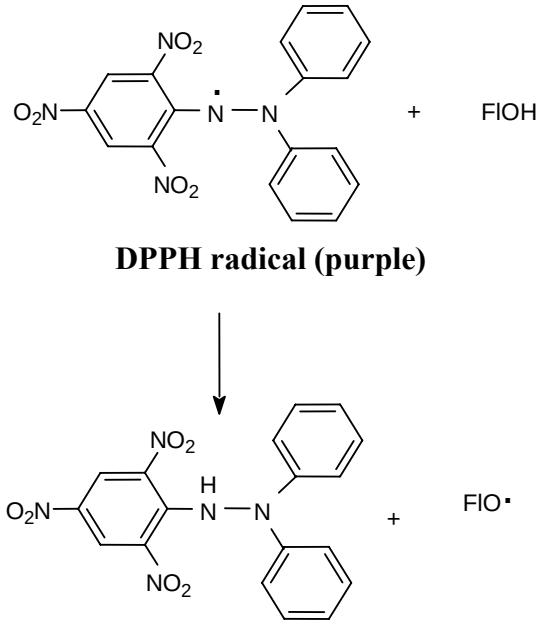


Figure 7.2: Reduction of the DPPH radical.

7.2.2.2. Thin layer chromatography

Thin layer chromatography was performed as a preliminary screening on silica gel 60 TLC plates (Alugram®, Germany). Dichloromethane and methanol extracts (1:1) of a

concentration 50mg/ml, were spotted individually onto a baseline drawn 1cm away from the bottom end of the TLC plate. Approximately 3µl of each extract was spotted. Rosmarinic acid was used as a standard since it is a potent anti-oxidant compound. The essential oils were diluted with hexane (1:7) and also spotted in the similar manner. The essential oil plate was developed in a TLC solvent system consisting of toluene and ethyl acetate (9.3: 7). The extract plate was developed in a TLC solvent system consisting of methanol, water, acetone, ethyl acetate and chloroform (1: 0.8: 3: 4: 1.2). Once the plates were dry, they were sprayed with a 0.4mM double strength DPPH solution and the colour changes were observed. The DPPH test performed directly on the TLC plates can be informative because it reveals contributions of different compounds separately, to the total anti-oxidant activity.

7.2.2.3. Spectrophotometric method

A 96µM DPPH (Fluka) solution was prepared in HPLC grade methanol and kept at 4°C in the dark. Test extracts and essential oils (10mg/ml) were dissolved in dimethyl sulfoxide (DMSO) (Saarchem) to obtain a stock solution of 10 000µg/ml. For the first dilution 50µl of the 10 000µg/ml stock solution was added to 950µl DMSO to obtain a concentration of 500µg/ml but a final concentration of 100µg/ml in the well. Serial dilutions (1:1) were thereafter performed using DMSO. Using a 96-well microtitre plate, 50µl of the initial stock solution and serial dilutions were plated out in triplicate from rows B to G. DMSO (50µl), for control purposes was plated out in rows A and H. HPLC grade methanol (200µl) was added to columns 2, 4, 6, 8, 10 and 12 while an equal volume of DPPH solution was plated out in columns 1, 3, 5, 7, 9, and 11. The microtitre

plate was then shaken for two min and left to stand in the dark at room temperature for 30 min. The absorbance was then read at 550 nm using a UV-VIS spectrophotometer (Labysystems Multiskan RC) linked to the computer equipped with GENESIS® software. The percentage decolourisation (free radical scavenging activity) of the test compound was calculated using the equation below. The IC_{50} values were calculated using Enzfitter® version 1.05 software. Vitamin C (ascorbic acid) was used as a positive control.

% decolourisation = 100 x (Abs contr – Av test Abs + Av Abs methanol) / Abs contr

Abs contr = Av Abs DPPH – Av Abs methanol

Av test Abs = Mean absorbance obtained in the well containing DPPH

Av Abs methanol = Mean absorbance obtained in the wells containing methanol

Abs = absorbance; contr = control; Av = average

7.2.3. The 2, 2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

7.2.3.1. Principle

The quenching of the ABTS radical cation ($ABTS^{\cdot+}$) forms the basis of a spectrophotometric method that allows the evaluation of both water soluble and lipid soluble anti-oxidants. The pre-formed $ABTS^{\cdot+}$ is generated by oxidation of ABTS with potassium persulfate ($K_2S_2O_8$) (Figure 7.3) and is reduced in the presence of such hydrogen-donating anti-oxidants. The influences of both the concentration of anti-oxidant and duration of reaction on the inhibition of the radical cation absorption are taken into account when determining the anti-oxidant activity. The method is a decolourisation

assay that results in the conversion of the colourless ABTS into the blue green $ABTS^{\cdot+}$. After the addition of an anti-oxidant, the reduction in absorbance at 734 nm of the $ABTS^{\cdot+}$ solution is measured, which in turn is proportional to the anti-oxidant concentration and activity calculated, in relation to the reactivity of a standard of Trolox analyzed under the same conditions (Pellegrini *et al*., 2003).

The assay clearly improves the original ferryl myoglobin assay for the determination of anti-oxidant activity in a number of ways. Firstly the chemistry involves the direct generation of the $ABTS^{\cdot+}$ with no involvement of an intermediary radical. Secondly, it is a decolourisation assay; thus the radical cation is pre-formed prior to the addition of an anti-oxidant test system, rather then the generation of the radical taking place continually in the presence of the anti-oxidant. Thirdly, the assay is applicable to both aqueous and lipophilic systems (Re *et al*., 1999).

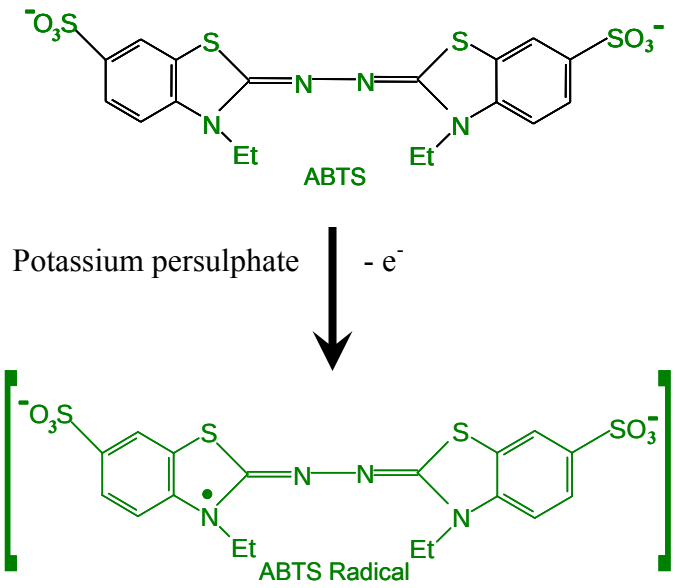


Figure 7.3: Generation of the ABTS radical.

7.2.3.2. Spectrophotometric method

Stock solutions (10mg/ml) of each of the species were prepared in DMSO. The assay was only performed on extracts due to insufficient quantities of the essential oils. Working solutions were prepared at nine different concentrations. A stock solution of Trolox was prepared in ethanol and this was diluted to obtain working solutions. A 7mM ABTS (Sigma Aldrich) stock solution was prepared in double distilled water. The $ABTS^{\cdot+}$ was produced by reacting 5ml of ABTS solution with 88µl of a 140mM potassium persulphate ($K_2S_2O_8$) (Fluka) solution and the mixture was allowed to stand in the dark for 12-16 h in order to stabilize. The radical solution is stable for 2-3 days in the dark.

The day of the assay, the $ABTS^{\cdot+}$ solution was diluted with cold ethanol to obtain an absorbance ranging between 0.68-0.72 at 732 nm in a 1cm cuvette. Ethanol was used as a negative control. The radical scavenging activity was quantified by reacting 1ml of $ABTS^{\cdot+}$ solution with 50µl of sample. The mixture was thereafter heated for four min, after which the absorbance was read at 734 nm on a Specord 40 spectrophotometer. Analysis was done in triplicate. The percentage inhibition was then plotted as a function of the concentration, from which the equation of the straight line was calculated. The concentration that produced 50% decolourisation (IC_{50}) was determined as well as the standard deviation. Trolox was used as a standard.

7.3. Results

7.3.1. Thin layer chromatography

The results from the TLC analysis are depicted in Figures 7.4 and 7.5. The TLC plates revealed that *Agathosma ovalifolia* was the only essential oil that showed distinct activity (Figure 7.4). The anti-oxidant compound appeared as a white spot against the purple background upon being sprayed with the DPPH radical. Most of the extracts showed good activity with the exception of *Agathosma bathii*, *A. betulina*, *A. capensis* (Besemfontein), *A*. *capensis* (Gamka) and *A. pungens* (Figure 7.5).

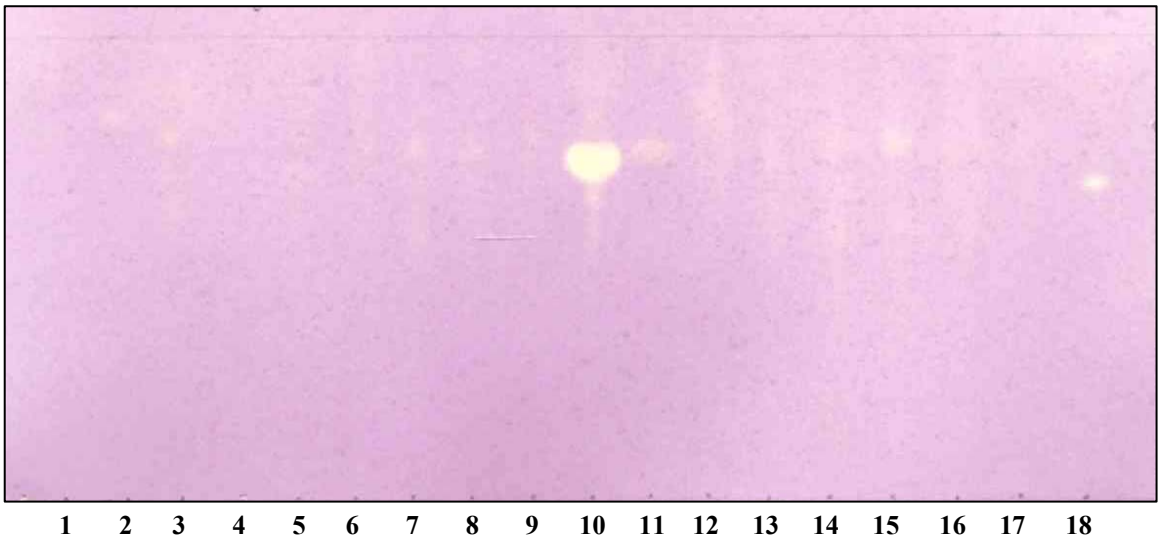
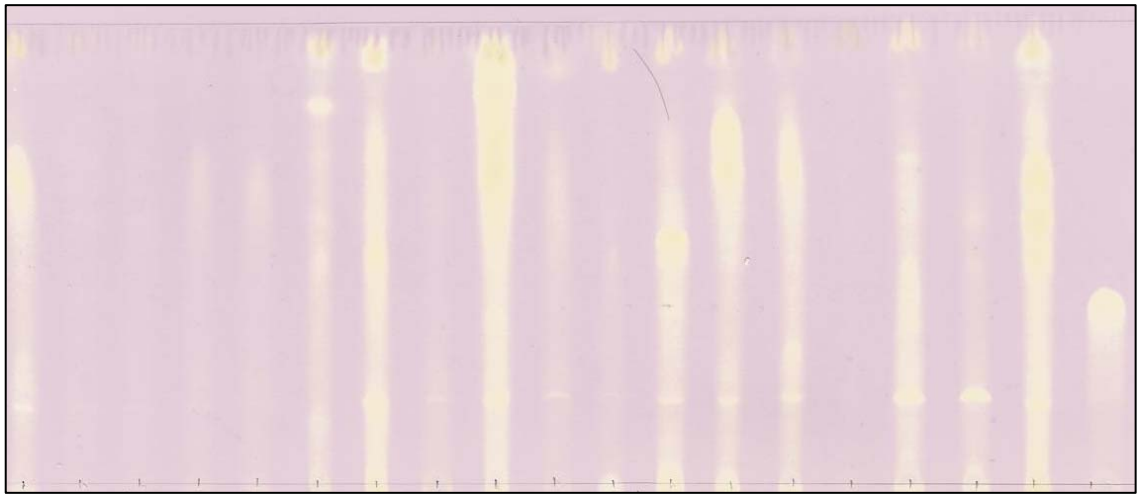


Figure 7.4: TLC screening of anti-oxidant compounds present in the essential oils of *Agathosma* species, using the DPPH spray reagent.

Key to samples:

1. *A. arida*
2. *A. bathii*
3. *A. betulina*
4. *A. capensis* (Besemfontein)
5. *A. capensis* (Gamka)

6. *A. collina*
7. *A. crenulata*
8. *A. hirsuta*
9. *A. lanata*
10. *A. ovalifolia*
11. *A. namaquensis*
12. *A. ovata* (round-leaf)
13. *A. parva*
14. *A. pubigera*
15. *A. pungens*
16. *A. roodebergensis*
17. *A. stipitata*
18. *A. zwartbergense*



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Figure 7.5: TLC screening of anti-oxidant compounds present in the dichloromethane and methanol (1:1) extracts of *Agathosma* species, using the DPPH spray reagent.

Key to samples:

1. *A. arida*
2. *A. bathii*
3. *A. betulina*
4. *A. capensis* (Besemfontein)
5. *A. capensis* (Gamka)
6. *A. crenulata*
7. *A. hirsuta*
8. *A. lanata*

9. *A. namaquensis*
10. *A. ovalifolia*
11. *A. ovata* (hook-leaf)
12. *A. ovata* (round-leaf)
13. *A. parva*
14. *A. pubigera*
15. *A. pungens*
16. *A. roodebergensis*
17. *A. stipitata*
18. *A. zwartbergense*
19. Rosmarinic acid

7.3.2. Spectrophotometry

Table 7.1 summarizes the radical scavenging activity of *Agathosma* species in the DPPH and ABTS anti-oxidant assays. The essential oils showed very poor activity in the DPPH assay, all having IC_{50} values > 100µg/ml. Most of the extracts portrayed moderate to poor activity in the DPPH assay with the exception of *Agathosma capensis* (Gamka) and *A. pubigera* which were two of the most active species in the assay (IC_{50} values of 24.08 $\pm$ 4.42µg/ml and 35.61 $\pm$ 0.86µg/ml), although not as active as vitamin C (IC_{50} value of 2.47 $\pm$ 0.178µg/ml). The results obtained from the ABTS assay differed from that of the DPPH assay. All extracts showed greater activity in this assay with *Agathosma namaquensis* and *A. capensis* (Besemfontein) being the most active species (IC_{50} values of 15.66 $\pm$ 4.57µg/ml and 19.84 $\pm$ 0.09µg/ml), although not as active as Trolox (IC_{50} value of 2.96 $\pm$ 0.001µg/ml). A bar graph comparing the results obtained from both assays is presented in Figure 7.6.

Table 7.1: *In vitro* anti-oxidant activity of indigenous *Agathosma* species.

Species	DPPH IC_{50} values of essential oils (µg/ml)	DPPH IC_{50} values of extracts (µg/ml)	ABTS IC_{50} values of extracts (µg/ml)
A. arida	> 100	40.86 ± 7.84	27.32 ± 0.66
A. bathii		> 100	29.25 ± 0.59
A. betulina		> 100	37.75 ± 0.54
A. capensis (Besemfontein)		30.79 ± 0.43	19.84 ± 0.09
A. capensis (Gamka)		24.08 ± 4.42	29.93 ± 1.04
A. collina		54.65 ± 6.34	39.98 ± 0.36
A. crenulata		> 100	33.32 ± 0.33
A. hirsuta		> 100	38.64 ± 0.25
A. lanata		> 100	26.30 ± 0.25
A. namaquensis		47.25 ± 7.47	15.66 ± 4.57
A. ovalifolia		52.84 ± 2.47	26.25 ± 0.21
A. ovata (hook-leaf)		51.45 ± 4.13	24.71 ± 0.19
A. ovata (round-leaf)		> 100	46.81 ± 1.54
A. parva		72.37 ± 3.06	25.45 ± 0.33
A. pubigera		35.61 ± 0.86	29.94 ± 0.39
A. pungens		94.65 ± 1.65	31.57 ± 0.82
A. roodebergensis		56.71 ± 4.76	29.63 ± 0.32
A. stipitata		> 100	28.20 ± 0.34
A. zwartbergense		> 100	31.73 ± 0.36
Vitamin C and Trolox	2.47 ± 0.178	2.47 ± 0.178	2.96 ± 0.001

n = 1 for essential oils

n = 3 for extracts

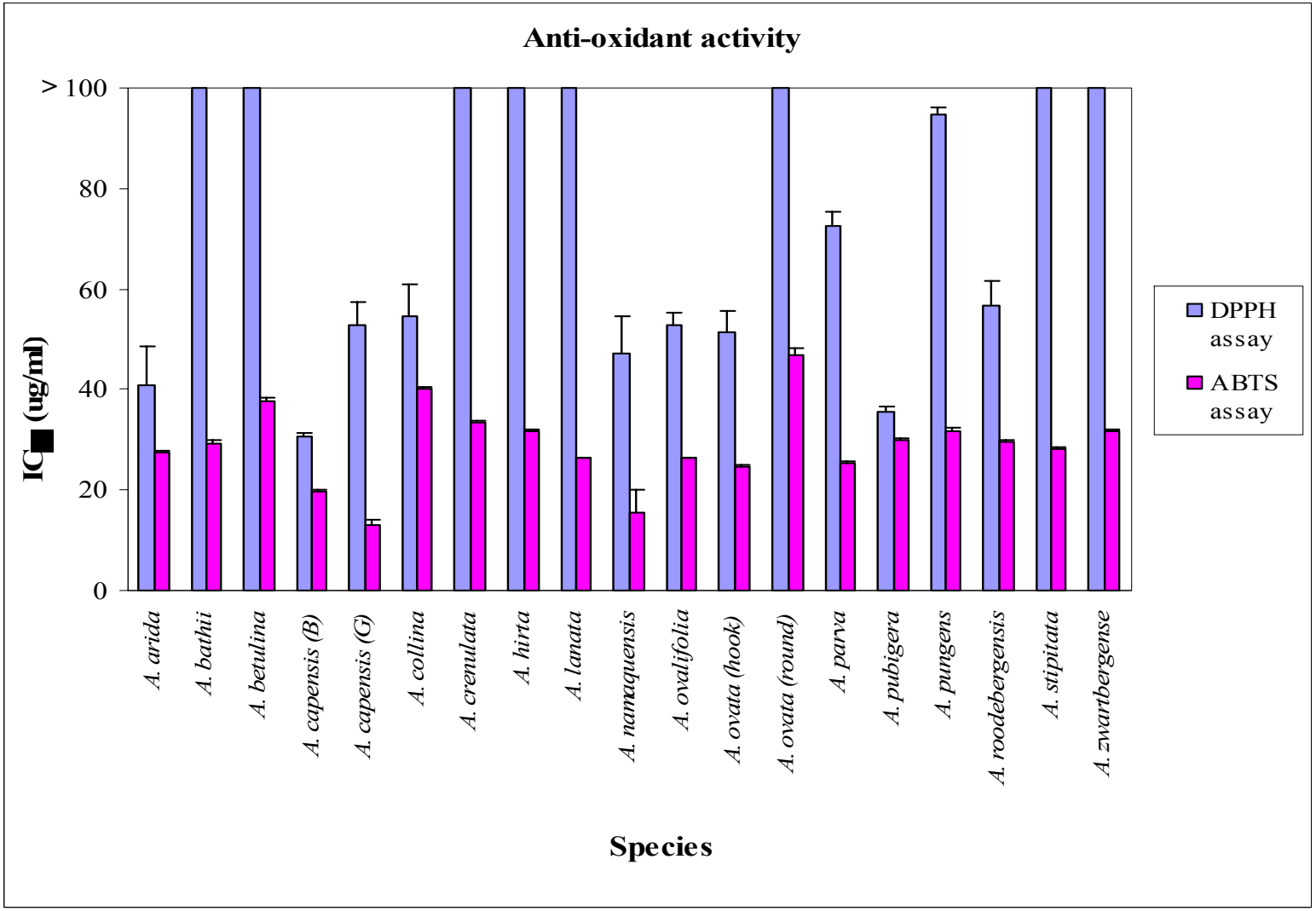


Figure 7.6: Bar graph showing a comparison of the IC_{50} values of the extracts of *Agathosma* species in the DPPH and ABTS anti-oxidant assays.

7.4. Discussion

7.4.1. Thin layer chromatography

A rapid evaluation for anti-oxidants using TLC screening and DPPH staining methods demonstrated that *Agathosma ovalifolia* was the only essential oil that showed distinct free radical scavenging capacity (Figure 7.4). GC-MS data has revealed that methyl eugenol (23.9%) is the major compound present in this species. The compound may contribute to its inherent anti-oxidant properties. Most of the plant extracts showed good activity. The stained silica layer revealed a purple background with yellow spots at the location of compounds which showed radical scavenging capacity (Figure 7.5). The intensity of the yellow colour depended upon the amount and the nature of the radical scavenger present in the samples. Most of the extracts showed numerous spots or bands, having strong intensities on the TLC plate. Anti-oxidants of several plant extracts do not all operate in the same way and some may be more effective against different free radicals. The anti-oxidant potential of the extracts and essential oils also corresponded with the results obtained in the DPPH and ABTS spectrophotometric assays with the exception of the essential oil of *Agathosma ovalifolia* which displayed poor activity in the DPPH spectrophotometric assay (IC_{50} value > 100µg/ml), but proved to be active once sprayed with DPPH on a TLC plate.

7.4.2. Spectrophotometry

The oxidative activity of the DPPH radical was inhibited by 11 of the extracts. *Agathosma capensis* (Gamka) exhibited the greatest anti-oxidant activity having an IC_{50} value of 24.08 $\pm$ 4.42µg/ml. *Agathosma bathii*, *A. betulina*, *A. crenulata*, *A. hirsuta*, *A.*

lanata, *A. ovata* (round-leaf), *A. stipitata* and *A. zwartbergense* showed weak anti-oxidant activity, all having IC_{50} values > 100µg/ml. The remaining species showed moderate to poor activity with IC_{50} values ranging between 30.79µg/ml and 94.65µg/ml. None of the extracts were found to exhibit radical scavenging activity equivalent to that of the standard, ascorbic acid (IC_{50} value of 2.47 ± 0.178µg/ml).

It is well known that plant polyphenolic extracts act as free radical scavengers and as anti-oxidants (Yen and Hsieh, 1998). Polyphenolic compounds have more than one mechanism of action for suppressing free radical reactions. It has been reported they act as anti-oxidants by virtue of the hydrogen-donating capacity of their phenolic groups. In addition, the metal chelating potential of polyphenols may also play a role in the protection against iron- and copper-induced free radical reactions (Yen and Hsieh, 1998). The relationship between the anti-oxidant or scavenging activity of a plant extract and its phenolic content is very difficult to establish because: (1) anti-oxidant properties of single compounds within a group can vary remarkably so that the same levels of phenolics do not necessarily correspond to the same anti-oxidant responses; (2) the different methods used to determine the anti-oxidant activity are sometimes based on different mechanisms of action so that they often give different results and (3) extracts are very complex mixtures of many different compounds with distinct polarities as well as anti-oxidant and pro-oxidant properties, sometimes showing synergistic actions by comparison with individual compounds (Parejo *et al*., 2002). Thus the scavenging activity of an extract cannot be predicted only on the basis of its total phenolic content.

HPLC analysis has revealed that *Agathosma* species are rich in flavonoids of which the anti-oxidant activities have been extensively reported (Chapter four). Flavonoids including quercetin, kaempferol, etc., are strong anti-oxidants that occur naturally in food and can inhibit carcinogenesis in rodents (Yen and Hsieh, 1998). The flavonoids appear to be mostly responsible for the anti-oxidant activity of the extracts. The 11 active species displayed a dose-dependant response in the DPPH assay such that increasing doses produced greater anti-oxidant activity. Since natural anti-oxidative substances usually have a high phenolic moiety in their molecular structure, it suggests that these plants contain high polyphenolic content such as flavonoids. In contrast the poor activity of eight of the species (IC_{50} values > 100µg/ml) may suggest a low anti-oxidant content within them. Differences in activity could be due to the content of anti-oxidant molecules within the plant and also the quantity of the molecules if they are present.

All of the extracts showed greater activity in the ABTS assay than in the DPPH assay with the extract of *Agathosma namaquensis* and *A. capensis* (Besemfontein) being the most active (IC_{50} values of 15.66 $\pm$ 4.57µg/ml and 19.84 $\pm$ 0.09µg/ml). The remaining species showed good activity with the IC_{50} values ranging between 24.71µg/ml and 46.81µg/ml. None was found to exhibit radical scavenging activity equivalent to that of the standard, Trolox (IC_{50} value of 2.96 $\pm$ 0.001µg/ml). In a study performed by Arts *et al*. (2004), HPLC analysis of the reaction mixture obtained after scavenging of the $ABTS^{\cdot+}$ by the flavonoid chrysin, revealed that a product was formed that also reacted with the $ABTS^{\cdot+}$. The product had a higher anti-oxidant capacity and reacted faster with the $ABTS^{\cdot+}$ then the parent compound chrysin. The study revealed that the activity was

due to the anti-oxidant capacity of the parent compound plus the potential anti-oxidant capacity of the reaction product(s). This could explain why species show much greater activity in the ABTS assay.

In general the ranges of the free radical scavenging activities of the extracts were dissimilar in both the methods and no correlation was found between the two (Figure 7.6). The results of the assays can be compared but the ABTS assay has an additional reaction system. Anti-oxidants can exercise their protective properties at different stages of the oxidation process and by different mechanisms. Furthermore the complex composition of the extracts could be responsible for certain interactions (synergistic, additive or antagonistic effects) between their components or the medium (Parejo *et al*., 2002). It has been reported that results from the ABTS assay does not have to correlate with anti-oxidant activity. An explanation for this discrepancy is that the ABTS assay measures the total amount of radical scavenged over a period of time. Reaction products and individual compounds within the extracts may contribute to the activity (Arts *et al*., 2003). Most anti-oxidant activity assays, however, determine the rate at which a radical is scavenged by an anti-oxidant. This is the activity of the extract itself with all compounds contributing to the activity. Previously it has been found that the total amount of $ABTS^{\cdot+}$ scavenged by a compound correlates with the biological activity in a selected group of flavonoids (Arts *et al*., 2003). In a study performed by Arts *et al*. (2003) is has been shown that the rate at which the $ABTS^{\cdot+}$ is scavenged shows a poorer correlation. The study demonstrates that reaction products can contribute to the activity.

The $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ are based on their ability to scavenge a proton from surrounding molecules resulting in a loss of colour by the radical which decreases the absorbance of the solution. The assays have the same mechanism of action but in most cases, the ABTS results are higher than those of the DPPH as obtained with these species. The ABTS radical may react with a molecule that has electron- or hydrogen- donating properties (Pellegrini *et al*., 1999). The electron donors undergo a rapid reaction with the $ABTS^{\cdot+}$ while the functional hydroxyl groups are slower reacting (Pannala *et al*., 2001). Thus the ability of the $ABTS^{\cdot+}$ to react via two mechanisms indicates that the activity displayed would be higher in this assay as compared to that of the DPPH assay which only reacts via the acceptance of a hydrogen from a suitable donor.

The differences in anti-oxidant activity in a particular assay are also largely a function of the ratio of hydrophilic and hydrophobic nature of the phenolics. The DPPH assay essentially measures the anti-oxidant activity of water soluble phenolics. Additional anti-oxidant assays need to be used in order to find the differences in phenolic profile-related anti-oxidant activities of different species. The ABTS assay is very sensitive towards water soluble anti-oxidants. Extracts generally have higher activity in this assay, indicating differences in the physico-chemical properties of the compounds within (Chun, 2005).

'Buchu' been used traditionally as a general tonic and medicine throughout South Africa. The results obtained from the study confirm that these species have anti-oxidant compounds which may contribute to their health benefit properties and hence promote the

general well being of the user. Although the leaves of *Agathosma* species have been found to contain anti-oxidant compounds such as flavonoids, further studies are required to reveal whether they contain other anti-oxidant compounds that may contribute to their activity. The extracts were found to exert a much lower activity then the standards. However we should note that these are not isolated compounds, hence they contain many more compounds that may or may not contribute to the total anti-oxidant activity. Isolation of pure compounds may provide results that could indicate equal or greater free radical scavenging activity than the standards. The results obtained from the two assays support the possibility that few of these plants can contribute to protective effects on human health. Further work on the characterization of specific phenolic components by HPLC needs to be performed in order to establish the connection between anti-oxidant activity and chemical composition.

CHAPTER 8: ANTI-INFLAMMATORY ACTIVITY

8.1. Introduction

'Buchu' has been used traditionally as an antipyretic, topically for the treatment of burns and wounds and for the relief of rheumatism, gout and bruises. The *in vivo* anti-inflammatory activity of many of the compounds present in these species (e.g. linalool, limonene, germacrene D, δ-3-carene, γ-terpinene, eugenol and α-pinene), have been reported previously and this may be relevant to the beneficial effects of *Agathosma* species in treating inflammation. In the continuing study aimed at relating the traditional use of these plants to the active compounds present, the anti-inflammatory activity of *Agathosma* species was investigated.

8.1.1. The inflammatory process

The word 'inflammation' is derived from a state of being 'inflamed'. To 'inflame' means 'to set fire,' which conjures up the colour red, a sense of heat and often pain (Trowbridge and Emling, 1989). It is a descriptive term for the physiological response of the body to injury and encroachment by external factors (Trowbridge and Emling, 1989). Inflammation is commonly divided into three phases: acute inflammation, the immune response and chronic inflammation. Acute inflammation is the initial response to injury; it is mediated by the release of autacoids and usually precedes the development of the immune response. It is a response that is abrupt in onset and of a short duration. The immune response occurs when immunologically competent cells are activated in response to foreign organisms or antigenic substances liberated during the acute or chronic

inflammatory response. The outcome for the host may be beneficial as when it causes invading organisms to be phagocytosed or neutralized. The outcome may also be deleterious if it leads to chronic inflammation without resolution of the underlying injurious process. Chronic inflammation involves the release of a number of mediators that are not prominent in the immune response. It is a proliferative response in which there is a proliferation of fibroblasts and vascular endothelium as well as lymphocytes, plasma cells and macrophages. An important condition involving these mediators is rheumatoid arthritis, in which chronic inflammation results in pain and destruction of bone and cartilage that can lead to severe disability and in which systemic changes occur that can result in shortening of life processes (Katzung, 2001).

The damage associated with inflammation acts on cell membranes to cause leucocytes to release lysosomal enzymes; arachidonic acid is then released from precursor compounds, and various eicosanoids are synthesized. Arachidonic acid, which is produced by the action of cellular phospholipases on phospholipids present in cell membranes, is the precursor of prostaglandins and leukotrienes (Figure 8.1), which are long-chain, lipid soluble hydroxyl fatty acids. The activation of neutrophil lysosomal phospholipase during inflammation is thought to be a major mechanism in initiating the formation of arachidonic acid. Once formed arachidonic acid metabolism proceeds along one of two different pathways, i.e. the cyclo-oxygenase (COX) pathway or the lipoxygenase (LOX) pathway (Figure 8.1).

The COX pathway of arachidonate metabolism produces prostaglandins, which have a variety of effects on blood vessels, on nerve endings, and on cells involved in inflammation. Aspirin and non-steroidal anti-inflammatory drugs (NSAID's) such as indomethicin, inhibit COX and thus suppress prostaglandin synthesis.

The LOX pathway of arachidonate metabolism yields leukotrienes (a group of biologically active unsaturated fatty acids), which have a powerful chemotactic effect on eosinophils, neutrophils and macrophages and promotes bronchoconstriction and alterations in vascular permeability (Katzung, 2001). When the mast cell is stimulated by an antigen, phospholipase A_2 is activated. It oxidizes arachidonic acid via this pathway, eventually giving rise to leukotrienes (LT's). The major leukotrienes are LTA_4, LTB_4, LTC_4, LTD_4 and LTE_4. LTB_4 causes adherence of neutrophils to the endothelium of venules. LTC_4 and LTD_4 cause vasodilation and increased venular permeability (Katzung, 2001). The LOX pathway of arachidonic metabolism produces reactive oxygen species and these reactive forms of oxygen and other arachidonic acid metabolites may play a role in inflammation and tumor promotion. The most physiologically important mammalian LOX has been shown to be the arachidonate 5-LOX. There are structural as well as mechanistic similarities between soybean LOX and mammalian LOX. The inhibition of soybean LOX, is therefore, used by scientists as an *in vitro* method for the screening of anti-inflammatory activity (Qinyun *et al*., 1992).

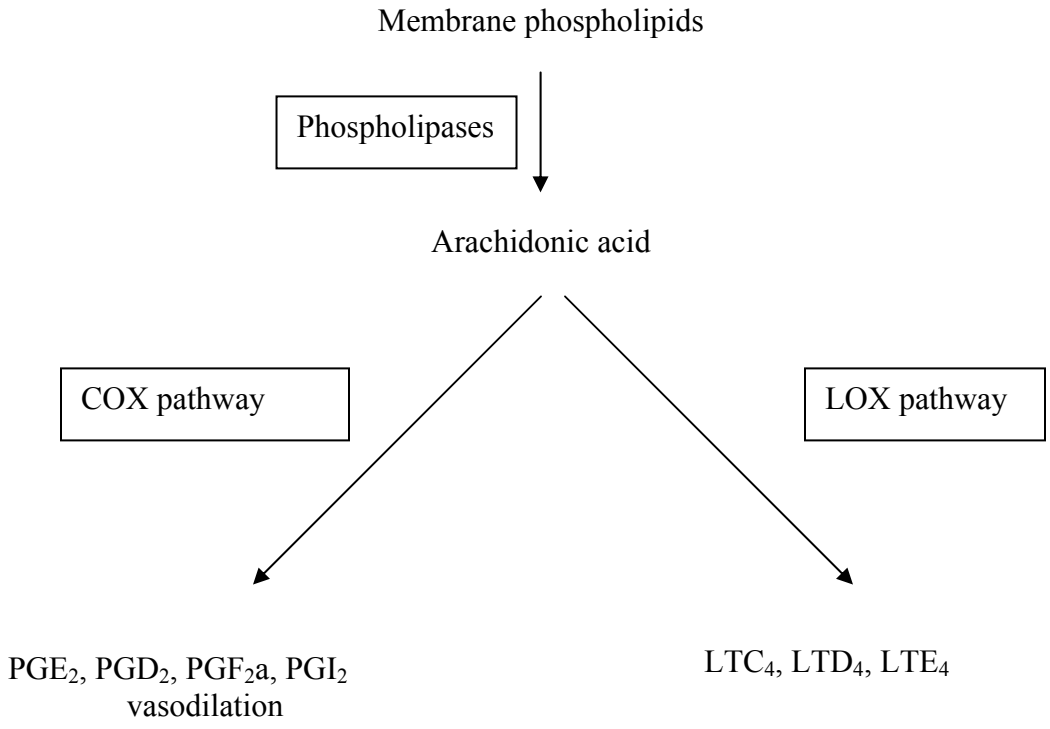


Figure 8.1: Mediators derived from arachidonic acid.

Classical NSAID's, such as aspirin and indomethicin cannot influence LOX activity at doses capable of inhibiting COX and the inflammatory reaction. Drugs that cause selective inhibition of LOX (with therapeutic value in anaphylaxis) as well as agents that are dual inhibitors of both COX and LOX, are capable of controlling inflammatory conditions with similar properties to the anti-inflammatory corticosteroids, but are devoid of the steroid-related toxicity (Maria *et al*., 1995).

8.1.2. Biology of the 5-lipoxygenase pathway

The oxidative metabolism of arachidonic acid is remarkably complex resulting in a number of substances which have a broad range of pathophysiological properties. At the time of the 5-LOX arm of arachidonic acid metabolism, the COX pathway had already

been shown to be important in inflammation and a significant target for clinical intervention. The clinical potential of the 5-LOX pathway came from the observation that arachidonic acid metabolism through 5-LOX was linked to the formation of slow reacting substances of anaphylaxis. The structures of leukotrienes were then established and then the potential utility of finding inhibitors of the formation of leukotrienes was thus established.

8.1.3. Inflammation and free radical damage

A direct result of inflammation is an increase in free radical production. Free radicals react with the polyunsaturated fatty acids of cell membranes leading to the eventual destruction of the cell. One single free radical can destroy an entire membrane through a self-propagating chain reaction. The body defends itself against free radical damage with an integrated anti-oxidant defense system that utilizes anti-oxidants produced naturally within the body and from anti-oxidants found within foods. During inflammation, the need for a variety of anti-oxidant nutrients may need to be increased (Percival, 1999).

8.1.4. Plants as anti-inflammatory agents

The treatment of inflammatory conditions with plants is widely reported. Natural products are already providing lead compounds in the search for inhibitory small molecules but only a few are beginning to be used commercially (Bremner and Heinrich, 2001). A large number of pure compounds have been shown to interfere with the cascade of events leading to inflammation. Notably these compounds generally come from medicinal plants used in indigenous or other medical systems. In traditional practice,

medicinal plants are used to control inflammation in many countries. This has caused an increase in the number of experimental and clinical investigations directed towards the validation of the anti-inflammatory properties which are putatively attributed to these remedies. The biological and cultural diversity has provided many exciting leads for developing useful pharmaceuticals.

The number of chemical compounds, called phytochemicals, found within the plant kingdom is truly vast and their range of activity is equally great. Some of the phytochemicals found in certain herbs and plants are reported to demonstrate pain and inflammation-reducing properties. Like aspirin, many are presumed to work by blocking the COX and LOX pathways and possibly by other mechanisms as well. Biflavonoids are a broad class of phytochemicals found largely in *citrus* fruits, tea and wine. Research suggests that biflavonoids, such as quercetin, may confer pain and inflammation reducing activity by inhibiting COX, LOX and phospholipase (Percival, 1999).

Anti-inflammatory activities of medicinal plants have been screened using a variety of *in vitro* or animal model systems. These include the testing of flavonoid components, e.g. a compound present in liquorice extract, glabridin, which has been shown to prevent inflammation of guinea pig skin, via inhibition of superoxide radical production and COX activity. Plants with potential anti-pruritic activity have been identified via inhibition of the substance P-induced itch-scratch response in mice. Lavender oil inhibits immediate type allergic reactions induced in mice or rats via mast cell degranulation (Mantle *et al*., 2001). Ginger (*Zingiber officinale*) and turmeric (*Curcuma longa*) are two popular spices

used within the East Indian system of medicine known as Ayurveda. Numerous studies have demonstrated significant anti-inflammatory activities for both. These studies suggest that both spices may block COX and LOX activity, thereby inhibiting prostaglandin and leukotriene release. In addition, turmeric may inhibit the release of histamine. Another compound capsaicin found in cayenne pepper (*Capsicum annuum*) was found to play a role in inhibiting prostaglandin synthesis by blocking COX activity. Boswellic acids derived from the gum resin of *Boswellia serata* have been found to inhibit LT synthesis by specifically inhibiting 5-LOX (Percival, 1999).

However, a number of important challenges still remain. Firstly, compounds which act only on a single target are unlikely to be identified because of the multiple effects generally observed. The pharmacological consequences of these actions have to be studied in detail. Secondly, *in vivo* studies on the pharmacological effects of the plants will be required to assure that the effects are truly of pharmacological relevance. Thirdly, natural products provide a particular challenge in the field of molecular biology. The information provided must include the characterized or quantified ingredients of an active species (providing at least an HPLC or GC-MS fingerprint). Finally, truly novel natural inhibitors of inflammation require appropriate mechanisms of benefit sharing between the original keepers of traditional knowledge and the investigators who further develop such products. The most important challenge remains the loss of cultural and biological diversity due to overexploitation of the environment and unsustainable use of natural and human resources as well as the enormous threat to the cultural diversity of the world (Bremner and Heinrich, 2001).

8.1.5. Wound healing properties of plants

Since antiquity, mankind has reached into the nearby environment for the means to treat wounds and topical infections that result from the vicissitudes of everyday living. Ethnobotanists in an analysis of the diverse uses of plants in traditional societies, point out that approximately one-third of traditional medicine are used for skin conditions and wounds, reflecting the widespread call for these remedies. At the same time they pointed out that a mere 1-3% of modern drugs are developed to address these conditions. Many of these are antibiotics and steroids, whose cost in industrial countries is high and, in non-industrial countries, is often prohibitive (Bodeker *et al*., 1999).

Topical inflammation generally involves some chemotactic and chemokinetic agents produced from arachidonic acid by LOX activity. These agents together with the prostaglandins and thromboxanes produced by COX activity participate in the onset of the inflammatory response of the skin. After this initial phase, LTB_4 is mainly responsible for the long-term maintenance of the inflammation; for this reason, the last several years have witnessed an increased interest in the role of LTB_4 such that it is now seen as something more than a mere chemotactic agent (Prieto *et al*., 2003).

Topical anti-inflammatory agents have been used as wound care agents. Wound healing is a fundamental response to tissue injury resulting in the restoration of tissue integrity. Wound care has existed almost certainly as long as *Homo sapiens*. Many treatments discovered by early civilizations were based on the use of local plants. Thus the largest class of research conducted to date is on herbal remedies used by different cultures

worldwide. Treatment of wounds and particularly burn injuries is a major problem in developing countries, due to the limited availability of conventional resources (Mantle *et al*., 2001). Plants used in traditional medicine of these countries therefore continue to play a significant role in the treatment of such skin injuries.

In traditional African medicine, many plant species have been described for their efficacy in promoting wound or burn healing. For example in The Gambia, the pulp of the papaya fruit (*Carica papaya*) is mashed and applied daily as a topical dressing to infected burns and is particularly well-tolerated in children. The preparation is effective in desloughing necrotic tissue (possibly via proteolytic action) and preventing burn wound infection (Mantle *et al*., 2001). In South America, dragons' blood is a traditional remedy used for wound healing. This is a blood red, viscous latex which is extracted from various *Croton* species (Euphorbiaceae) by slashing the bark. Experiments have shown that it stimulates contraction of wounds, crust formation, formation of new collagen and epithelial regeneration. Pro-anthocyanidins were found within and were shown to be responsible for the properties (Bodeker *et al*., 1999). In the West Indies, in Jamaica, the herbaceous plant *Justica pectoralis* (Acanthaceae family) is used in folk medicine to treat cuts and wounds. The leaves are bruised, alone or with rum, and applied as a plaster. Preliminary investigations showed that coumarins were a major component of an acetone extract of the plant. Fresh wounds created on rats and treated with the extract rich in coumarins showed attenuated inflammation processes and significantly enhanced healing of wounds (Bodeker *et al*., 1999). *Aloe vera* (*Aloe barbadensis* Miller.), has been used worldwide to treat wounds and skin conditions. Its beneficial effects have been demonstrated in *in vitro*

and *in vivo* studies. Extracts have been found to penetrate tissue, have anaesthetic properties, have antibacterial, antifungal and antiviral properties and serve as an anti-inflammatory agent (Bodeker *et al*., 1999). *In vivo* analysis of burn injuries show that the mediator of progressive tissue damage was thromboxane A2. *Aloe* extracts have been shown to inhibit thromboxane A2 and also maintain homeostasis within the vascular endothelium as well as in surrounding tissue (Bodeker *et al*., 1999).

8.2. Materials and methods

8.2.1. 5-Lipoxygenase assay

The anti-inflammatory activity of *Agathosma* species was determined *in vitro* using the 5-LOX assay (Baylac and Racine, 2003).

8.2.1.1. Principle of the method

The enzyme 5-LOX is known to catalyze the oxidation of unsaturated fatty acids containing 1,4-pentadiene structures. Arachidonic acid is the biological substrate for the enzyme 5-LOX in the body, but the enzyme accepts linoleic acid too which was the substrate chosen for the study since it is easy to handle. *In vitro* 5-LOX oxidizes linoleic acid into a conjugated diene that absorbs at 234 nm. The initial reaction rate is measured by spectrophotometry and the inhibitory activity of a substance is measured by the decrease of this initial rate. The assay aims at detecting the 5-LOX inhibitory activity of test compounds, which therefore inhibits the formation of the conjugated diene that is detected by spectrophotometry at 234 nm.

Arachidonic acid is metabolized by LOX in addition to COX. Hence it is of value to examine the effects on a LOX enzyme by all the species. Potato LOX was used for the assay since it is a commercially available enzyme source and it provides a rapid method to evaluate a large number of compounds for their effects on LOX activity. It cannot be assumed that the results reported here can be extrapolated to a mammalian LOX. There are several LOX's with different specificities, and any effects of an inhibitor on one LOX cannot be extrapolated to an effect on a different LOX (Sircar *et al*., 1983).

Essential oils are not very water soluble hence the assay was performed in a phosphate buffer. A small amount of nonionic surfactant (Tween 20) was necessary to disperse the oil in this aqueous medium. Preliminary assays had determined the maximum concentration of surfactant required that did not interfere with the enzyme kinetics (Baylac and Racine, 2003).

8.2.1.2. Method

The essential oils and extracts were dissolved in DMSO (Saarchem) and Tween® 20 (Merck) to obtain a starting concentration of 100µg/ml. Serial dilutions were performed with DMSO for species that were active at 100µg/ml, in order to obtain working solutions of 50µg/ml and 25µg/ml. In a 3ml cuvette maintained at 25°C in a water bath, 10µl of test compound was mixed with 2.95ml of 0.1M potassium phosphate buffer (pH 6.3) and 45µl linoleic acid (≥99%, Fluka). The enzymatic reaction was initiated by adding 100U of 5-lipoxygenase (Cayman) diluted with an equal volume of 0.1M potassium phosphate buffer (pH 6.3) which was stored at 4°C until required. The increase in absorption at 234

nm arising from the modification of the unsaturation site of linoleic acid (1,4-diene to 1,3-diene) was measured for 10 min at 25°C using spectrophotometry (UV-VIS spectrophotometer, Analytikjena Specord 40) connected to the computer equipped with Winaspect® software. The initial reaction rate was determined from the slope of the straight line portion of the curve and the percentage inhibition of enzyme activity was calculated by comparison to the control. The concentration that gave 50% inhibition (IC_{50}) was calculated using Enzfitter® version 1.05 software. Nordihydroguaiaretic acid (NDGA) was used as a positive control.

8.3. Results

The species which displayed 5-LOX inhibitory activity and their corresponding IC_{50} values are shown Figure 8.2. All of the essential oils exhibited *in vitro* 5-LOX anti-inflammatory activity with the exception of *Agathosma stipitata* which was UV active and showed interference. Hence the IC_{50} value of this species could not be calculated. All of the species were active in the assay at a starting concentration of 100µg/ml, hence serial dilutions were performed with DMSO. The results revealed that *Agathosma collina* displayed the most promising activity (IC_{50} value of 25.98 ± 1.83µg/ml). The essential oil of *Agathosma bathii* showed the least activity (IC_{50} value of 76.58 ± 5.44µg/ml), while the remaining species displayed good to moderate activity. The 5-LOX inhibitor NDGA, which represented the positive control and putatively blocked the formation of 5-LOX products, had an IC_{50} value of 2.39 ± 0.71µg/ml. The extracts displayed very poor activity. All had IC_{50} values > 100µg/ml.

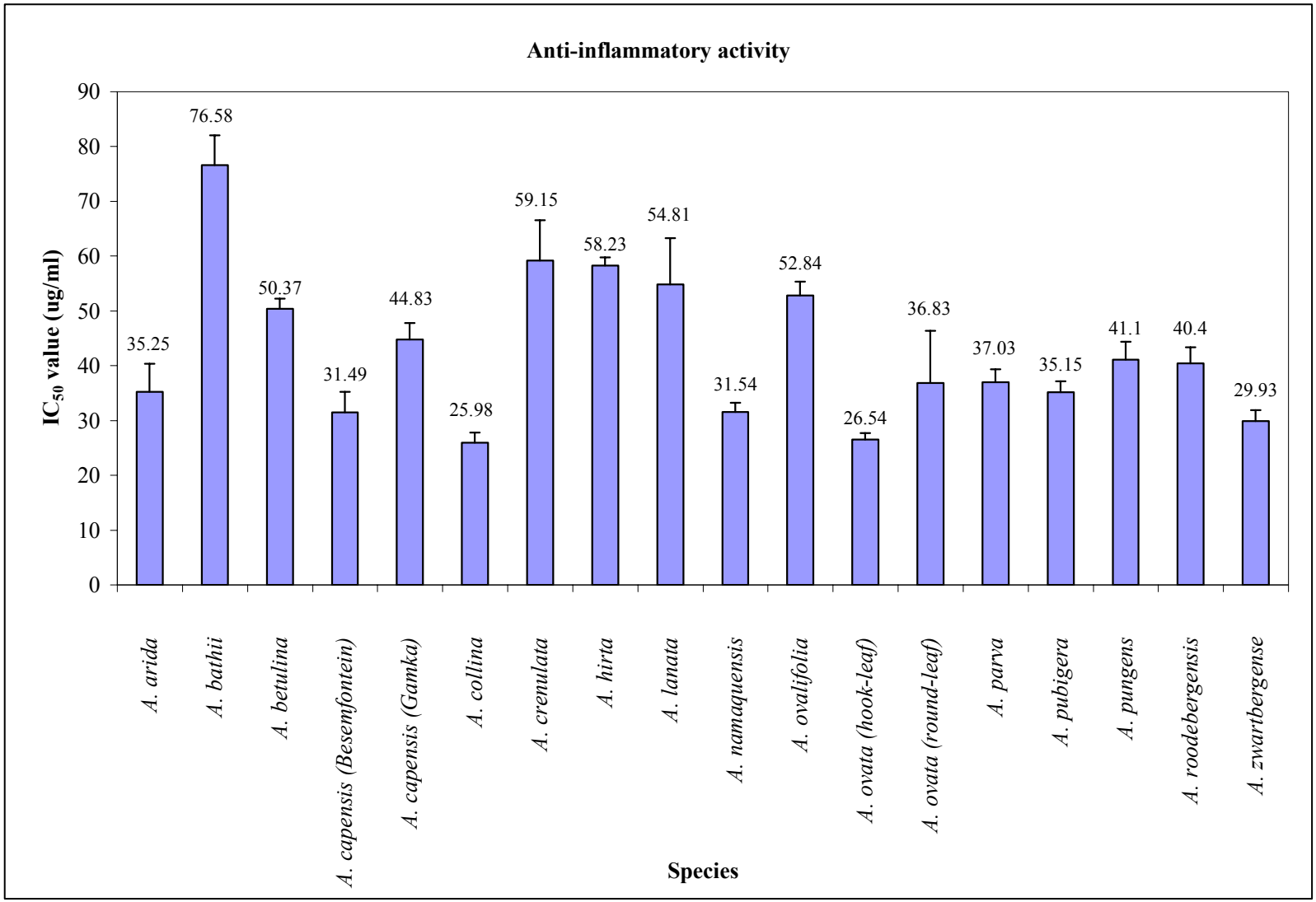


Figure 8.2: Bar graph depicting the *in vitro* anti-inflammatory activity of the essential oils of indigenous *Agathosma* species. n = 1

8.4. Discussion

The anti-inflammatory activities of *Agathosma* species were compared by means of their IC_{50} values, defined as the concentration of test substance necessary for 50% inhibition of the enzyme reaction. The results varied and the study revealed that all species had affinity for the enzyme equal to or greater than the substrate, linoleic acid. The most potent inhibitor of the enzyme was *Agathosma collina* (IC_{50} value of 25.98 ± 1.83µg/ml). Two of the species portrayed excellent activity; *Agathosma ovata* (hook-leaf) (IC_{50} value of 26.54 ± 1.18µg/ml) and *A. zwartbergense* (IC_{50} value of 29.93 ± 1.99µg/ml). In a summary all species with the exception of *Agathosma stipitata*, showed inhibitory activity. According to Baylac and Racine (2003), essential oils rich in citral, have been reported to have anti-inflammatory activity that could not be evaluated due to a strong absorption of citral (neral-geranial) at 234 nm, which renders the spectrophotometric measurement impossible. GC-MS data has revealed that the major compounds in *Agathosma stipitata* are neral (39.9%) and geranial (10.1%), which explains why the IC_{50} value could not be determined.

The same authors have reported a good correlation between the activity of the terpene, *d*-limonene and those of *citrus* oils evaluated. Although not tested, it is expected that essential oils rich in *d*-limonene, like grapefruit, lime and celery will also be good inhibitors of 5-LOX. GC-MS data has revealed the presence of limonene in all of the species with the exception of *Agathosma arida*. It is also the major compound in the most active species, *Agathosma collina* (30.9%) and is reported to have an IC_{50} value ranging

between 10µg/ml and 30µg/ml; hence the presence of this compound may contribute to activity.

Linalool is a principle component of many essential oils known to possess several biological activities attributable to this monoterpene. A number of linalool producing species are used in traditional medicine systems to relieve symptoms and cure a variety of ailments, both acute and chronic (Peana *et al*., 2002). Their pharmacological activities are attributable to the content of alcohols like linalool and its corresponding ester (linalyl acetate) (Peana *et al*., 2002). Linalool was evaluated recently for its psychopharmacological activity in mice, revealing marked dose-dependant sedative effects in the central nervous system, including protecting against picrotoxin and transcorneal electroshock-induced convulsions (Peana *et al*., 2002). It has also been reported that linalool modulated glutamate activation expression *in vitro* and *in vivo*. The mechanism by which the anti-inflammatory effect occurs remains to be determined, although several observations suggest a possible involvement of N-methyl-D-aspartic acid (NMDA) receptors. It has been reported that linalool is a competitive NMDA receptor antagonist and the administration of excitatory amino acid receptor antagonists selectively attenuates carrageenan-induced behavioral hyperalgesia in rats (Peana *et al*., 2002). Moreover, it has been reported that linalool, as well as some terpenes, could enhance the permeability of a number of drugs through biological tissues like the skin or mucus membranes. A study performed by Peana *et al*. (2002) has shown that (-) linalool, a natural occurring enantiomer in essential oils and its racemate form, possess anti-inflammatory and antinociceptive properties. Pretreatment with (-) linalool (50-

150mg/kg) inhibited the development of acute hyperalgesia induced by carrageenan in the injected rat paw, with no effect in the contralateral paw. The administration of higher doses resulted in greater inhibitory effects. The correlation between the anti-inflammatory effect and administered doses could suggest a dose-dependant effect. This observation is consistent with the possibility of a saturation of the receptors involved in the inflammatory reaction. The results obtained from the study indicate that linalool plays a major role in the anti-inflammatory activity displayed by essential oils containing the compound and provides further evidence suggesting that linalool producing species are potential anti-inflammatory agents. GC-MS data has revealed its presence in all of the species except *Agathosma roodebergensis*.

The presence of aliphatic aldehydes, dodecanal (0.2%) and decanal (0.1%) in *Agathosma roodebergensis* may contribute to its activity since these aldehydes have been reported to have activity (Baylac and Racine, 2003). Dodecanal has an IC_{50} value ranging between 10µg/ml and 30µg/ml while decanal has an IC_{50} value ranging between 31µg/ml and 50µg/ml (Baylac and Racine, 2003).

In a study performed by Ocete *et al*. (1989), it was found that the δ-3-carene component in the essential oil of *Bupleurum gibraltaricum* was responsible for the species showing considerable anti-inflammatory activity against carrageenan-induced pedal edema in rats. The essential oil and the δ-3-carene component both produced qualitatively similar changes in rat uterine contractions caused by oxytocin and acetylcholine. The activity of *Agathosma capensis* (Besemfontein) (0.2%), *A. capensis* (Gamka) (0.9%), *A. hirsuta*

(trace), *A. namaquensis* (0.1%), *A. pubigera* (0.5%) and *A. zwartbergense* (trace) could be due to the presence of δ-3-carene, in addition to other components. Acute inflammation such as carrageenan-induced edema involves the synthesis and release of mediators at the injured site. These mediators include prostaglandins, especially bradykinins, leukotrienes and serotonine, all of which cause pain and fever. Inhibition of these mediators from reaching the injured site or from bringing out their pharmacological effects will normally ameliorate the inflammation and other symptoms (Asongalem *et al*., 2004).

The activity of *Agathosma hirsuta* could be attributed to the presence of the sesquiterpene, β-caryophyllene (0.1%), which has an IC_{50} value ranging between 10μg/ml and 30μg/ml and has been found to strongly inhibit 5-LOX (Baylac and Racine, 2003). The compound γ-terpinene (IC_{50} value ranging between 10μg/ml and 30μg/ml), is present in many of the species and has been reported to have good anti-inflammatory activity (Baylac and Racine, 2003). The terpene, α-pinene (IC_{50} value ranging between 31μg/ml and 50μg/ml), present in all of the species in varying concentrations, was also found to have activity (Baylac and Racine, 2003). These compounds may contribute to the activities of these species.

The activity of *Agathosma betulina* and *A. capensis* (Besemfontein) could be attributed to the presence of the *o*-methoxyphenol, eugenol, which has been found to exhibit anti-inflammatory activity (Murakami *et al*., 2005). *o*-Methoxyphenols such as eugenol and isoeugenol are components of clove oil, which is commonly used as a flavouring agent in

cosmetics and food products. Although these compounds have a beneficial anti-inflammatory property, high concentrations have been found to cause inflammatory and allergic reactions (Murakami *et al*., 2005). In another study performed by Saeed *et al*. (1995), the effect of eugenol on human platelets, arachidonic acid and carrageenan-induced paw edema was investigated. Eugenol was found to significantly inhibit arachidonic acid and platelet activating factor induced platelet aggregation, and at higher doses also inhibited collagen-induced aggregation. Eugenol inhibited arachidonic acid metabolism by human platelets by acting against COX and LOX enzymes. *In vivo* experiments revealed that eugenol gave 100% protection against arachidonic acid- or platelet activating factor- induced death. It also inhibited the inflammation and paw edema and was five times more potent then aspirin. The results from the study demonstrated that eugenol is a dual antagonist of arachidonic acid and platelet activating factor.

Variable anti-inflammatory activity has been reported for esters (Baylac and Racine, 2003). The presence of citronellyl acetate in *Agathosma hirsuta* (0.9%), *A. namaquensis* (0.4%) and *A. ovata* (trace) may contribute to their activities since the compound has been reported to have activity (IC_{50} value ranging between 10µg/ml and 30µg/ml). Similarly, the presence of methyl benzoate in *Agathosma namaquensis* (0.2%) and *A. zwartbergense* (0.2%) may contribute to their activities. Methyl benzoate has a reported IC_{50} value ranging between 31µg/ml and 50µg/ml (Baylac and Racine, 2003).

In a study performed by Ceschel *et al*. (2000) involving porcine buccal mucosa it was found that when comparing the partition co-efficients (K_p) of single components of an essential oil, the terpenic components (β-pinene, cineole, terpineol and linalool) had a higher K_p when compared to the other components (linalyl acetate and α-terpinil acetate). The authors report that the terpenic components are usually used as enhancers in percutaneous absorption but are also good permeants of the buccal mucosa. 'Buchu' has been used traditionally topically to treat inflammation, burns, bruises and skin diseases (Watt and Breyer-Brandwijk, 1962); and GC-MS data has revealed that most of the *Agathosma* species are rich in terpenes, which explains why they diffuse through the skin barrier easily and are hence effective when used topically.

The compound *p*-methylacetophenone present in *Agathosma bathii* (0.1%), *A. capensis* (Gamka) (0.1%), *A. ovata* (0.1%) and *A. pubigera* (0.1%) may contribute to their activities since a study performed by Sala *et al*. (2003) involving six acetophenones demonstrated that 4-hydroxy-3-(3-methyl-2-butenyl)acetophenone was an inhibitor of both COX and 5-LOX, whereas 4-hydroxy-3-(2-hydroxy-3-isopentenyl)acetophenone was a selective inhibitor of 5-LOX. Some acetophenones (e.g. hydroxyacetophenone derivatives) have been described as inhibitors of chemotaxis of polymorphonuclear granulocytes. Methyl or methoxy group substitution at C-3 has resulted in pronounced inhibitory effects (Sala *et al*., 2003).

The sesquiterpene germacrene D, is also reported by Baylac and Racine (2003) to possess good anti-inflammatory activity (IC_{50} value ranging between 10µg/ml and 30µg/ml). The

compound may contribute to the overall activity of *Agathosma capensis* (Besemfontein) and *A. roodebergensis*.

The results obtained from the study lead us to conclude that different compounds contribute to the activity of *Agathosma* species and that a correlation between the effects observed and the chemical profiles exhibited by each of the species, exists. Further studies are required to establish the mechanism of the anti-inflammatory effects, the structure of the active compounds, the effectiveness of the interaction with other pro-inflammatory biochemical pathways and their possible structure-activity relationships, so that the mode of action of these compounds can be clarified. Inflammation is a very complex process and the essential oils may exhibit variable activity in other assays (e.g. COX-1 and COX-2 inhibition). The data clearly indicates that *Agathosma* species block the synthesis of 5-LOX products *in vitro*, but are not as effective as the positive control NDGA, a blocking agent for 5-LOX products formation. Since leukotrienes, for which 5-LOX is the key enzyme, are considered to be involved in the initiation and maintenance of a variety of inflammatory diseases, it may be reasonable to state that the inhibition of leukotriene synthesis may, at least in part, be responsible for the anti-inflammatory action of these species. These results also suggest that in the future further pharmacologically effective compounds in species like these may find therapeutic application.

The discovery and steady exploration of many of the oxygenation products that participate in the arachidonic cascade has been one of the outstanding advances in biomedical research in the last two decades. Although clinical interventions that act primarily on the COX products of the cascade associated with inflammation and pain,

such as aspirin and NSAID's, have been widely studied, clinical studies of the 5-LOX cascade products, the leukotrienes, that mediate inflammation and have vasoactive effects are just being started. The accumulating evidence that the secretion of leukotrienes may initiate a chain of biochemical events that amplify inflammatory responses poses a challenge for those attempting to devise appropriate pharmacological interventions because the complex of reactions may have both pathologic and homeostatic consequences. In this decade, basic science data and clinical evidence on the modes of action and clinical effects of the leukotrienes are beginning to come together. The more specific our knowledge of biochemical changes becomes, the more likely it is that specific interventions producing more benefit then harm in reducing leukotriene-induced inflammation, vasodilation and edema will be found.

'Buchu' has been used traditionally by the Khoi-San in the treatment of a number of inflammatory conditions, including rheumatoid arthritis, burns, bruises and inflammatory respiratory conditions (Watt and Breyer-Brandwijk, 1962). The results obtained from the study confirm that *Agathosma* species inhibit 5-LOX, consequently providing scientific evidence (albeit *in vitro*) justifying the use of 'buchu' traditionally.

CHAPTER 9: TOXICITY

9.1. Introduction

The natural world is not entirely pleasant and we hardly expect to find plants that are harmful to us. Even so, our earliest human ancestors, about half a million years ago, approached it cautiously. Ages passed while they tried out various plants, noted successes and failures and gradually learned what they could eat or smoke, smear on arrows or rub on a wound. From those earliest beginnings to the present day, human beings have owed their entire existence to nature. A surprisingly large number of the world's plants contain toxic substances that can kill any creature that eats enough of them. The toxins are an incidental by-product of the growing and fruiting process, but they have become a protection against animals and people (Dowden, 1994).

9.1.1. Toxicity of plants

Toxicity can be divided into topical effects (skin, mucous membranes, eye irritation, phototoxicity, skin sensitization and photosensitization) and systemic effects (mutagenicity, carcinogenicity, embryo toxicity, reproductive toxicity and effects on specific organs). The toxicity of individual species is influenced by various compounds present within and more importantly the size of the dose (Burfield, 2000).

Adverse reactions of skin to plants, referred to as phytodermatitis, may result from mechanical injury, toxicologic effects (e.g. alkaloids), pharmacologic effects (e.g. stinging nettle causing liberation of histamine and acetylcholine), contact dermatitis by

irritant chemicals or delayed hypersensitivity reactions involving immunologic mechanisms. Phytodermatitis evoked by several furanocoumarins (psoralens) produced by such plant families as Umbelliferae, Leguminosae and Rutaceae, occurs only after contact with a plant (e.g. sap and leaves) and subsequent exposure of the skin to long-wave ultraviolet radiation (Pathak, 1986). Coumarins have been detected previously in several *Agathosma* species hence the possibility of these species being toxic needs to be investigated. The subject and literature concerning dermatitis and allergic plant dermatitis is voluminous, and our knowledge of the chemical nature of allergens present in plants is still limited. Existing data gathered from the published reports do not necessarily establish that the plants mentioned in the literature are indeed capable of inducing phytodermatitis; some plants may concomitantly produce reactions of the skin by direct mechanical trauma, contact irritation, allergic sensitization, photosensitization or any combination of these factors (Pathak, 1986).

At least 80% of the poisonous components of plants are cardio-active components and are mostly cardiac glycosides. The main plant sources in reference to these are; *Acokanthera*, *Parquetina*, *Strophanthus* and scattered or in more special areas *Adenium*, *Mansonia*, *Calotropis*, *Pergularia*, *Corchorus* and *Erythrophleum* (Neuwinger, 1996). Several other large groups of poisonous principles are alkaloids, triterpenoids, especially saponins and diterpenoids. Most saponins are able to cause haemolysis of erythrocytes with only a few mg/ml (Neuwinger, 1996). Alkaloids with an extreme diversity of structure types are known to have a wide range of pharmacological activities. The muscle relaxant, respiratory and central nervous system activities are mainly derived from alkaloids.

On the other hand, throughout the ages, almost all poison based biologically active plants have also found application in traditional medicine. Some of our most valuable drugs were discovered by primitive man. The dreaded arrow poisons have also provided modern scientific medicine with effective therapeutics, or have been applied as tools in research; the best known examples are ouabain and *k*-strophanthin for acute cardiac insufficiency, physostigmine for the treatment of glaucoma and myasthenia gravis, *d*-tubocurarine as a muscle-relaxant in general anaesthesia, reserpine as an antihypertensive and psychotropic drug, and ajmaline for cases of cardiac rhythm disturbances (Neuwinger, 1996).

9.1.2. Essential oils and toxicity

As with most medicinal drugs, whether of a 'synthetic' or a 'natural' origin, the compounds present in essential oils have the potential to create serious, even fatal toxic effects, if ingested in overly large quantities. There are numerous cases reported in toxicological literature regarding both serious (non-fatal) and fatal outcomes of essential oil ingestion in both children and adults. These cases are generally due to accidental ingestion by young children, attempts at creating abortions in past years and the use of essential oils for suicide attempts. There are rare cases of toxic effects due to overly large doses of specific essential oils being 'self-prescribed', 'prescribed' to children by parents or prescribed to clients by ill-informed therapists. Most essential oil compounds have a 'non-specific' toxic effect, whereby the absorption of these lipophilic compounds into cellular membranes can eventually lead to disruption of membrane permeability. The primary toxic outcome is that of the disruption of ion channel function in nerve cells, first

affecting the heart and central nervous system, leading to cardiac and respiratory depression (Burfield, 2000).

Certain aromatic compounds, most notably 1,8-cineole (as in many *Eucalyptus* species), camphor (borneone) (as an isolated compound or as in *Rosmarinus officinalus*) and methyl salicylate (as a synthetically derived compound or as in *Gaultheria procumbens*) have specific toxic effects at much lower doses. These compounds make up the bulk of both serious and fatal poisonings in children and adults, due not just to their toxicity, but to the common availability of products containing these compounds and their reputed beneficial properties (Burfield, 2000).

Various types of hazards exist and the following may apply to certain essential oils: carcinogenic (e.g. Calamus oil, *Acorus calamus*), mutagenic, teratogenic (e.g. Savin oil from *Juniperus sabina*), harmful (e.g. *Mentha pulegium* and *Hedeoma pulegoides*), irritant (e.g. Winter Savoury oil, *Satureia montana*), phototoxic (e.g. Rue oil, *Ruta graveolens*), reproductive toxicity (e.g. *Melaleuca bracteata* assumed genotoxic from methyl eugenol content) and environmental toxin (e.g. Wintergreen oil, *Gaultheria procumbens*) (Burfield, 2000).

Many plants are known to be toxic when used in overdose or in combination with other medicine hence many studies only focus on this, assuming that there are no levels of toxicity when used at lower doses or singularly. For this reason, research needs to be

carried out in order to determine the pharmacological action and the toxicity of medicinal plants.

9.2. Materials and methods

9.2.1. Microculture tetrazolium cellular viability assay

The toxicity profile of the extracts and essential oils of indigenous *Agathosma* species was assessed on transformed human kidney epithelium (Graham) cells using the microculture tetrazolium (MTT) cellular viability assay (Mosmann, 1983).

9.2.2. Principle of the method

MTT is a tetrazolium dye [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] which is used to measure the cellular conversion of the dye into an insoluble formazan product by the action of NADH-generating (reduced form of nicotinamide adenine dinucleotide) dehydrogenases present in the active mitochondria of living cells. The mitochondrial dehydrogenase activity is assessed by the reductive cleavage of the tetrazolium salt MTT due to the succinic dehydrogenase present in living cells to yield a formazan dye. The conversion of the tetrazolium compound to a formazan product is monitored by a shift in absorbance and there is a linear relationship between cell number and the amount of formazan product produced. The MTT assay is performed in order to evaluate cellular viability in the presence of different concentrations of test samples.

9.2.3. Cell culture

The Graham cells were maintained in culture medium, which consisted of Ham F10 solution, 5% fetal calf serum (FCS) and 50mg/ml gentamicin sulfate. The Ham F10 solution consisted of 9.38g of Ham's F10 medium (Highveld Biological (PTY) LTD, South Africa) and 1.18g of $NaHCO_3$ (Saarchem) in a total volume of 1L sterile water. Before use, the FCS was inactivated at 56°C for one hour. The culture medium was replaced every second day and when the cells were confluent, they were trypsinised with 4ml of 0.25% Trypsin / 0.1% Versene EDTA (Highveld Biological (PTY) LTD, South Africa) at an ambient temperature. Once the cells had been resuspended in experimental medium, 1ml of the suspended cells was used to seed a new culture and the remaining cell suspension was used in the MTT assay. The experimental medium was prepared in the same way as the culture medium but did not contain gentamicin sulfate.

9.2.4. Method

The dichloromethane and methanol extracts (1:1), were dissolved in DMSO to prepare a stock solution of 10mg/ml. Dilutions were made using experimental medium in order to obtain the following final concentrations, with a ten times dilution factor taken into consideration: 100µg/ml, 10µg/ml, 1µg/ml, 0.1µg/ml, 0.01µg/ml, 0.001µg/ml and 0.0001µg/ml. Essential oils (10µl) were diluted in 90µl of DMSO to make a stock solution and thereafter serial dilutions were made. The control, quinine, was dissolved in the experimental medium to obtain concentrations ranging from 12.722µg/ml to 12.722ηg/ml.

The trypsinised cell suspension was stained with 0.2% Trypan blue (Sigma®) in a 1:1 ratio and cell counts were performed under a microscope using a haemocytometer. The cell suspension (> 95% cell viability) was then adjusted with culture medium to 0.25 million cells/ml and 180µl was plated out and incubated under humidified conditions at 37°C in 5% CO_2 for six hours to allow the cells to adhere to the 96-well flat bottomed plates. Thereafter the different concentrations of test compounds (20µl for extracts and controls, and 2µl for essential oils) were plated out in triplicate in 96-well microtitre plates. The experimental medium (18µl) was added to each of the wells containing essential oil to ensure a total volume of 200µl per well. Each plate contained 10 wells for the untreated cell control and two wells for the blank cell-free control. After 44 h of incubation, 40µl of a 12mM MTT (USB™) solution prepared with phosphate buffered saline (pH 7.4) was added to each well and the plates were re-incubated for a further four hours. The supernatant (180µl) was removed from each well and replaced with 150µl DMSO to stop the reaction and solubilize the formazan crystals. The plates were shaken for four min and the absorbance was read at a test wavelength of 540 nm and a reference wavelength of 690 nm using a microplate reader (Labsystems iEMS Reader MF) connected to the computer equipped with Ascent® software. The results were expressed as percentage cellular viability, with the appropriate controls taken into account. The percentage cellular viability was plotted against concentration and sigmoid curves were obtained after logarithmic transformation of the concentration. IC_{50} values were determined from these dose-response curves. The experiment was repeated four times for each test substance.

9.3. Results

The toxicity of indigenous *Agathosma* species was evaluated using the MTT assay and the results obtained are presented in Figure 9.1 below. The extracts of *Agathosma lanata* (IC_{50} value of 26.17 ± 9.58µg/ml) and *A. ovata* (round-leaf) (IC_{50} value of 25.20 ± 6.30µg/ml) proved to be the most toxic. The extracts of *Agathosma arida*, *A. capensis* (Gamka), *A. collina*, *A. hirsuta*, *A. ovalifolia*, *A. ovata* (hook-leaf), *A. parva*, *A. pubigera*, *A. pungens*, *A. roodebergensis*, *A. stipitata* and *A. zwartbergense* also proved to be toxic at the concentrations tested. Another finding from the results obtained was that the extracts of *Agathosma bathii*, *A. capensis* (Besemfontein), *A. betulina*, *A. crenulata* and *A. namaquensis* were not toxic at the concentrations tested and had IC_{50} values > 100µg/ml. The essential oils of all 19 species proved to be much more toxic (IC_{50} values < 0.0001µg/ml) than a plant-derived compound that is considered relatively safe, namely quinine (IC_{50} value of 136.06 ± 4.06µg/ml).

9.4. Discussion

Serial dilutions displayed different inhibitions of cell growth and the samples proved to be toxic in a dose-dependant manner. The toxicity profiles of the extracts of *Agathosma arida*, *A. collina*, *A. hirsuta*, *A. roodebergensis*, *A. stipitata* and *A. zwartbergense* were similar to one another.

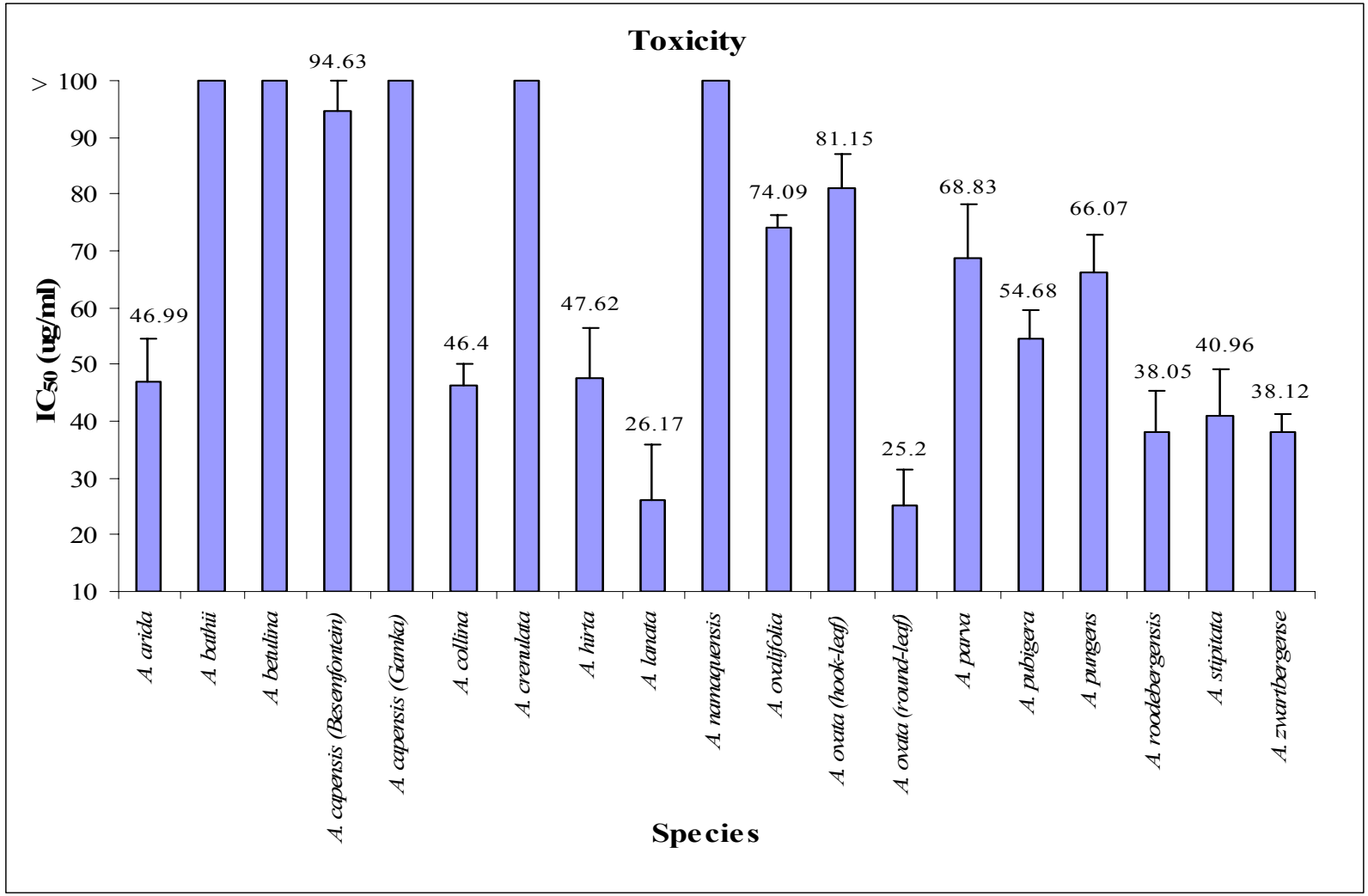


Figure 9.1: Bar graph depicting the toxicity of the extracts of indigenous *Agathosma* species.

n = 4

Dittany (*Dictamnus albus* Linnaeus) is a perennial plant that belongs to the Rutaceae or *citrus* family. Dittany like rue and *citrus*, produces phototoxic furanocoumarins. Specifically, dittany contains 5-methoxypsoralen (bergapten or 5-MOP) and xanthotoxin (8-MOP) which cause phytodermatitis. The mechanism of toxicity to humans depends upon the chemicals' exposure to high energy photons (300 to 350 nm UV). A rash shows up about 24 h after contact with the furanocoumarins and exposure to sunlight (Vandaveer, 2002).

Dictamnus (*Dictamnus dasycarpus*) is one of the most commonly used Chinese herbs for the treatment of skin diseases, especially those with itching, such as eczema and other forms of atopic dermatitis and psoriasis. It was found to be a major ingredient in the formulas taken by six patients who had reported hepatic reactions. This plant was suspected as being the cause of the rare herb-induced hepatitis, as all these cases had dictamnus in common. The implication of dictamnus as a potential culprit in the liver toxicity cases in England was relayed in a recent British review article (McRae *et al*., 2002). The chemical composition reveals a very broad range of potentially active components including *citrus* limonoids (e.g. limonin) and sesquiterpenes (e.g. dictamnol). GC-MS data reveals the presence of dictamnol in *Agathosma roodebergensis* (14.2%) in addition to other species. It also reveals the presence of 8-epi-dictamnol in *Agathosma hirsuta* (trace), *A. roodebergensis* (0.7%) and *A. stipitata* (0.2%). *Agathosma* species, like *Dictamnus dasycarpus*, are also used topically to treat skin diseases and eczema's and the presence of dictamnol may contribute to the toxicity of these species.

Methyl chavicol (= estragole) is present in many species including *Agathosma capensis* (Besemfontein) (2.8%), however there is not enough data to predict its carcinogenic effects. When used as a component of perfumes and cosmetics, geraniol has been classed as a sensitizer since it constantly causes irritation; but it is only found in low levels (less than 1.2%) in the tested *Agathosma* species (Nakayama, 1974).

Methyl eugenol present in amongst others, *Agathosma ovalifolia* (23.0%), has been classed as a hazard since it is genotoxic. It occurs in a few oils as a major component (e.g. *Melaleuca bracteata*, 95% methyl eugenol). Investigations have confirmed genotoxicity and carcinogenicity in rats, probably due to strong DNA-binding reactions (Chan and Caldwell, 1992). Many perfume companies impose in-house restrictions on the use of this material in perfume formulations. Eugenol has frequently induced allergic reactions when used in fragrance formulations and is a component of *Agathosma betulina* and *A. capensis* (Besemfontein) (Loveless *et al*., 1996).

R-(+)-Pulegone is known to be a hepatotoxic compound, where large oral doses have been shown to deplete glutathione, which is needed in one of several detoxification steps. This depletion along with excess pulegone leads to centrilobar hepatocellular necrosis. The acute oral LD_{50} for rats consuming Pennyroyal oil which contains this compound is 0.5g/kg (Burfield, 2000). The compound *cis*-isopulegone present in some *Agathosma* species may contribute to their toxicities.

The compound methyl salicylate present in *Agathosma bathii* (0.1%), *A. capensis* (Besemfontein) (0.1%) and *A. pungens* (0.2%) has been found to be toxic to man (Burfield, 2000). It is used as a counter-irritant in many over-the-counter preparations. The toxicity is of concern especially where children are a consideration, as the substance directly interferes with glucose metabolism, and exhibits central nervous system (CNS) toxicity. The lethal dose for a 70kg man is between 5ml and 30ml. It has been noted that children under five years of age are especially susceptible to salicylate poisoning, and can quickly show physiological symptoms associated with advanced poisoning. There are a large number of studies on absorption of methyl salicylate through the skin which is much more rapid than intestinal absorption (Burfield, 2000). Evidence suggests that blood salicylate levels are highest 20-30 min after application. In a study performed by Collins *et al*. (1984) on the topical absorption of 'Deep-Heat®', an aerosol preparation containing methyl and ethyl salicylate for the relief of rheumatic pain, was analyzed. After a one-shot 500µl spray on the forearm, erythema production was correlated with salicylate concentration and blood salicylate levels reached a maximum after 20 min. The study revealed that methyl salicylate is a CNS poison with acute salicylate poisoning resulting in disorientation, irritability, hallucinations, stupor and coma. The manifestations of salicylism are mainly related to patients with a history of aspirin use (Burfield, 2000).

Ruta graveolens is a bitter herb or garden rue that belongs to the Rutaceae family. It is characterized by a particularly wide spectrum of different plant constituents which belong to four major classes of substances, which may be present in variable amounts in the aerial parts of the plant (European agency for the evaluation of medicinal products, 1999). They include: alkaloids like quinoline, quinolinone (e.g. graveoline),

furoquinoline (e.g. dictamine, γ-fagarine, skimmianine), pyranoquinoline (e.g. rutalinium) and acridone (e.g. furacridone); coumarins like coumarin, dicoumarins, furanocoumarins (e.g. bergapten), pyranocoumarins and coumarin-naphthoquinone-compounds; flavonoids (e.g. rutin) and essential oil components (e.g. 2-nonanone, 2-nonyl acetate, 2-undecyl acetate). Limited data on the acute toxicity of *Ruta graveolens* is available. For the essential oil, acute toxicity was low with oral LD_{50} values slightly above 2g/kg body weight in mice and above 5g/kg body weight in rats. The dermal LD_{50} was found to be higher than 5g/kg body weight in rabbits (European agency for the evaluation of medicinal products, 1999). Deaths have been reported in humans after oral use of the oil or plant extracts for the purpose of abortion (European agency for the evaluation of medicinal products, 1999). A decrease in implantation rate by approximately 90% has been observed in rats after intramuscular application of 40mg and 80mg/kg body weight of an ethanolic extract of dry matter of the plant on day one *post coitum*. Repeated oral administration of approximately 1ml/kg body weight of the essential oil lead to abortion in pregnant guinea pigs, possibly induced by general toxicity (European agency for the evaluation of medicinal products, 1999). Hot water extracts of the plant have been used orally in the form of tea infusions as an abortifacient in humans. In a study performed by Prakash *et al*. (1985), the extracts demonstrated an anti-implantation effect in Albino rats, inhibiting pregnancy in 50-60% of the rats. The mechanism is reported to be due to a compound methyl nonyl ketone (2-undecanone), which induces uterine contractions and pelvic congestion, leading to uterine haemorrhage. The compound was not detected in any of the species investigated in this study.

In terms of carcinogenicity studies, not much has been done but in general, furanocoumarin derivatives (e.g. bergapten) have been shown to increase the incidence of squamous cell carcinoma and other skin cancers like melanoma in human patients treated over long periods against psoriasis. The species is also a photosensitizer in humans. Topical as well as oral exposure may lead to severe photodermatitis. Psoralens or furanocoumarins are photoactive chemicals that applied to the skin and exposed to sunlight produce redness, hyperpigmentation and blistering (Heskel *et al*., 1983). The essential oil is a local irritant. In the European Union the sum of furanocoumarins (e.g. psoralen and bergapten) is not allowed to exceed 1mg/kg in sun protection and bronzing products (European agency for the evaluation of medicinal products, 1999). Phototoxicity has also been found experimentally, in bacteria, fungi and animal ovarian cells, in which mitosis is inhibited and gross chromosomal changes occur. Furoquinolones and canthinones also produce phototoxic effects for which the target seems to be the cell nucleus (Towers and Abramowsky, 1983).

A commercial tincture prepared from Rutae Herba (*Ruta graveolens* L.) exhibited moderate photomutagenicity in a strain of *Chlamydomonas reinhardtii* (Schimmer and Kühne, 1990). In the tincture, some furanocoumarins and furoquinoline alkaloids (e.g. skimmianine) were detected. All compounds revealed photomutagenic properties. The furoquinoline alkaloids were less phototoxic and photomutagenic and this may be due to the fact that furoquinolines form only monoadducts with DNA in the presence of UV-A in contrast to furanocoumarins which also form biadducts (Schimmer and Kühne, 1990).

Limonoids are described as modified triterpenes (Ahn, 1994). Current literature for the last two years is devoted to the identification of the Rutaceae and Meliaceae species containing limonoids (Ahn, 1994). Some of the most exciting applications of limonoids and compounds derived from them are their use in the treatment of specific cancers (Ahn, 1994). Limonoids such as limonin, nomilin, isofraxinellone and their derivatives have been successful treatments when used in *in vitro* bioassays with human tumor cell lines (Ahn, 1994). Limonin and nomilin have been found to induce activity of the detoxifying enzyme glutathione-*S*-transferase. The increased enzyme activity was correlated with the ability of these compounds to inhibit chemically-induced carcinogenesis in laboratory animals. Topical application of limonoids was found to inhibit both the initiation and promotion phases of carcinogenesis of the skin of mice. Limonin appeared to be a potent inhibitor during the promotion phase of the carcinogenesis (Ahn, 1994). This and other findings (Miller *et al*., 1994; Miyazawa *et al*., 1995) suggest that *citrus* limonoids may be useful as cancer chemo-preventative agents.

The aerial parts of selected *Agathosma* species were screened for alkaloids in a study performed by Campbell *et al*. (1987). Positive results were obtained for five species including *Agathosma capensis*, and the compounds halfordamine and skimmianine were identified. Quinoline alkaloids were also detected in *Agathosma barosmaefolia* (Campbell and Bean, 1996). Seven alkaloids including skimmianine were tested against the A2780 human ovarian cancer cell line, and all proved to have weak cytotoxic activity (Chaturvedula *et al*., 2003). A study performed by Cheng *et al*. (1990) found skimmianine to have a selective inhibitory effect on the 5-hydroxytryptamine-induced vasopressor responses of rats. It produced a non-specific

blockade of cardiovascular function at higher concentrations. It was also found to have a significant inhibitory effect on spontaneous motor activity, exploratory behaviour, cataleptogenic activity, conditioned avoidance response and long-term isolation-induced fighting of animals (Cheng, 1986). A mild antimethamphetamine effect was also observed and its neuroleptic activity was found to be less potent than that of chlorpromazine (Cheng, 1986).

Seven naturally occurring furoquinoline alkaloids were investigated for their photobiological activity using cells of *Chlamydomonas reinhardtii* (Schimmer and Kühne, 1991). Dictamnine was found to be the strongest mutagen as well as the most toxic compound of the group. Skimmianine also proved to be mutagenic but not as potent as the others (Schimmer and Kühne, 1991).

Zanthoxylum gilletii and *Zanthoxylum chalybeum* are two species belonging to the Rutaceae family known to be included in arrow poisons (Neuwinger, 1996). *Zanthoxylum gilletii* is characterized by a large content of isoquinoline alkaloids: quinolines, furoquinolines, benzylisoquinolines, benzophenanthridines, aporphines and acridones. Further characteristic constituents are coumarins and isobutylamides. The similarity of alkaloid patterns in all species examined up to now is striking. The occurrence of the furoquinoline nucleus, in particular, can be regarded as a characteristic of the Rutaceae. The quaternary alkaloids are responsible for the high toxicity of these plants (Neuwinger, 1996). It has been found that *Zanthoxylum* species are undoubtedly toxic as shown in *in vivo* studies (Neuwinger, 1996). *Zanthoxylum viridis* killed 100% of the mice with a dose of 10g of bark/kg administered subcutaneously, in 15 min (Neuwinger, 1996). The leaves of

Zanthoxylum parvifolia, which are low in alkaloid content, killed 100% of the mice with a dose of 10g/kg in only 10 min. The aqueous root extract of *Zanthoxylum zanthoxyloides* had a LD_{50} of 8g of extract/kg mice administered intravenously; where the animals died in a clonic convulsive state (Neuwinger, 1996). Intraperitoneal administration of 20g of extract/kg body weight killed 50% of the mice, 60% had clonic convulsions and 1/3 of these mice died of respiratory arrest (Neuwinger, 1996). *Zanthoxylum chalybeum* was found to contain skimmianine, chelerythrine and nitidine. The quaternary alkaloids N-methylcorydine and N-methylisocorydine, magnoflorine and candicine were found to be responsible for the high toxicity of this plant (Neuwinger, 1996). Candicine causes nicotine-like effects in the autonomic nervous system. When administered intravenously to dogs, it produced a rise in blood pressure due to vasoconstriction. High doses produced a curare-like effect and death occurred from respiratory paralysis. It is one of the most poisonous *Zanthoxylum* principles (Neuwinger, 1996).

Overall, previous studies reveal that the alkaloid components of these species are most probably responsible for the toxicities of the extracts, even though a study performed by Olila *et al*. (2002), found skimmianine to have minimal toxicity against VERO cell lines (monkey cell lines). Although determining the presence of alkaloids in these species, isolating them and evaluating their toxicity profiles was not part of the research study, the possibility of alkaloids being present and contributing to the toxicities of the extracts is probable.

It is well known that many herbal medicines can have adverse effects, in which case it is convenient to evaluate the benefit-risk profile. However the toxic effects of

Agathosma species have been poorly studied and no information is available in this regard. The evaluation of the toxic action of plant extracts and essential oils is indispensable in order to consider a treatment safe; it enables the evaluation of the intrinsic toxicity of the plant and the effects of acute overdose. The testing of samples in increasing amounts enables the evaluation of the toxicity limits, and the test should be carried out in at least three doses. In general the MTT assay is useful for the screening of plants extracts and essential oils in order to predict their toxicities. The method offers advantages such as speed, simplicity, lack of radioactivity and animal use and a lower cost. It is a reliable short term assay that can be performed easily. The results obtained indicate that it is a valuable tool for evaluating the toxicity of natural products widely used in popular medicine.

It can be concluded that *Agathosma* species show toxic effects at the concentrations tested. Further studies are necessary to determine whether the MTT cytotoxicity assay truly reflects the likely toxicity of *Agathosma* species *in vivo* and studies need to be performed in order to determine the chemical characterization of the active principles. It would also be interesting to determine the toxicity of pure alkaloids and coumarins *in vivo* and *in vitro*, and to relate this to the toxicity of plants containing the same compounds, hence performing more extensive biological evaluations.

CONCLUSION

- The chemical composition of the essential oils showed qualitative and quantitative differences amongst the 17 species (18 samples) investigated. GC-MS analysis resulted in the identification of 333 compounds in 18 of the samples. The essential oils of *Agathosma betulina* and *A. crenulata* showed notable similarities in their chemical composition. The essential oils of *A. capensis* (Besemfontein) and *A. capensis* (Gamka) also showed remarkable qualitative and quantitative similarities in their chemical composition.

- There was considerable variation in the percentage oil yield of the essential oils. *Agathosma hirsuta* produced the highest yield (1.15%) whilst *A. ovalifolia* produced the lowest yield (0.16%). *Agathosma capensis* (Besemfontein) had a greater oil yield (0.86%) then *Agathosma capensis* (Gamka) (0.68%).

- HPLC analysis revealed that all of the species are rich in flavonoids (i.e. flavones and flavonols). Many of the compounds detected are common to most of the species. A pure coumarin puberulin, was identified in the diethyl ether extract of *Agathosma ovata* (round-leaf). It was detected in the dichloromethane and methanol (1:1) extract of *Agathosma namaquensis*.

- The extracts exhibited greater antimicrobial activity than the essential oils. In general, all of the extracts were active against the four pathogens tested with the exception of *Agathosma bathii* which displayed very poor activity against

Klebsiella pneumoniae (MIC value of 32mg/ml). The MIC values of the extracts ranged between 0.125mg/ml and 32mg/ml, whereas those of the essential oils ranged between 3mg/ml and 32mg/ml. Both the essential oils and extracts exhibited greater activity towards the Gram-positive bacteria than the Gram-negative bacterium, with the extract of *Agathosma ovata* (round-leaf) displaying the greatest activity against *Staphylococcus aureus* (MIC value of 0.156mg/ml) and *Bacillus cereus* (MIC value of 0.125mg/ml). The extract of *Agathosma parva* displayed the greatest activity against *Candida albicans* and *Klebsiella pneumoniae* (MIC value of 1.5mg/ml). Amongst the essential oils, *Agathosma pungens* proved to be the most active against the Gram-positive pathogen, *Bacillus cereus* (MIC value of 3mg/ml). *Agathosma collina* was the most active against *Candida albicans* (MIC value of 3mg/ml). The essential oil of *Agathosma zwartbergense* proved to be the least active against most of the tested bacteria. The antimicrobial activity of the essential oils may be ascribed to oxygenated constituents, such as 1,8-cineole, linalool and carvacrol. The activity of the extracts may be ascribed to constituents such as flavonoids, coumarins and alkaloids.

- The seasonal study of *Agathosma ovata* revealed a substantial variation in the oil yield throughout the year, ranging from 0.23% in early Spring to 0.85% in late Autumn. A higher yield was observed during the flowering season as compared to the non-flowering season. Overall the yields were dependant on the season harvested and proportion of plant parts distilled.

- GC-MS analysis resulted in the identification of 145 compounds in the seasonal variation study. All samples contained a large number of common monoterpenes and had very similar compositions, with minor quantitative variation. Some components common to all samples include: sabinene, *p*-cymene, β-pinene, α-pinene, α-thujene, myrcene, limonene, linalool and terpinen-4-ol. Many of the changes were associated with flowering and the results obtained revealed that the chemical composition of the essential oil of *Agathosma ovata* is subject to seasonal variation.

- The seasonal variation study demonstrated differences in the potency of antimicrobial activity of the essential oils distilled each month. The Winter samples were more active against the Gram-positive bacteria *Bacillus cereus*, *Staphylococcus aureus* and the Gram-negative bacterium *Klebsiella pneumoniae*. Activity in mid Spring was greater against *Staphyloccocus aureus* (MIC value of 3mg/ml) and *Klebsiella pneumoniae* (MIC value of 3mg/ml), whilst activity decreased in Summer. *Bacillus cereus* and *Candida albicans* were not drastically affected. Overall the antimicrobial activity of the essential oil was subject to seasonal variation. The results reveal that the antimicrobial activity of the essential oil of *Agathosma ovata* may not depend on the level of one component but rather the ratio of several components.

- Most of the extracts portrayed moderate to poor activity in the DPPH assay with the exception of *Agathosma capensis* (Gamka) (IC_{50} value of 24.08 ± 4.42μg/ml) and *A. pubigera* (IC_{50} value of 35.61 ± 0.86μg/ml) which were two of the most active species. The essential oils showed very poor activity in the

DPPH assay, all having IC_{50} values > 100µg/ml. The results from the ABTS assay differed from that of the DPPH assay. All extracts showed higher activity in this assay with *Agathosma namaquensis* (IC_{50} value of 15.66 ± 4.57µg/ml) and *A. capensis* (Besemfontein) (IC_{50} value of 19.84 ± 0.09µg/ml) being the most active species. This may be due to the ABTS assay having an additional reaction system. *Agathosma* species are rich in flavonoids which are the compounds most probably responsible for their *in vitro* anti-oxidant activity.

- All of the essential oils exhibited *in vitro* 5-LOX anti-inflammatory activity with the exception of *Agathosma stipitata* which was UV active and showed interference. This was due to its major compounds neral (39.9%) and geranial (10.1%) which absorbed strongly at 234 nm and hence rendered its spectrophotometric measurement impossible. The essential oil of *Agathosma collina* proved to be the most active (IC_{50} value of 25.98 ± 1.83µg/ml) whilst that of *A. bathii* proved to be the least active (IC_{50} value of 76.58 ± 5.44µg/ml). The inhibition of enzyme activity exhibited by the essential oils may be ascribed to the terpenes and sesquiterpenes. The extracts proved to be inactive at 100µg/ml.

- The extracts of *Agathosma lanata* (IC_{50} value of 26.17 ± 9.58µg/ml) and *A. ovata* (round-leaf) (IC_{50} value of 25.20 ± 6.30µg/ml) proved to be the most toxic. The extracts of *Agathosma arida*, *A. capensis* (Gamka), *A. collina*, *A. hirsuta*, *A. ovalifolia*, *A. ovata* (hook-leaf), *A. parva*, *A. pubigera*, *A. pungens*, *A. roodebergensis*, *A. stipitata* and *A. zwartbergense* also proved to be toxic at

the concentrations tested. Another finding from the results obtained was that the extracts of *Agathosma bathii*, *A. capensis* (Besemfontein), *A. betulina*, *A. crenulata* and *A. namaquensis* were not toxic at the concentrations tested. The essential oils of all 19 species proved to be much more toxic (IC_{50} values < 0.0001µg/ml) than a plant-derived compound that is considered relatively safe, namely quinine (IC_{50} value of 136.06 ± 4.06µg/ml). The toxicities of the essential oils may be due to compounds like methyl chavicol, eugenol, methyl eugenol, pulegone and methyl salicylate whilst the toxicities of the extracts may be due to the alkaloid and coumarin components.

The selected indigenous *Agathosma* species investigated displayed the *in vitro* biological activities stated in the objectives. The results emerging from the study may provide scientific evidence for the ethnobotanical uses of *Agathosma* species.

RECOMMENDATIONS

- Determine whether the MTT cytotoxicity assay truly reflects the likely toxicity of *Agathosma* species *in vivo*. Determine the toxicity of the active principle of the most toxic species and relate this to the toxicity of plants containing the same compound.
- Isolate the active principles of the most active species in each of the assays and determine their chemical structures.
- Determine if coumarins and alkaloids are present in these species, isolate them, determine their chemical structures and investigate their biological properties in order to determine if they contribute to the overall biological effects.

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APPENDIX I

MONOGRAPHS

1. *Agathosma arida* P.A. Bean

1. Botanical description
A single-stemmed, round shrublet that grows to a height of 40cm. It has a sweet herb-scent when crushed. The pink or violet flowers are found in terminal clusters, the fruits are three chambered and the ovary is usually three lobed.

2. Distribution
It is found growing in gravelly loam and in the karoo-fynbos ecotone. This species is restricted to the Little Karoo, specifically the northern slopes of Langeberg and Outeniqua Mountains (Goldblatt and Manning, 2000).

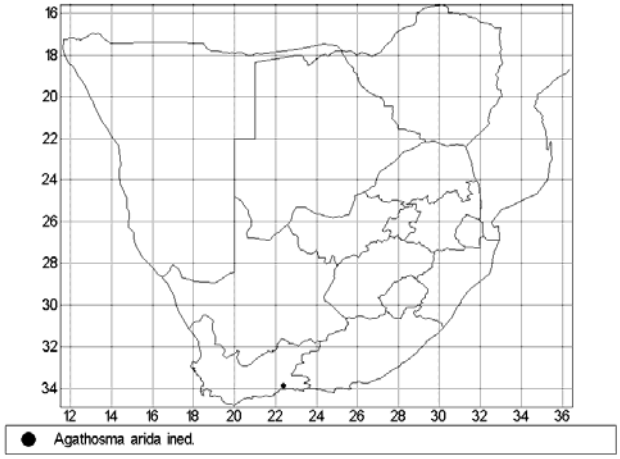


Figure 1: Geographical distribution of *A. arida*.

3. Origin: Rooiberg (TTS 241).

4. Essential oil composition
4.1. Essential oil yield: 0.61% (dry weight).

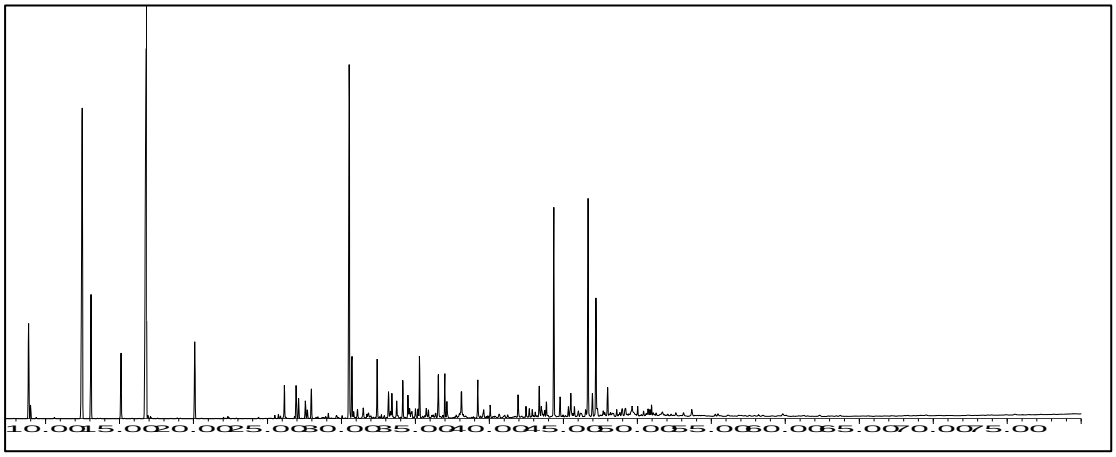


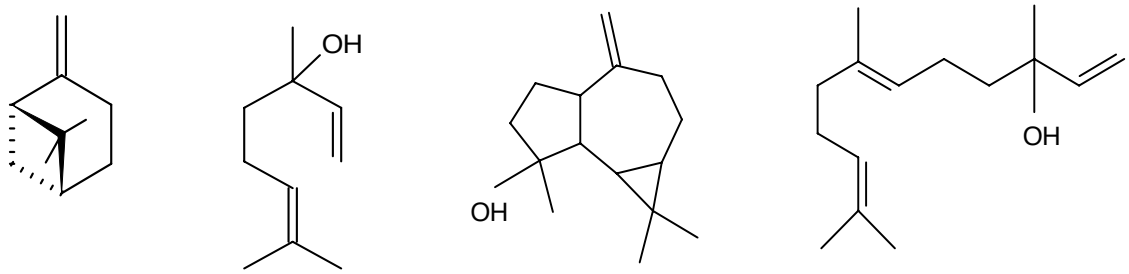
Figure 2: GC-MS chromatogram of *A. arida*.

Table 1: Compounds identified in the essential oil of *A. arida*.

RRI	Compound	%
1032	α-pinene	2.5
1035	α-thujene	0.4
1048	2-methyl-3-buten-2-ol	0.1
1076	camphene	tr
1118	β-pinene	11.4
1132	sabinene	3.6
1174	myrcene	1.9
1280	*p*-cymene	2.0
1429	perillene	0.9
1450	*trans*-linalool oxide (furanoid)	1.0
1458	*cis*-1,2-limonene epoxide	0.5
1468	*trans*-1,2-limonene epoxide	0.4
1478	*cis*-linalool oxide (furanoid)	0.7
1553	linalool	10.1
1562	isopinocamphone	1.7
1571	*trans-p*-menth-2-en-1-ol	0.3
1580	nopinone	0.2
1586	pinocarvone	0.4
1611	terpinen-4-ol	1.4
1639	*trans-p*-mentha-2,8-dien-1-ol	0.7
1648	myrtenal	0.7
1657	umbellulone	0.5
1664	*trans*-pinocarveol	0.9
1678	*cis-p*-mentha-2,8-dien-1-ol	0.6
1687	methyl chavicol	0.3
1700	limonen-4-ol	0.3
1704	methyl geranate	0.3
1706	α-terpineol	1.5
1729	*cis*-1,2-epoxyterpinen-4-ol	0.2
1751	carvone	1.3
1766	decanol	1.0
1772	citonellol	0.5
1804	myrtenol	0.7
1845	*trans*-carveol	0.9
1864	*p*-cymen-8-ol	0.3
1882	*cis*-carveol	0.3
1900	epi-cubebol	0.2
1949	(*Z*)-3-hexenyl nonaoate	0.6
1973	dodecanol	tr
2001	isocaryophyllene oxide	0.1
2008	caryophyllene oxide	0.8
2050	(*E*)-nerolidol	4.7
2071	humulene epoxide II	0.5
2096	elemol	0.7

RRI	Compound	%
2100	4-hydroxy-4-methylcyclohex-2-enone	0.2
2144	spathulenol	5.3
Total		**63.6**

β-pinene (11.4%) and linalool (10.1%) are the major compounds present in the essential oil of *A. arida*. Spathulenol and (*E*)-nerolidol represent 5.3% and 4.7% of the total composition.



β-pinene linalool spathulenol or ledol (*E*)-nerolidol

Figure 3: Structures of the major compounds present in the essential oil of *A. arida*.

5. Non-volatile compounds

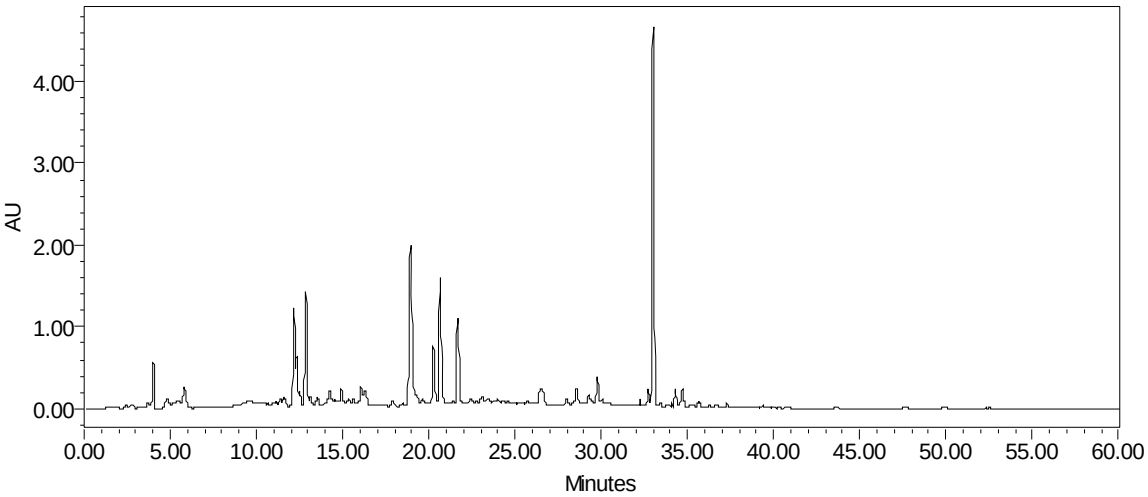


Figure 4: HPLC chromatogram of the dichloromethane and methanol (1:1) extract of *A. arida*.

Table 2: Compounds detected in the crude extract of *A. arida*.

R_t	UV max and / tentative identification	%
4.02	203.8	2.20
5.43	278.1	1.58
5.81	221.5 and 275.8	2.25
9.57	208.5, 321.0 and 370.9	2.76
12.18	258.0	6.16
12.33	212.0 and 258.0	2.60

R_t	UV max and / tentative identification	%
12.88	221.5 and 266.3	7.54
14.25	205.0, 274.6 and 329.3	1.72
14.92	210.9 and 316.2	1.50
16.05	208.5, 260.4 and 352.0 (flavonol)	1.46
16.30	206.2, 267.5 and 326.9	1.79
18.93	203.8, 255.7 and 355.6 (flavonol)	15.51
20.27	206.2, 255.7 and 353.2 (flavonol)	4.30
20.65	203.8, 255.7 and 355.6 (flavonol)	9.85
21.67	201.5, 284.1 and 328.1 (flavanone)	6.90
23.07	208.5 and 324.5	1.43
26.47	212.0, 281.7 and 323.3	3.74
28.56	216.7, 278.1 and 334.1 (flavanone)	2.08
29.27	214.4, 282.9 and 322.1 (flavanone)	1.83
29.77	213.2, 249.7 and 321.0	2.25
33.00	222.6, 341.2 and 360.9	18.79
34.70	235.6 and 341.2	1.77

6. Biological activity

- The extract was the most active against the yeast *Candida albicans* (MIC value of 0.375mg/ml) and also displayed good activity against *Staphylococcus aureus* (MIC value of 0.75mg/ml).
- The essential oil displayed good activity in the anti-inflammatory assay (IC_{50} value of 35.25 ± 5.07µg/ml). The extract was inactive at 100µg/ml.
- The extract was active in both the anti-oxidant assays (IC_{50} value of 40.86 $\pm$ 7.84µg/ml in the DPPH assay and 27.32 $\pm$ 7.84µg/ml in the ABTS assay). The essential oil was inactive at 100µg/ml.
- Both the extract (IC_{50} value 46.99 ± 7.44µg/ml) and essential oil (IC_{50} value < 0.0001µg/ml) were toxic in the MTT assay.

7. References

- Goldblatt P. and Manning J. 2000. Cape Plants: A Conspectus of the Cape Flora of South Africa. National Botanical Institute of South Africa, Pretoria.

2. ***Agathosma bathii*** (Dummer) Pillans

1. Common name
Zebrabuchu.

2. Botanical description
A single-stemmed, broad-leaved shrub that grows to a height of 1m. The flowers have five carpels, are white in colour and dark-spotted.

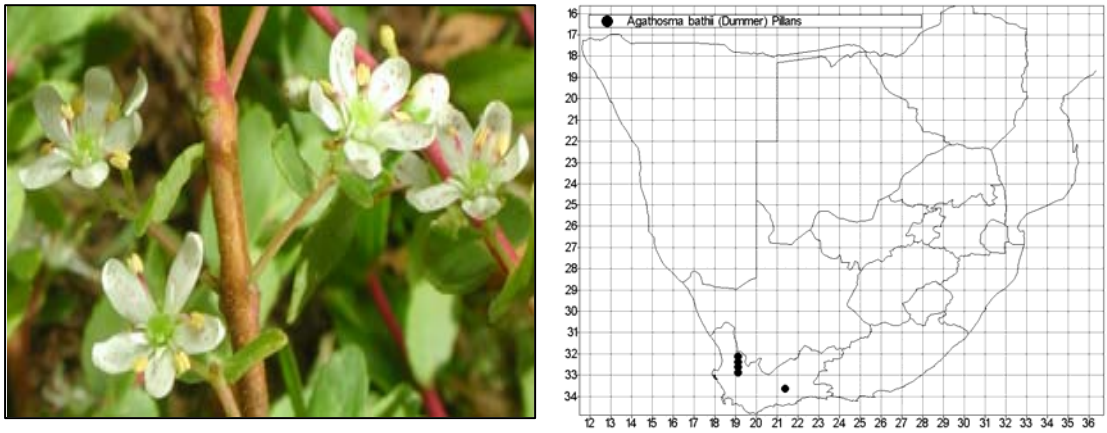


Figure 5: Flower and geographical distribution of *A. bathii*.

3. Distribution
The rocky middle to upper slopes of the north western Cape region (Cederberg Mountains) (Goldblatt and Manning, 2000).

4. Origin: Kleinplaas (AV 1013).

5. Essential oil composition
5.1. Essential oil yield: 0.76% (dry weight).

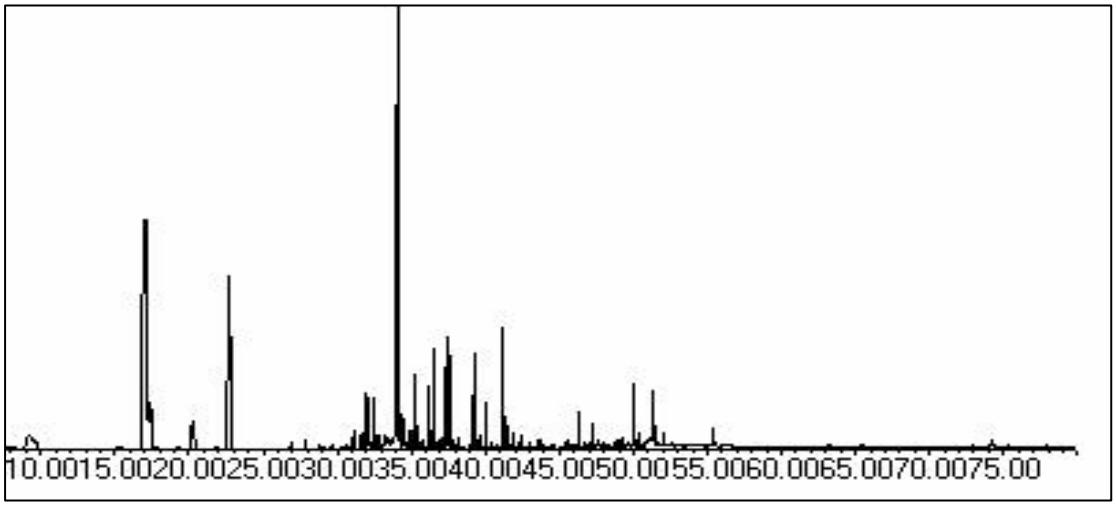


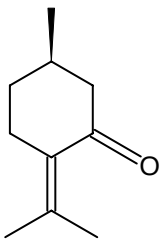
Figure 6: GC-MS chromatogram of *A. bathii*.

Table 3: Compounds identified in the essential oil of *A. bathii*.

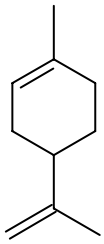
RRI	Compound	%
1032	α-pinene	2.0
1118	β-pinene	0.2
1203	limonene	25.6
1213	1,8-cineole	3.1
1222	*p*-mentha-1,3,8-triene	0.1
1280	*p*-cymene	1.9
1377	3-methylcyclohexanone	9.3
1441	α-*p*-dimethylstyrene	0.2
1475	menthone	0.3
1503	isomenthone	0.2
1536	pinocamphone	0.2
1553	linalool	0.1
1562	isopinocamphone	0.1
1571	methyl citronellate	0.4
1583	*cis*-isopulegone	0.3
1591	α-fenchyl alcohol	0.1
1598	*trans*-isopulegone	1.2
1611	terpinen-4-ol	1.3
1624	*trans*-dihydrocarvone	0.3
1662	pulegone	28.7
1678	*cis*-*p*-mentha-2,8-dien-1-ol	0.2
1690	cryptone	0.1
1700	limonen-4-ol	0.3
1704	methyl geranate	0.3
1706	α-terpineol	1.1
1719	borneol	0.1
1725	verbenone	0.3
1733	pseudodiosphenol	1.3
1748	piperitone	0.1
1751	carvone	1.8
1758	*cis*-piperitol	0.2
1772	citonellol	0.2
1775	8-hydroxymenthone	2.1
1797	*p*-methylacetophenone	0.1
1798	methyl salicylate	0.1
1807	perillaldehyde	0.1
1811	*trans*-*p*-mentha-1(7),8-dien-2-ol	0.1
1834	pulegone epoxide	0.1
1845	*trans*-carveol	1.7
1850	carvone-1,2-epoxide	0.1
1864	*p*-cymen-8-ol	0.3
1882	*cis*-carveol	0.7
1896	*cis*-*p*-mentha-1(7),8-dien-2-ol	0.1
1917	*trans*-*p*-mentha-8-methylthio-3-one	2.1

RRI	Compound	%
1930	*cis*-*p*-mentha-8-methylthio-3-one	0.5
1949	piperitenone	0.3
1989	evodone	0.1
2008	*p*-mentha-1,8-dien-10-ol	0.1
2029	perilla alcohol	0.1
2056	*p*-menth-1-ene-4,8-diol	0.1
2094	*p*-cresol	0.1
2096	elemol	0.1
2124	thymohydroquinone	0.6
2152	dictamnol	0.1
2144	spathulenol	0.1
2176	*trans*-2-oxo-*p*-menthan-8-thiol acetate	0.1
2187	*τ*-cadinol	0.4
2198	*cis*-3-oxo-*p*-menthan-8-thiol acetate	0.1
2239	carvacrol	0.1
2344	menthofurolactone	1.3
Total		**93.4**

Pulegone (28.7%) and limonene (25.6%) are the major compounds present in the essential oil of *A. bathii*. 3-Methylcyclohexanone represents 9.3% of the total composition.



pulegone



limonene

Figure 7: Structures of the major compounds present in the essential oil of *A. bathii*.

6. Non-volatile compounds

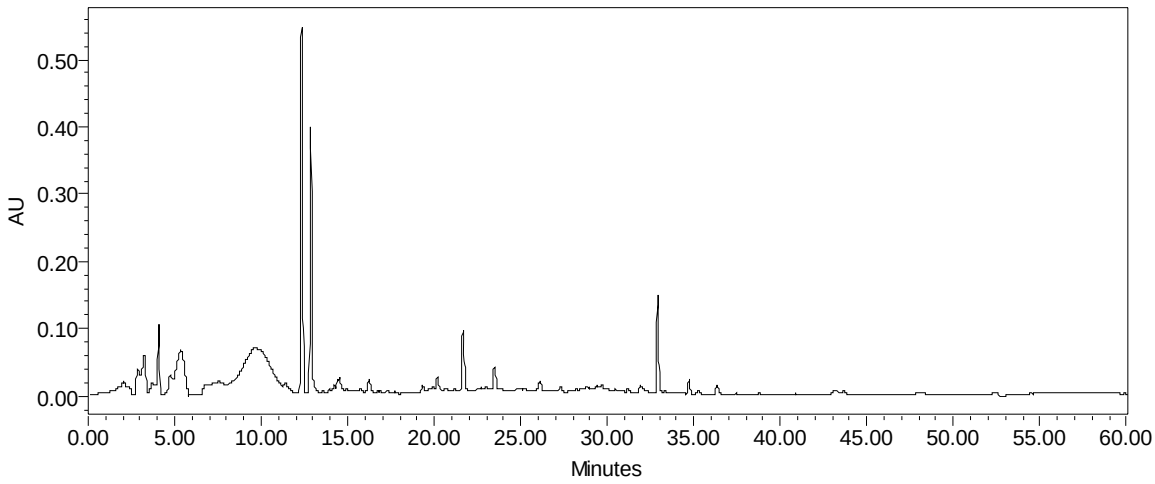


Figure 8: HPLC chromatogram of the dichloromethane and methanol (1:1) extract of *A. bathii*.

Table 4: Compounds detected in the crude extract of *A. bathii*.

R_t	UV max and / tentative identification	%
1.81	209.7, 291.2 and 396.2	2.20
2.04	209.7 and 291.2	2.21
2.87	209.7, 303.1, 329.3, 366.1 and 396.2	1.79
3.23	208.5, 313.8 and 366.1	3.44
3.68	209.7, 303.1, 321.0, 366.1 and 398.6	1.08
4.08	207.3 and 347.2	3.10
4.75	208.5, 303.1, 356.8 and 391.3	1.68
5.33	209.7, 303.1, 330.5 and 356.8	8.20
6.97	209.7, 303.1, 321.0, 356.8 and 391.3	1.25
7.59	207.3, 303.1, 321.0 and 366.1	4.06
9.64	206.2 and 370.9	29.46
11.40	209.7 and 324.5	1.66
12.33	212.0 and 258.0	14.06
12.86	220.3 and 265.1	11.06
14.52	236.8 and 328.1	1.01
16.22	230.9 and 343.6	1.10
20.18	214.4, 274.6 and 324.5	1.13
21.66	284.1 and 326.9 (flavanone)	3.37
23.48	241.5 and 329.3	1.69
26.11	229.7, 282.9, 331.7 and 397.4 (flavanone)	0.94
28.79	206.2, 282.9 and 324.5	0.80
29.67	206.2, 269.9 and 334.1	0.73
32.91	254.5 and 370.9	3.99

7. Biological activity

- The extract and essential oil displayed average activity in the antimicrobial assay. Both were poorly active against the Gram-negative pathogen, *Klebsiella pneumoniae*.

- The essential oil was active in the anti-inflammatory assay (IC_{50} value of 76.58 ± 5.44µg/ml). The extract did not display any activity at 100µg/ml.
- Both the extract and essential oil were inactive at 100µg/ml in the DPPH assay. However, the extract was active in the ABTS assay (IC_{50} value of 29.25 ± 0.59µg/ml).
- The extract did not display toxicity in the MTT assay at the concentrations tested (IC_{50} value > 100µg/ml), however the essential oil was found to be toxic (IC_{50} value < 0.0001µg/ml).

8. References

- Germishuizen G. and Meyer N.L. 2003. Plants of Southern Africa: an annotated checklist. *Strelitzia* **14**. National Botanical Institute, Pretoria, South Africa.
- Goldblatt P. and Manning J. 2000. Cape Plants: A Conspectus of the Cape Flora of South Africa. National Botanical Institute of South Africa, Pretoria.
- Pillans N. 1950. A revision of the genus *Agathosma* (Rutaceae). Journal of South African Botany, **16**: 55.

3. *Agathosma betulina* (P.J. Berguis) Pillans

1. Common name
Buchu, 'Bergboegoe', Round-leaf buchu, Short buchu.

2. Botanical description
A resprouting, broad-leaved, fragrant shrub that grows to a height of two meters. The leaves are of a pale green colour, 20mm long, leathery and glossy; with a blunt, strongly curved tip and a finely toothed margin. Conspicuous round oil glands are scattered throughout the leaf (along the margins and lower surfaces). The leaves are less than twice as long as broad. They are strongly aromatic and the oil is golden in colour, with a strong-sweetish, peppermint-like odour. The flowers are large, star-shaped, five petalled, usually solitary, axillary, and white to purplish pink in color. The brownish fruits are five chambered.

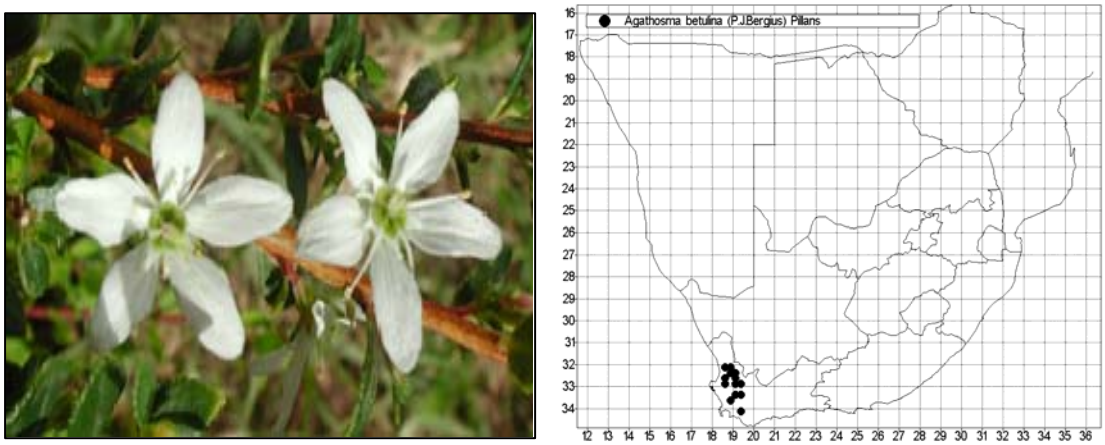


Figure 9: Flower and geographical distribution of *A. betulina*.

3. Distribution
It is particularly adapted to dry conditions, and can be found on sunny hillsides where other crops will not succeed. It can be found on the rocky sandstone slopes, of the north western Cape region (Bokkeveld to Grootwinterhoek Mountains) (Goldblatt and Manning, 2000).

4. Origin: Landmeterskop, Middelberg (AV 852).

5. Traditional uses
The indigenous South African people used the leaves medicinally as an infusion; and powdered and mixed it with sheep fat, to anoint their bodies for cosmetic reasons and as an antibiotic protectant. The leaves were chewed to relieve stomach complaints. An infusion of the leaves in brandy, known as 'Buchu' brandy or 'boegoebrandewyn', is used in the Cape as a stimulant tonic and a remedy for stomach complaints. 'Buchu' vinegar ('boegoe-asyn') was highly regarded for the washing and cleaning of wounds. It has been used to treat kidney and urinary tract diseases, for the symptomatic relief of rheumatism, and also for the external application on wounds and bruises. It has also been used as a tonic to treat minor digestive disturbances (van Wyk and Wink, 2003).

6. Essential oil composition

6.1. Essential oil yield: purchased sample (Afriplex).

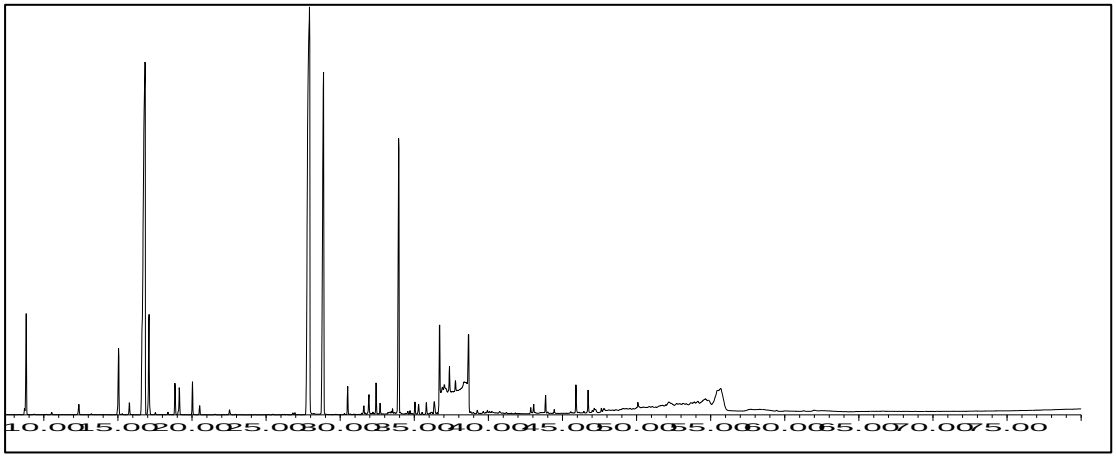


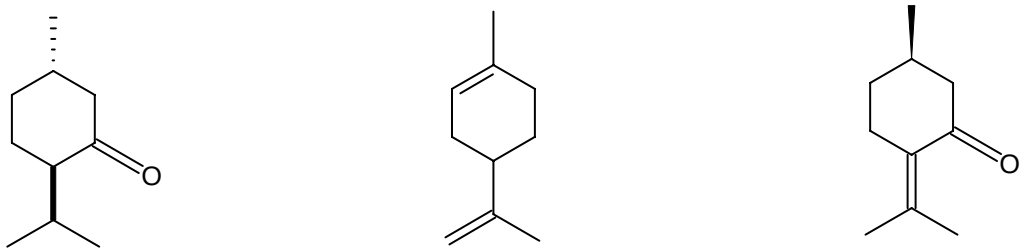
Figure 10: GC-MS chromatogram of *A. betulina*.

Table 5: Compounds identified in the essential oil of *A. betulina*.

RRI	Compound	%
1014	tricyclene	tr
1032	α-pinene	2.4
1040	2-methyl-3-buten-2-one	tr
1076	camphene	0.1
1118	β-pinene	0.3
1141	thuja-2,4(10)-diene	tr
1174	myrcene	1.8
1183	*p*-mentha-1(7),8-diene (= pseudolimonene)	tr
1188	α-terpinene	0.3
1203	limonene	23.7
1213	1,8-cineole	2.7
1215	*p*-mentha-1,3,6-triene	0.1
1246	(*Z*)-β-ocimene	0.1
1255	γ-terpinene	0.7
1266	(*E*)-β-ocimene	0.7
1280	*p*-cymene	0.8
1290	terpinolene	0.2
1329	3-methylcyclohexanone	0.1
1398	(*E*)-4,8-dimethyl-1,3,7-nonatriene (tentative identification)	tr
1437	α-thujone	0.1
1441	α, *p*-dimethylstyrene	0.1
1475	menthone	29.2
1503	isomenthone	14.2
1553	linalool	0.5
1562	isopinocamphone	tr
1576	*cis*-isopulegone	0.2

RRI	Compound	%
1588	*trans*-isopulegone	0.5
1597	neomenthol	tr
1611	terpinen-4-ol	0.7
1614	*trans*-dihydrocarvone	0.2
1631	neoisomenthol	0.1
1641	menthol	0.1
1654	pulegone	8.4
1682	δ-terpineol	0.1
1684	*p*-vinylanisole	tr
1690	cryptone	0.1
1704	myrtenyl acetate	0.3
1706	α-terpineol	0.3
1719	borneol	0.1
1733	neo-dihydrocarveol	0.1
1737	neryl acetate	tr
1748	piperitone + dihydroisomenthone (= *p*-menth-3-en-5-one)	0.4
1751	carvone	tr
1751	*psi*-diosphenol (= pseudo-diosphenol)	2.9
1776	8-hydroxymenthone	0.6
1789	2-hydroxy isomenthone	0.3
1823	diosphenol	2.5
1845	*trans*-carveol	0.1
1864	*p*-cymen-8-ol	0.1
1867	*trans*-8-mercapto-*p*-menthan-3-one	0.1
1871	*p*-mentha-1,8-dien-10-yl acetate	tr
1882	*cis*-carveol	0.1
1900	*cis*-8-mercapto-*p*-menthan-3-one	0.1
1916	*trans*-8-methylthiomenth-3-one	tr
1928	*cis*-8-methylthiomenth-3-one	tr
1949	piperitenone	tr
1984	*trans*-2-acetoxyisomenthone	0.2
1992	*trans*-2-acetoxymenthone	0.2
2008	*p*-mentha-1,8-dien-10-ol	tr
2029	methyl eugenol	0.4
2100	4-hydroxy-4-methyl-cyclohex-2-enone	tr
2104	dimethyl anthranilate	tr
2104	1-hydroxy-pseudo-diosphenol	0.7
2140	4-hydroxy-diosphenol	0.6
2179	*trans*-*p*-menthone-8-thioacetate	0.1
2179	4-vinyl guaiacol + *cis*-*p*-menthon-8-thiolacetate	tr
2184	*cis*-*p*-menth-3-ene-1,2-diol	0.1
2186	eugenol	tr
Total		**98.8**

The major compounds present in the essential oil of *A. betulina* include menthone (29.2%) and limonene (23.7%). Isomenthone and pulegone represent 14.2% and 8.4% of the total composition.



menthone limonene pulegone

Figure 11: Structures of the major compounds present in the essential oil of *A. betulina*.

7. Non-volatile compounds

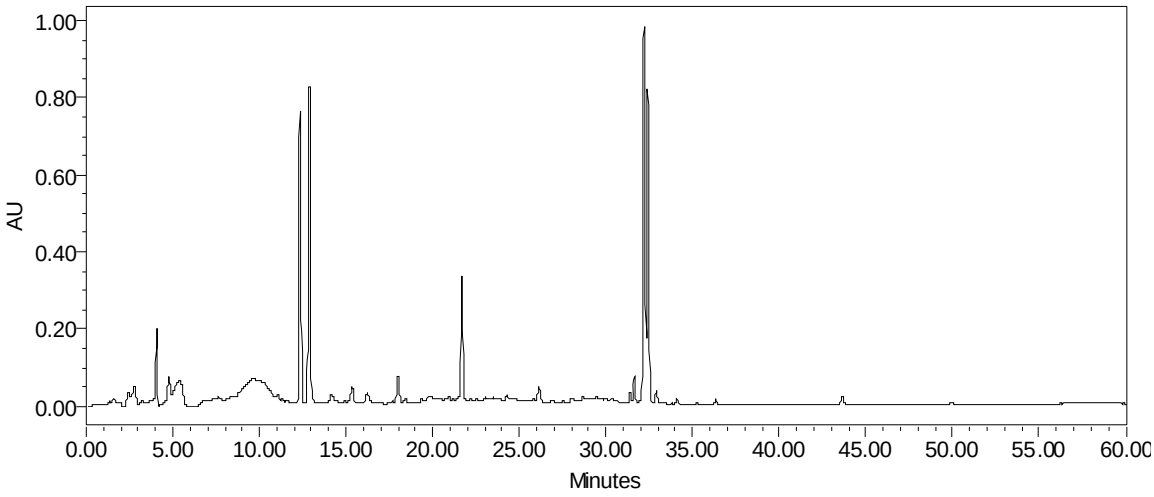


Figure 12: HPLC chromatogram of the dichloromethane and methanol (1:1) extract of *A. betulina*.

Table 6: Compounds detected in the crude extract of *A. betulina*.

R_t	UV max and / tentative identification	%
1.59	203.8, 285.3 and 386.5	0.93
2.79	208.5, 304.3, 338.9, 369.7 and 395.0	1.64
4.06	206.2 and 370.9	2.73
4.77	205.0, 265.1 305.5, 349.6, 369.7 and 395.0	2.12
5.35	208.5, 293.6, 349.6, 369.7 and 387.7	4.29
7.61	207.3, 293.6, 340.1, 359.0, 369.7 and 387.7	2.03
8.32	208.5, 305.5, 319.8, 349.6, 369.7 and 395.0	1.01
12.34	213.2 and 258.0	10.87
12.88	220.3 and 266.3	12.97
14.14	209.7 and 300.7	1.67

R_t	UV max and / tentative identification	%
15.35	210.9, 271.0 and 331.7 (flavone)	1.38
16.23	209.7, 269.9 and 323.3	1.24
17.99	208.5, 255.7 and 354.4 (flavonol)	1.72
19.79	209.7 and 324.5	0.87
20.93	209.7 and 342.4	0.96
21.68	202.7 and 284.1 (flavanone)	5.26
23.04	209.7, 267.5 and 326.9	0.92
23.50	209.7 and 325.7	1.66
26.13	210.9 and 282.9 (flavanone)	1.23
28.71	209.7	1.20
29.46	208.5 and 272.2	1.10
31.63	214.4, 269.9 and 317.4	1.17
32.20	273.4 and 398.6	14.60
32.42	274.6 and 398.6	12.16

8. Biological activity

- The extract was active against the yeast *Candida albicans* in the antimicrobial assay (MIC value of 2mg/ml).
- The essential oil was active in the anti-inflammatory assay (IC_{50} value of 50.37 ± 1.87µg/ml). The extract was inactive at 100µg/ml.
- Both the extract and essential oil were inactive at 100µg/ml in the DPPH assay. However, the extract was active in the ABTS assay (IC_{50} value of 37.75 ± 0.54µg/ml).
- The extract was not toxic in the MTT assay at the concentrations tested (IC_{50} value > 100µg/ml), however the essential oil was found to be toxic (IC_{50} value < 0.0001µg/ml).

9. References

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4. ***Agathosma capensis*** (L.) Dummer

1. Common name
'Boegoe', 'Steenbokboegoe'.

2. Botanical description
A resprouting, multi-stemmed shrub that grows to a height of 90cm and is sweetly spice-scented when crushed. The lower stems are woody while the upper, softer stems are completely clothed with very tiny aromatic leaves. The leaves are needle-like to narrowly elliptical. The inflorescence is a rounded head consisting of small five-petalled flowers with prominent stamens. The flowers are found in lax terminal clusters; white, pink and purple in colour and 8mm in diameter. Flowering occurs throughout the year, peaking between August and November. The fruits are three chambered and the ovary is usually three lobed (Manning, 2003).

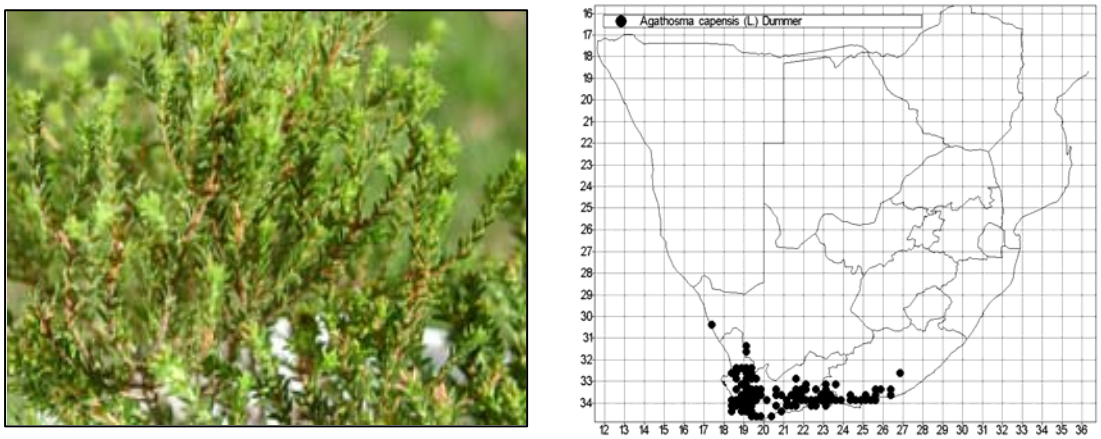


Figure 13: Leaves and stems and geographical distribution of *A. capensis*.

3. Distribution
It is frequently found in soils derived from mineral-rich rocks, and on the slopes and flats from Niewoudtville to Grahamstown. It is also found on slopes and flats on shale, granite or coastal sands, and is found less than often on acid sand. They are usually found resprouting from a persistant rootstock after a fire and are common on sunny mountain slopes and lower down in the coastal scrub. This species is distributed from Namaqualand to Port Elizabeth (Goldblatt and Manning, 2000).

4. Origin: Besemfontein (TTS 348)
Gamka Mountains (JEV 164).

5. Essential oil composition
Two samples of *A. capensis* from different localities were collected and analyzed in order to determine the effect of geographical variation on their chemical compositions. The samples were collected from Besemfontein and Gamka Mountains.

5.1. Essential oil yield:
5.1.1. *A. capensis* (Besemfontein): 0.86% (dry weight).
5.1.2. *A. capensis* (Gamka): 0.68% (dry weight).

A. capensis (Besemfontein) produced a greater oil yield (0.86%) then *Agathosma capensis* (Gamka) (0.68%) which may be attributed to different localities and hence the plants growing under different conditions (e.g. temperature, soil type and climate).

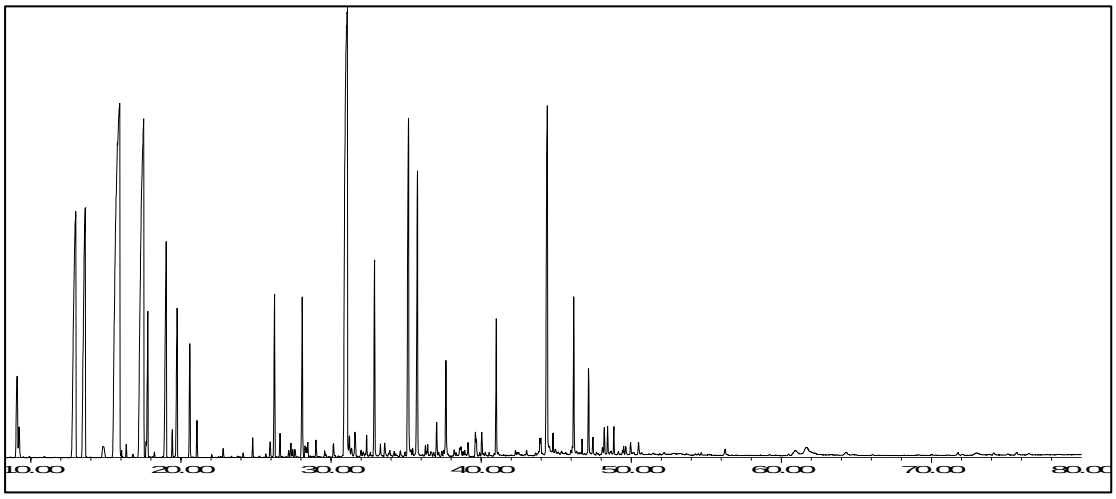


Figure 14: GC-MS chromatogram of *A. capensis* (Besemfontein).

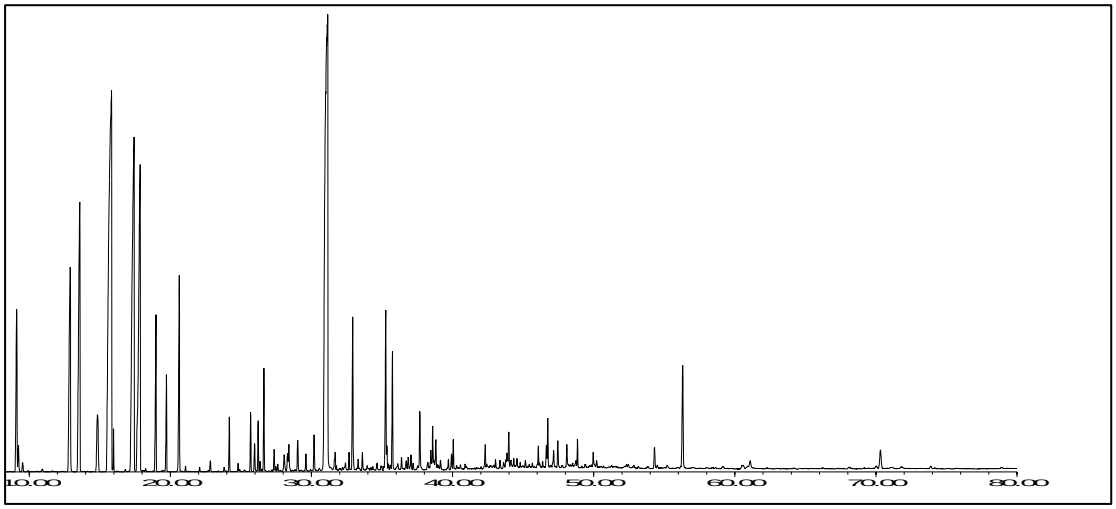


Figure 15: GC-MS chromatogram of *A. capensis* (Gamka).

Table 7: Compounds identified in the essential oils of *A. capensis*.

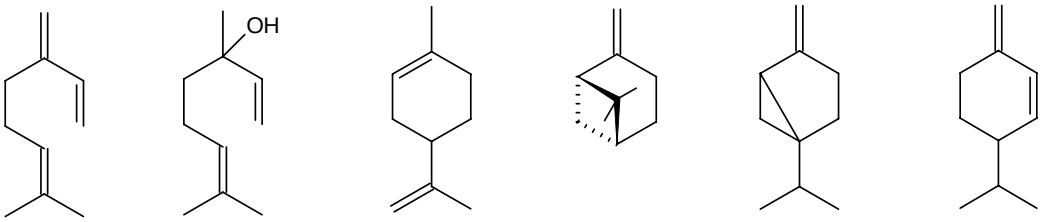
RRI	Compound	% (Besemfontein)	% (Gamka)
1032	α-pinene	1.0	1.6
1035	α-thujene	0.6	0.2
1048	2-methyl-3-buten-2-ol		0.2
1076	camphene		tr
1118	β-pinene	7.9	3.3
1132	sabinene	6.0	4.4
1159	δ-3-carene	0.2	0.9

RRI	Compound	% (Besemfontein)	% (Gamka)
1174	myrcene	26.5	19.3
1183	pseudolimonene	tr	0.2
1188	α-terpinene	0.1	
1195	dehydro-1,8-cineole	tr	
1203	limonene	12.5	9.8
1213	1,8-cineole	1.5	
1218	β-phellandrene	1.1	6.6
1225	(*Z*)-3-hexenal	0.1	
1246	(*Z*)-β-ocimene	2.7	1.5
1255	γ-terpinene	0.2	
1266	(*E*)-β-ocimene	1.3	0.8
1280	*p*-cymene	0.7	1.9
1290	terpinolene	0.2	tr
1319	(*E*)-2,6-dimethyl-1,3,7-nonatriene	tr	tr
1337	geijerene	0.1	0.1
1372	(*E*)-3-hexen-1-ol		0.4
1382	allo-ocimene		0.1
1398	(*E*)-4,8-dimethyl-1,3,7-nonatriene (tentative identification)		0.2
1424	*o*-methylanisole		0.1
1429	perillene	0.1	0.8
1450	*trans*-linalool oxide (furanoid)	tr	0.1
1451	β-thujone	tr	tr
1458	*cis*-1,2-limonene epoxide	0.1	0.1
1468	*trans*-1,2-limonene epoxide	1.1	tr
1476	(*Z*)-β-ocimene epoxide	0.1	0.1
1478	*cis*-linalool oxide (furanoid)	0.1	0.2
1498	(*E*)-β-ocimene epoxide	0.1	0.2
1552	8,9-limonene epoxide	0.1	
1553	linalool	17.1	33.3
1562	isopinocamphone	0.1	
1570	methyl citronellate	0.1	
1571	*trans*-*p*-menth-2-en-1-ol	0.1	0.2
1576	*cis*-isopulegone	tr	
1583	myrcenone	0.2	0.1
1586	pinocarvone	0.1	
1602	6-methyl-3,5-heptadien-2-one		0.1
1611	terpinen-4-ol	1.2	1.2
1616	hotrienol	0.1	
1626	2-methyl-6-methylene-3,7-octadien-2-ol		0.1
1638	*cis*-*p*-menth-2-en-1-ol	0.1	0.2
1648	myrtenal	tr	tr

RRI	Compound	% (Besemfontein)	% (Gamka)
1657	umbellulone	tr	
1664	*trans*-pinocarveol	0.1	0.1
1678	*cis*-*p*-mentha-2,8-dien-1-ol	tr	
1687	methyl chavicol	2.8	
1687	sylveterpineol		tr
1690	cryptone		1.5
1700	limonen-4-ol	tr	tr
1706	α-terpineol	2.1	0.8
1718	(*Z*,*E*)-α-farnesene	tr	
1719	borneol		0.1
1726	germacrene D	0.1	
1729	*cis*-1,2-epoxyterpinen-4-ol	tr	0.1
1744	phellandral		0.1
1748	piperitone	tr	0.1
1751	carvone	0.1	0.1
1758	*cis*-piperitol	tr	tr
1763	naphthalene	0.1	
1772	citronellol	0.6	0.5
1797	*p*-methylacetophenone		0.1
1798	methyl salicylate	0.1	
1802	cuminaldehyde		0.3
1808	nerol		0.1
1828	(*E*)-anethiole	0.2	
1830	2,6-dimethyl-3(*E*),5(*E*),7-octatriene-2-ol	0.2	0.1
1845	*trans*-carveol	tr	0.1
1851	traginone	tr	
1857	geraniol	0.1	0.1
1864	*p*-cymen-8-ol	0.1	0.2
1876	safrole	0.6	
1882	*cis*-carveol	tr	tr
1949	(*Z*)-3-hexenyl nonaoate	0.1	0.3
2008	caryophyllene oxide	0.1	
2029	methyl eugenol	3.8	0.1
2050	(*E*)-nerolidol	0.1	
2053	anisaldehyde	tr	
2096	elemol	tr	
2096	(*E*)-methyl cinnamate	0.7	
2113	cumin alcohol		0.2
2144	spathulenol	0.4	0.1
2148	dictamnol	0.1	0.2
2186	eugenol	0.2	
2219	dimyrcene IIa	0.1	0.1
2239	carvacrol	tr	
2245	elemicin	0.1	

RRI	Compound	% (Besemfontein)	% (Gamka)
2255	α-cadinol	tr	
2269	dimyrcene IIb	tr	tr
	myristicine-like		0.8
Total		**96.4**	**94.5**

Myrcene, linalool, limonene, β-pinene, sabinene and β-phellandrene are the major compounds present in the essential oils of *A. capensis*.



myrcene linalool limonene β-pinene sabinene β-phellandrene

Figure 16: Structures of the major compounds present in the essential oils of *A. capensis*.

TLC analysis has revealed that the essential oils of *A. capensis* (Besemfontein) and *A. capensis* (Gamka) have an almost identical chemical constitution (Chapter three). Both species are very similar in terms of their compositions with the exception of some compounds which are only present in either one of the species (e.g. 2-methyl-3-buten-2-ol, α-terpinene, (*Z*)-3-hexenal, γ-terpinene, allo-ocimene and few other compounds). Although it cannot be certain, the qualitative and quantitative differences in the chemical compositions of the leaf oils of *Agathosma capensis* (Gamka) and *A. capensis* (Besemfontein) may be attributed to the plants growing in different localities and hence under different conditions (e.g. temperature, soil type and climate) . The small differences could also be ascribed to either a genetic or phenetic divergence, or to environmental differences. The similarities are also revealed in the dendrogram obtained from the cluster analysis in which the two samples are closely related (Chapter three).

6. Non-volatile compounds

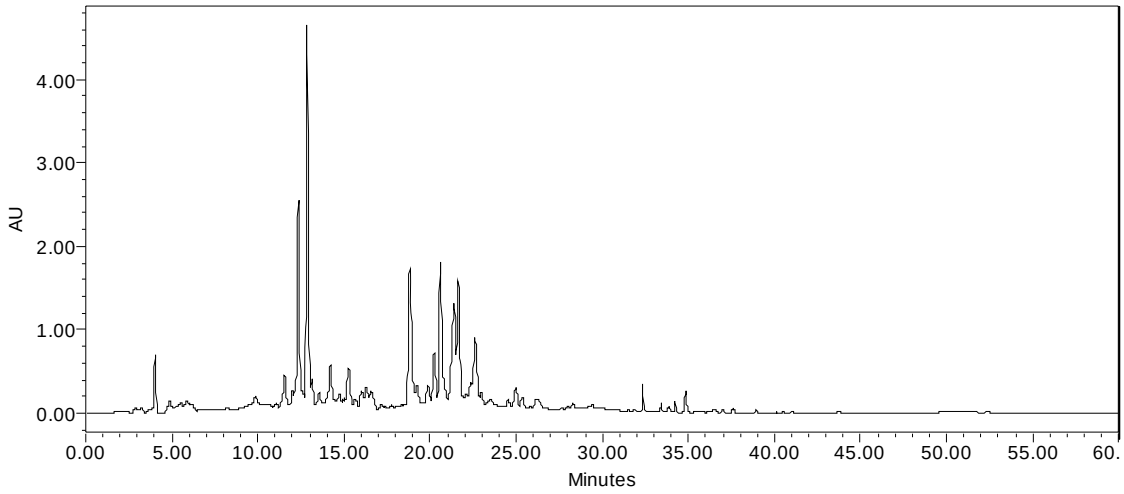


Figure 17: HPLC chromatogram of the dichloromethane and methanol (1:1) extract of *A. capensis* (Besemfontein).

Table 8: Compounds detected in the crude extract of *A. capensis* (Besemfontein).

R_t	UV max and / tentative identification	%
4.03	205.0	2.03
9.55	207.3 and 297.1	1.58
9.86	210.9, 253.3 and 291.2	1.65
11.55	205.0, 266.3 and 296.0	2.80
12.34	210.9 and 258.0	9.59
12.87	210.9 and 268.7 (flavanone)	13.84
13.14	205.0 and 266.3	1.70
13.58	210.9 and 324.5	1.62
14.21	202.7, 258.0 and 330.5 (flavone)	4.38
15.27	205.0, 271.0 and 332.9 (flavone)	2.97
16.30	206.2 and 267.5	1.66
16.58	208.5 and 259.2	1.74
18.82	202.7, 255.7 and 355.6 (flavonol)	9.74
19.24	207.3, 254.5, 299.5 and 352.0	1.96
19.88	206.2 and 296.0	2.07
20.24	205.0, 256.8, 299.5 and 354.4	3.55
20.59	202.7, 255.7 and 355.6 (flavonol)	8.93
21.39	202.7, 255.7 and 350.8 (flavonol)	7.98
21.64	202.7, 284.1 and 332.9 (flavanone)	7.51
22.37	206.2, 255.7, 298.3 and 348.4	1.73
22.61	205.0, 256.8, 297.1 and 352.0	5.26
23.51	206.2 and 324.5	2.23
24.97	207.3 and 319.8	1.77
29.39	207.3 and 293.6	1.71

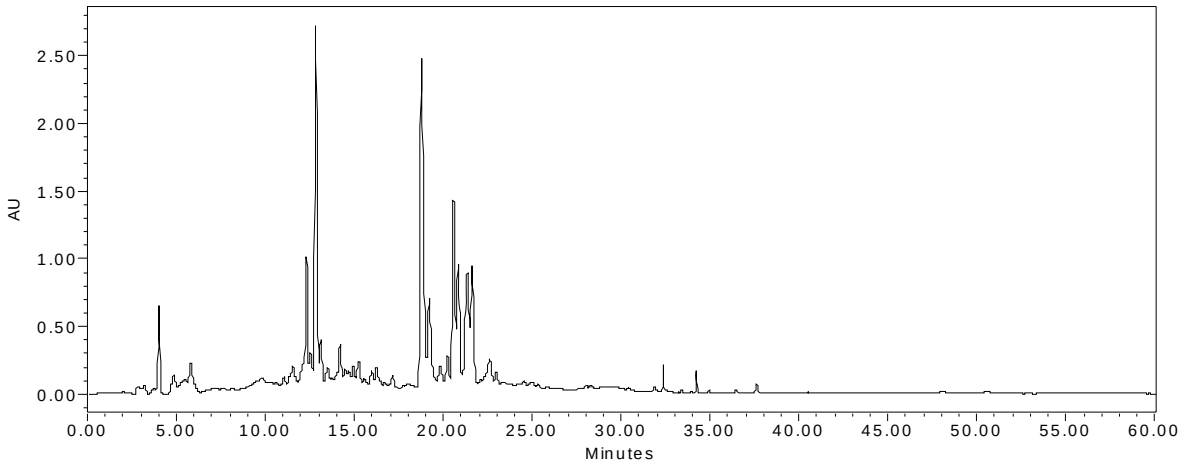


Figure 18: HPLC chromatogram of the dichloromethane and methanol (1:1) extract of *A. capensis* (Gamka).

Table 9: Compounds detected in the crude extract of *A. capensis* (Gamka).

R_t	UV max and / tentative identification	%
4.02	206.2	2.48
5.81	275.8	2.18
9.51	207.3	2.10
11.53	207.3 and 266.3	2.63
12.31	212.0 and 258.0	6.44
12.52	207.3 and 259.2	1.96
12.84	222.6 and 267.5	12.68
13.12	206.2 and 265.1	2.26
13.49	209.7 and 273.4	1.78
14.20	25tr, 275.8 and 332.9 (flavanone)	3.13
15.24	206.2, 271.0 and 329.3 (flavone)	1.88
16.25	207.3 and 267.5	1.81
18.79	201.5, 255.7 and 354.4 (flavonol)	17.65
19.22	206.2, 254.5 and 254.4 (flavonol)	6.01
19.83	207.3 and 296.0	1.93
20.24	206.2, 258.0 and 340.1	2.03
20.57	206.2, 255.7 and 354.4 (flavonol)	7.86
20.84	266.3 and 331.7	5.97
21.35	203.8, 255.7 and 349.6 (flavone)	7.69
21.62	201.5, 284.1 and 332.9 (flavanone)	6.18
22.60	206.2, 255.7, 297.1 and 340.1	3.37

HPLC analysis revealed that both the extracts are rich in flavonoids.

7. Biological activity

- Both the essential oils displayed similar activities in the antimicrobial assay. Both the extracts were active against all pathogens tested.
- Both the essentials oils were active in the anti-inflammatory assay (IC_{50} value of 31.49 ± 3.73µg/ml (Besemfontein) and 44.83 ± 2.98µg/ml (Gamka)). The extracts did not display any activity at 100µg/ml.
- Both the extracts were active in both the anti-oxidant assays (IC_{50} values of 30.79 $\pm$ 0.43µg/ml (Besemfontein) and 24.08 $\pm$ 4.42µg/ml (Gamka) in the DPPH assay; and IC_{50} values of 19.84 $\pm$ 0.09µg/ml (Besemfontein) and 29.93 $\pm$ 1.04µg/ml (Gamka) in the ABTS assay). The essential oils were inactive at 100µg/ml.
- The extract from Besemfontein was not toxic in the MTT assay at the concentrations tested (IC_{50} value > 100µg/ml), however the extract from Gamka Mountains (IC_{50} value of 94.63 ± 5.41µg/ml) and both the essential oils (IC_{50} value < 0.0001µg/ml) were found to be toxic.

8. References

- Campbell W.E., Finch K.P., Bean P.A. and Finkelstein N. 1987. Alkaloids of the Rutoideae: tribe Diosmeae. Phytochemistry, **26**(2): 433.
- Campbell W.E., Majal T. and Bean P.A. 1986. Coumarins of the Rutoideae: tribe Diosmae. Phytochemistry, **25**(3): 655.
- Campbell W.E. and Williamson B.K. 1991. Composition of *Agathosma capensis* essential oil. Planta Medica, **57**: 291.
- Germishuizen G. and Meyer N.L. 2003. Plants of Southern Africa: an annotated checklist. *Strelitzia* **14**. National Botanical Institute, Pretoria, South Africa.
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- Manning J. 2003. Photographic Guide to the Wildflowers of South Africa. Briza Publications, Pretoria, South Africa.
- Pillans N. 1950. A revision of the genus *Agathosma* (Rutaceae). Journal of South African Botany, **16**: 55.
- van Rooyen G. and Steyn H. 1999. South African Wild Flower Guide 10: Cederberg. Botanical Society of South Africa, Cape Town.

5. *Agathosma collina* Ecklon and Zeyher

1. Botanical description
A dense, round, single-stemmed, yellow-green shrub that grows to a height of 1m. The shrub is mildly aromatic and has white flowers that are found in dense terminal clusters. The fruits are three chambered.

2. Distribution
It is found in stabilized dunes, from Agulhas to Stilbaai (Goldblatt and Manning, 2000).

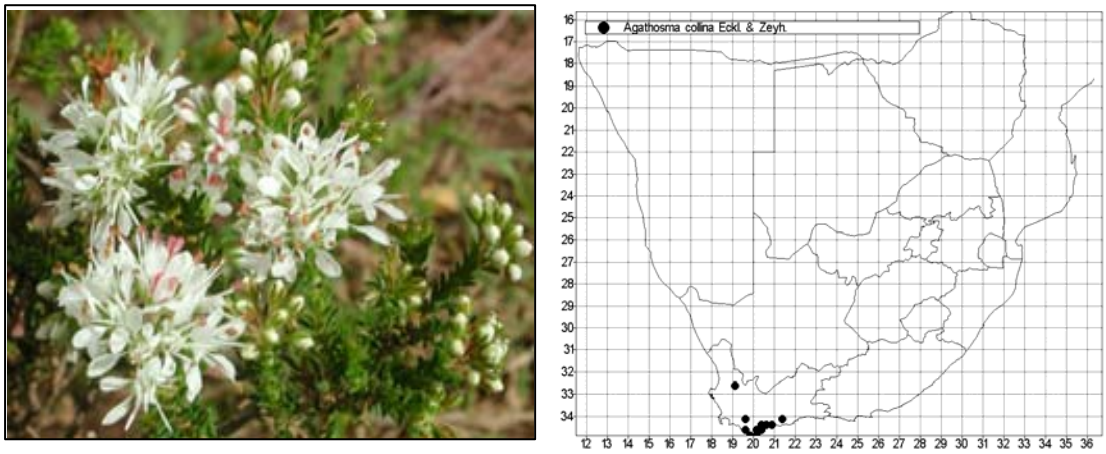


Figure 19: Flower and geographical distribution of *A. collina*.

3. Origin: De Hoop (TTS 328).

4. Essential oil composition
4.1. Essential oil yield: 0.45% (dry weight).

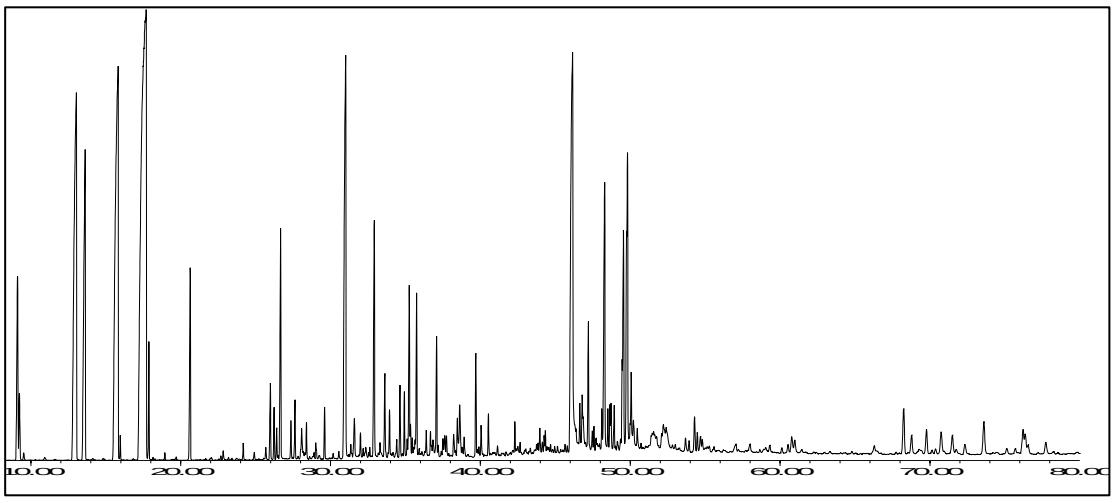


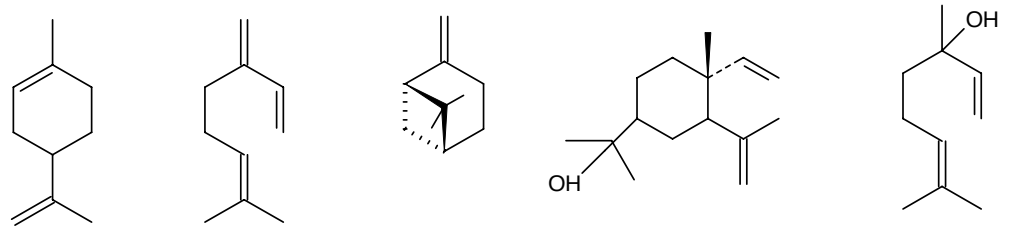
Figure 20: GC-MS chromatogram of *A. collina*.

Table 10: Compounds identified in the essential oil of *A. collina*.

RRI	Compound	%
1032	α-pinene	1.8
1035	α-thujene	0.5
1118	ß-pinene	9.6
1132	sabinene	4.7
1174	myrcene	14.8
1183	pseudolimonene	0.1
1203	limonene	30.9
1218	β-phellandrene	0.5
1280	*p*-cymene	1.2
1384	α-pinene oxide	0.1
1429	perillene	1.3
1450	*trans*-linalool oxide (furanoid)	0.1
1458	*cis*-1,2-limonene epoxide	0.2
1469	*trans*-1,2-limonene epoxide	0.2
1478	*cis*-linalool oxide (furanoid)	0.1
1498	(*E*)-β-ocimene epoxide	0.1
1552	8,9-limonene epoxide I	0.1
1553	linalool	6.0
1560	8,9-limonene epoxide II	0.2
1586	pinocarvone	0.1
1580	nopinone	0.1
1611	terpinen-4-ol	1.4
1626	2-methyl-6-methylene-3,7-octadien-2-ol	0.1
1639	*trans*-*p*-mentha-2,8-dien-1-ol	0.4
1648	myrtenal	0.2
1661	*trans*-pinocarvyl acetate	0.1
1664	*trans*-pinocarveol	0.3
1678	*cis*-*p*-mentha-2,8-dien-1-ol	0.2
1687	methyl chavicol	tr
1690	cryptone	0.7
1700	limonen-4-ol	0.1
1704	γ-muurolene	tr
1706	α-terpineol	0.7
1729	*cis*-1,2-epoxyterpinen-4-ol	0.1
1740	α-muurolene	0.1
1751	carvone	0.6
1758	*cis*-piperitol	tr
1773	δ-cadinene	0.1
1776	γ-cadinene	0.1
1802	cuminaldehyde	0.1
1804	myrtenol	0.3
1845	*trans*-carveol	0.4
1864	*p*-cymen-8-ol	0.1
1882	*cis*-carveol	0.1

RRI	Compound	%
1900	epi-cubebol	tr
1949	(*Z*)-3-hexenyl nonaoate	0.1
2029	methyl eugenol	0.1
2029	perilla alcohol	tr
2096	elemol	6.2
2113	cumin alcohol	0.1
2144	spathulenol	0.5
2148	dictamnol	0.1
2158	eremoligenol	0.1
2157	5-epi-7-epi-α-eudesmol	0.1
2185	γ-eudesmol	2.1
2209	τ-muurolol	tr
2103	α-guaiaol	0.1
2219	dimyrcene IIa	0.2
2225	guaiol	tr
2248	elemicin	0.1
2250	α-eudesmol	1.3
2255	α-cadinol	tr
2257	β-eudesmol	2.3
2393	veratraldehyde	tr
2950	carrisone	tr
Total		**92.3**

Limonene (30.9%) and myrcene (14.8%) are the major compounds present in the essential oil of *A. collina*. β-pinene, elemol and linalool respresent 9.6%, 6.2% and 6.0% of the total composition.



limonene myrcene β-pinene elemol linalool

Figure 21: Structures of the major compounds present in the essential oil of *A. collina*.

5. Non-volatile compounds

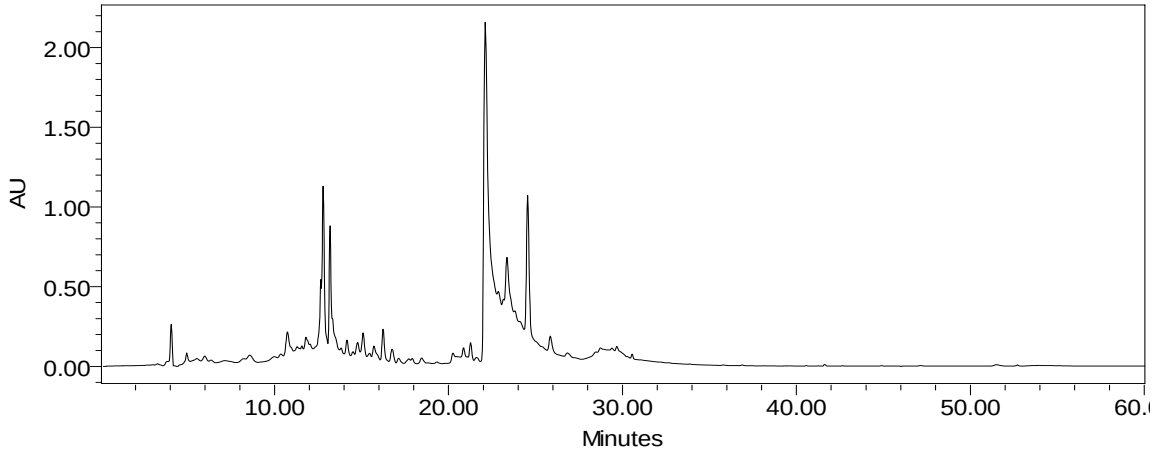


Figure 22: HPLC chromatogram of the dichloromethane and methanol (1:1) extract of *A. collina*.

Table 11: Compounds detected in the crude extract of *A. collina*.

R_t	UV max and / tentative identification	%
4.06	206.3, 278.0 and 371.6	1.27
7.13	207.4, 308.8 and 387.2	0.99
8.57	202.8, 273.3 and 390.8	1.02
9.99	207.4 and 308.8	1.33
10.74	207.4 and 269.7	2.42
11.30	207.4, 269.7 and 308.8	1.31
11.81	207.4, 267.4 and 296.9	1.56
12.67	210.9 and 257.9	3.13
12.79	273.3	6.04
13.19	220.3 and 269.7	6.18
14.16	278.0	1.22
14.78	203.9, 173.3 and 331.3 (flavone)	1.07
15.09	202.8 and 285.1 (flavanone)	1.55
15.71	203.9, 286.3 and 324.2 (flavanone)	1.23
16.24	202.8 and 276.8 (flavanone)	1.47
20.86	206.3 and 272.1	0.95
22.11	207.4, 283.9 and 331.3 (flavanone)	25.37
22.86	201.6, 282.7 and 333.7 (flavanone)	3.85
23.16	201.6 and 282.7 (flavanone)	1.69
23.36	201.6, 283.9 and 329.0 (flavanone)	8.35
23.81	201.6, 282.7 and 331.3 (flavanone)	2.94
24.09	201.6, 283.9 and 332.5 (flavanone)	2.54
24.55	226.2 and 278.0	10.90
25.85	202.8, 278.0 and 327.8 (flavanone)	2.87
26.84	202.8, 283.9 and 330.1 (flavanone)	1.66
28.47	202.8, 282.7 and 333.7 (flavanone)	1.63
28.74	202.8, 283.9 and 340.9 (flavanone)	2.21
29.68	202.8, 283.9 and 330.1	2.11

R_t	UV max and / tentative identification	%
31.04	202.8, 282.7 and 332.5 (flavanone)	1.14

6. Biological activity

- The extract displayed good activity against *Klebsiella pneumoniae* in the antimicrobial assay (MIC value of 2mg/ml). The essential oil was active against *Candida albicans* (MIC value of 3mg/ml).
- The essential oil was the most active in the anti-inflammatory assay (IC_{50} value of 25.98 ± 1.83µg/ml). The extract did not display any activity at 100µg/ml.
- The extract was active in both the anti-oxidant assays (IC_{50} value of 54.65 $\pm$ 6.34µg/ml in the DPPH assay and 39.98 $\pm$ 0.36µg/ml in the ABTS assay). The essential oil was inactive at 100µg/ml.
- Both the extract (IC_{50} value of 46.40 ± 3.77µg/ml) and essential oil (IC_{50} value < 0.0001µg/ml) were toxic in the MTT assay.

7. References

- Campbell W.E., Finch K.P., Bean P.A. and Finkelstein N. 1987. Alkaloids of the Rutoideae: tribe Diosmeae. Phytochemistry, **26**(2): 433.
- Campbell W.E., Majal T. and Bean P.A. 1986. Coumarins of the Rutoideae: tribe Diosmae. Phytochemistry, **25**(3): 655.
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- Goldblatt P. and Manning J. 2000. Cape Plants: A Conspectus of the Cape Flora of South Africa. National Botanical Institute of South Africa, Pretoria.
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- Schwegler M. 2003. Medicinal and Other Uses of Southern Overberg Fynbos Plants. Durban, South Africa.

6. *Agathosma crenulata* (L.) Pillans

1. Common name
Buchu, 'Anysboegoe', Long-leaf buchu.

2. Botanical description
An intensely aromatic, woody, single-stemmed shrub which reaches a height of 2.5m.The glossy, dark green leaves are more than twice as long as they are broad and have oil glands throughout them. The delicate stems bear one to three, relatively large white or mauve flowers in the leaf axils. The flowers have five carpels. Flowering occurs between June and November. The oil is pale in colour with a sharp pulegone note (van Rooyen and Steyn, 1999).

3. Distribution
It is found growing on the damp lower and middle slopes and valleys, from Ceres to Swellendam (Goldblatt and Manning, 2000).



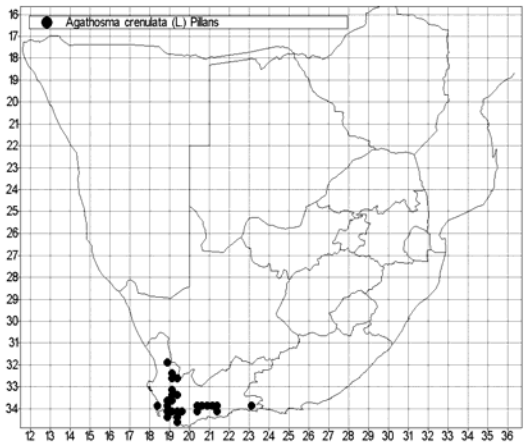


Figure 23: Flower and geographical distribution of *A. crenulata*.

4. Origin: Welbedacht, Tulbagh (AV 853).

5. Traditional uses
It has been used as a stimulant tonic and soothing stomach remedy. In a vinegar based lotion, the oil has been used to treat bruises and sprains. When the leaves are brittle after being dried, they possess a strong aromatic, black currant-like aroma and can be used to make a tea that is useful for burning urination, urinary tract infections, digestive problems, gout, rheumatism, coughs, and colds.

6. Essential oil composition
6.1. Essential oil yield: Purchased (Afriplex).

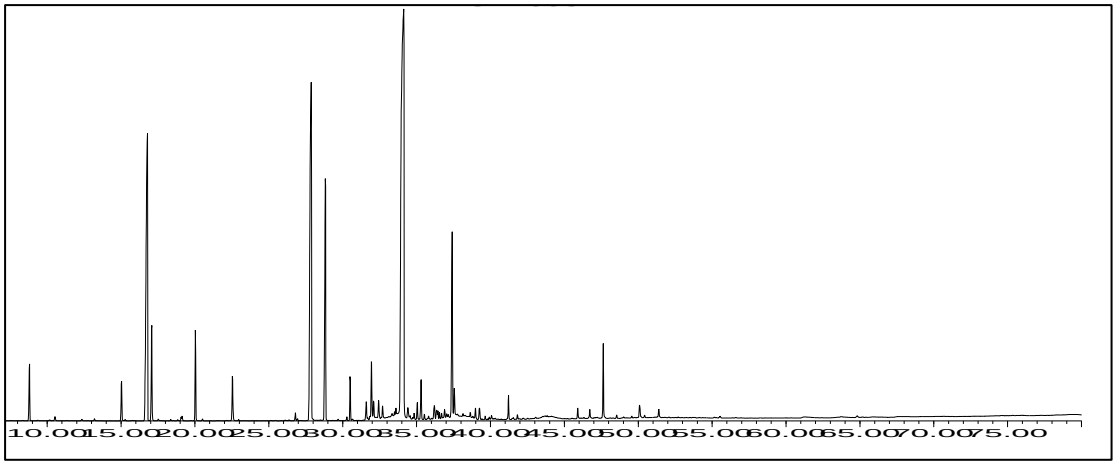


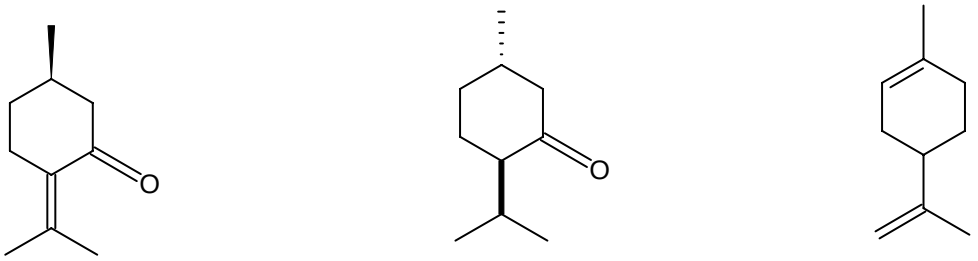
Figure 24: GC-MS chromatogram of *A. crenulata*.

Table 12: Compounds identified in the essential oil of *A. crenulata*.

RRI	Compound	%
1032	α-pinene	1.3
1040	2-methyl-3-buten-2-one	tr
1061	α-fenchene	tr
1076	camphene	0.1
1118	β-pinene	0.1
1141	thuja-2,4(10)-diene	0.1
1174	myrcene	0.9
1183	*p*-mentha-1(7),8-diene (= pseudolimonene)	tr
1203	limonene	13.4
1213	1,8-cineole	2.4
1224	*o*-mentha-1(7),5,8-triene	0.1
1246	(*Z*)-β-ocimene	tr
1250	menthatriene isomer (correct isomer not identified)	0.1
1255	γ-terpinene	tr
1266	(*E*)-β-ocimene	0.1
1280	*p*-cymene	2.0
1290	terpinolene	tr
1329	3-methylcyclohexanone	1.2
1429	perillene	tr
1437	α-thujone	tr
1441	α, *p*-dimethylstyrene	0.2
1475	menthone	16.6
1503	isomenthone	7.3
1532	camphor	tr
1553	linalool	0.9
1562	isopinocamphone	0.1
1562	menthyl acetate	tr
1576	*cis*-isopulegone	0.5
1588	*trans*-isopulegone	1.6

RRI	Compound	%
1611	terpinen-4-ol	0.5
1614	*trans*-dihydrocarvone	0.4
1634	*cis*-isodihydrocarvone	0.1
1654	pulegone	34.9
1682	δ-terpineol	0.1
1690	cryptone	tr
1704	myrtenyl acetate	0.4
1706	α-terpineol	1.0
1719	borneol	0.1
1725	verbenone	0.1
1733	*cis*-piperitone oxide	0.5
1748	piperitone	0.3
1751	carvone	0.1
1751	*psi*-diosphenol (= pseudo-diosphenol)	0.1
1772	citronellol	0.1
1776	8-hydroxymenthone	4.9
1804	myrtenol	0.1
1823	diosphenol	0.1
1834	8-hydroxy isomenthone	0.3
1845	*trans*-carveol	0.4
1857	geraniol	tr
1864	*p*-cymen-8-ol	0.1
1867	*trans*-8-mercapto-*p*-menthan-3-one	0.1
1871	*p*-mentha-1,8-dien-10-yl acetate	tr
1882	*cis*-carveol	0.1
1882	*cis*-myrtanol	tr
1900	*cis*-8-mercapto-*p*-menthan-3-one	tr
1916	*trans*-8-methylthiomenth-3-one	0.5
1928	*cis*-8-methylthiomenth-3-one	tr
1949	piperitenone	0.1
2008	*p*-mentha-1,8-dien-10-ol	tr
2029	methyl eugenol	tr
2104	1-hydroxy-pseudo-diosphenol	0.3
2140	4-hydroxy-diosphenol	0.2
2179	*trans*-*p*-menthon-8-thioacetate	1.7
2183	*cis*-*p*-menthon-8-thioacetate	0.1
2184	*cis*-*p*-menth-3-ene-1,2-diol	0.1
2247	*trans*-α-bergamotol	tr
2344	menthofurolactone	0.2
2426	8-(3-oxo-2-*p*-menthanylthio)-3-*p*-menthanone-I	tr
2453	8-(3-oxo-2-*p*-menthanylthio)-3-*p*-menthanone-II	tr
Total		**97.0**

Pulegone (34.9%) and menthone (16.6%) are the major compounds present in the essential oil of *A. crenulata*. Limonene and isomenthone represent 13.4% and 7.3% of the total composition.



pulegone menthone limonene

Figure 25: Structures of the major compounds present in the essential oil of *A. crenulata*.

7. Non-volatile compounds

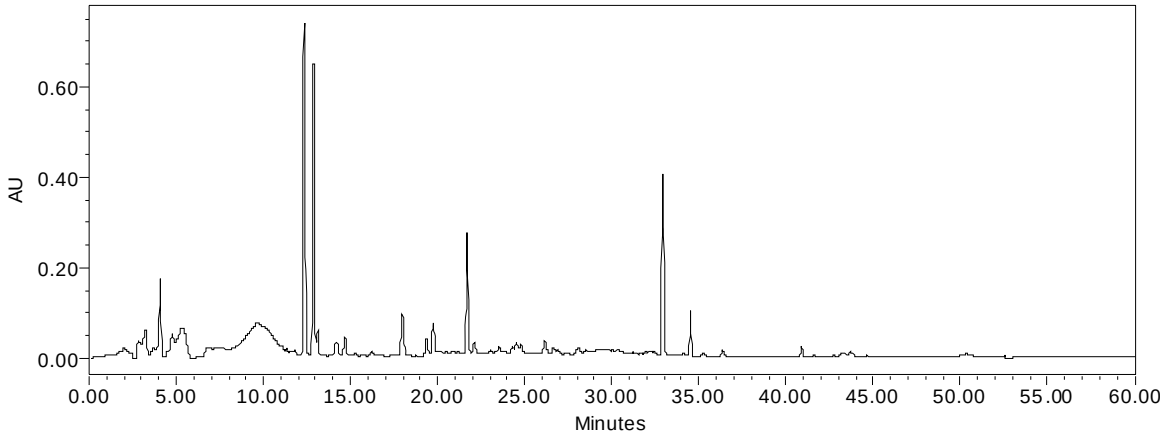


Figure 26: HPLC chromatogram of the dichloromethane and methanol (1:1) extract of *A. crenulata*.

Table 13: Compounds detected in the crude extract of *A. crenulata*.

R_t	UV max and / tentative identification	%
1.79	210.9, 287.6 and 399.8	1.32
2.01	203.8, 287.6 and 399.8	1.73
2.84	208.5, 301.9, 331.7, 346.0 and 370.9	1.33
3.23	207.3, 297.1, 350.8, 375.7 and 398.6	2.64
4.08	202.7, 274.6 and 370.9	3.97
4.77	206.2, 261.6, 324.5, 350.8, 375.7 and 398.6	2.22
5.36	207.3, 300.7, 331.7, 367.3 and 393.8	6.21
6.84	203.8, 286.5, 331.7, 350.8, 367.3 and 398.6	1.74
7.32	207.3, 331.7, 350.8, 375.7 and 398.6	1.19
7.62	207.3, 331.7, 350.8, 375.7 and 398.6	1.74
9.65	206.2, 373.3 and 396.2	23.03
12.34	212.0 and 258.0	14.46
12.88	220.3 and 265.1	12.91
13.12	203.8 and 293.6	1.10

R_t	UV max and / tentative identification	%
14.21	222.6, 271.0 and 366.1 (flavonol)	1.27
14.68	234.4 and 398.6	1.27
17.97	205.0, 255.7 and 354.4 (flavonol)	2.88
19.75	214.4 and 323.3	2.22
21.69	284.1 and 329.3 (flavanone)	5.55
32.92	254.5	8.27
34.52	233.2 and 366.1	1.64
55.51	212.0 and 366.1	1.32

8. Biological activity

- The extract displayed good activity in the antimicrobial assay (MIC value of 2mg/ml against *Bacillus cereus*, *Candida albicans* and *Staphylococcus aureus*).
- The essential oil was active in the anti-inflammatory assay (IC_{50} value of 59.15 ± 7.44µg/ml). The extract did not display any activity at 100µg/ml.
- Both the extract and essential oil were inactive at 100µg/ml in the DPPH assay. However, the extract was active in the ABTS assay (IC_{50} value of 33.32 ± 0.33µg/ml).
- The extract was not toxic in the MTT assay at the concentrations tested (IC_{50} value > 100µg/ml), but the essential oil was found to be toxic (IC_{50} value < 0.0001µg/ml).

9. References

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7. *Agathosma hirsuta* (Lam.) Bartl. and H.L.Wendl.

1. Botanical description
A resprouting, dense, leafy shrublet that grows to a height of 60cm. The white flowers are located in terminal clusters. The fruits are three chambered.

2. Distribution
It is found in seasonal seeps and lower slopes and dunes, from Humansdorp to Port Elizabeth (Goldblatt and Manning, 2000).

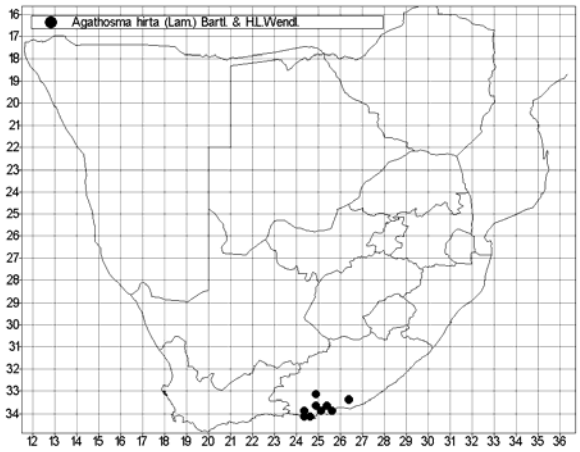


Figure 27: Geographical distribution of *A. hirsuta*.

3. Origin: Khamiesberg (TTS 310).

4. Essential oil composition
4.1. Essential oil yield: 1.15% (dry weight).

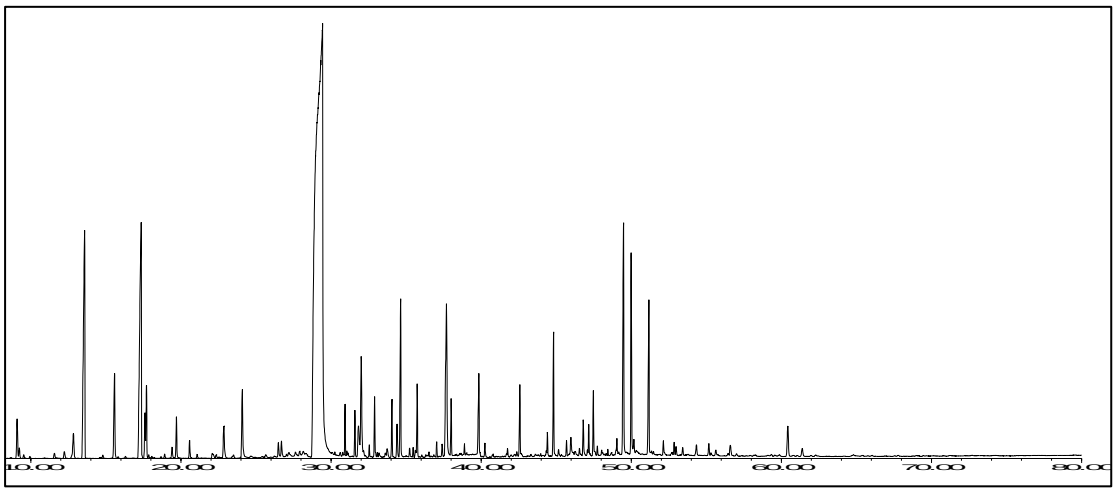


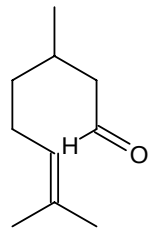
Figure 28: GC-MS chromatogram of *A. hirsuta*.

Table 14: Compounds identified in the essential oil of *A. hirsuta*.

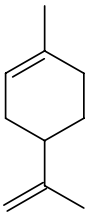
RRI	Compound	%
1032	α-pinene	0.3
1035	α-thujene	0.1
1118	ß-pinene	0.3
1132	sabinene	3.2
1159	δ-3-carene	tr
1174	myrcene	0.8
1203	limonene	3.8
1213	1,8-cineole	0.3
1218	β-phellandrene	0.8
1246	(*Z*)-β-ocimene	tr
1255	γ-terpinene	tr
1266	(*E*)-β-ocimene	0.3
1280	*p*-cymene	0.1
1290	terpinolene	tr
1319	(*E*)-2,6-dimethyl-1,3,7-nonatriene	0.1
1337	geijerene	0.3
1365	melonal	0.6
1458	*cis*-1,2-limonene epoxide	tr
1459	(*Z*)-3-hexenyl 2-methylbutyrate	0.1
1474	*trans*-sabinene hydrate	0.1
1487	citronellal	72.5
1490	isogeijerene	tr
1553	linalool	0.3
1556	*cis*-sabinene hydrate	0.1
1564	neoisopulegol	0.4
1570	methyl citronellate	0.2
1583	isopulegol	1.0
1611	terpinen-4-ol	0.3
1612	β-caryophyllene	0.1
1612	guai-6,9-diene	tr
1638	*cis*-*p*-menth-2-en-1-ol	tr
1650	γ-elemene	0.4
1655	2,6-dimethyl-5-hepten-1-ol	0.4
1668	citronellyl acetate	0.9
1682	δ-terpineol	0.1
1690	cryptone	0.1
1706	α-terpineol	0.4
1737	neryl acetate	tr
1755	bicyclogermacrene	0.1
1772	citronellol	1.3
1786	kessane	0.5
1815	2-tridecanone	tr
1854	germacrene B	0.5
1911	2-dodecanol	0.1

RRI	Compound	%
2019	2-pentadecanone	0.1
2029	methyl eugenol	tr
2050	(*E*)-nerolidol	0.6
2069	germacrene-D-4-ol	tr
2071	*cis*-*p*-menthane-1,8-diol	0.1
2095	methyl *p*-methoxybenzoate	tr
2098	globulol	0.1
2117	guaiol acetate	0.2
2131	8-epi-dictamnol	tr
2245	elemicin	1.8
2254	citronellic acid	0.8
2269	guaia-6,10(14)-dien-4β-ol	0.4
	a fatty acid ester	0.9
2465	methyl veratrate	tr
2620	methyl 3,4,5-trimethoxybenzoate	tr
Total		**95.9**

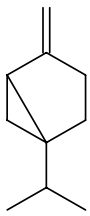
Citronellal (72.5%) is the major compound present in the essential oil of *A. hirsuta*. Limonene and sabinene represent 3.8% and 3.2% of the total composition.



citronellal



limonene



sabinene

Figure 29: Structures of the major compounds present in the essential oil of *A. hirsuta*.

5. Non-volatile compounds

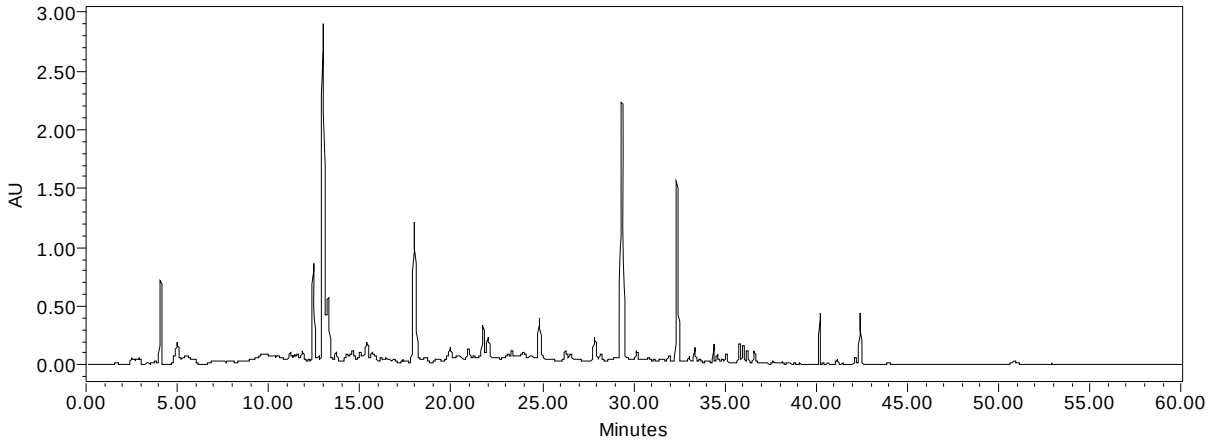


Figure 30: HPLC chromatogram of the dichloromethane and methanol (1:1) extract of *A. hirsuta*.

Table 15: Compounds detected in the crude extract of *A. hirsuta*.

R_t	UV max and / tentative identification	%
4.10	203.8 and 269.6	3.56
4.99	203.8 and 267.5	2.06
5.57	208.5, 280.5, 373.3 and 395.0	1.61
9.72	208.5 and 317.4	4.59
12.45	212.0 and 258.0	4.98
12.98	216.7 and 265.1	19.23
13.26	216.7 and 287.6 (flavanone)	4.70
14.61	209.7 and 326.9	1.39
15.39	214.4, 271.0 and 331.7 (flavone)	1.98
17.98	206.2, 255.7 and 354.4 (flavonol)	10.17
19.95	206.2 and 322.1	1.78
20.47	208.5, 266.3 and 326.9	1.41
21.75	203.8, 284.1 and 332.9 (flavanone)	2.18
22.02	216.7 and 326.9	2.33
23.09	209.7 and 326.9	1.41
23.97	210.9 and 322.1	1.71
24.84	213.2 and 259.2	3.42
27.86	217.9, 261.6 and 292.4	2.08
29.35	208.5 and 265.1	17.00
32.37	208.5, 268.7, 326.9 and 398.6	8.74
40.19	242.7 and 275.8	1.39
42.40	232.0 and 317.4	2.29

6. Biological activity

- The extract displayed good activity in the antimicrobial assay against *Bacillus cereus* and *Staphylococcus aureus* (MIC values of 0.75mg/ml and 0.25mg/ml).
- The essential oil was active in the anti-inflammatory assay (IC_{50} value of 58.23 ± 1.54µg/ml). The extract did not display any activity at 100µg/ml.

- Both the extract and essential oil were inactive at 100µg/ml in the DPPH assay. However, the extract was active in the ABTS assay (IC_{50} value of 26.30 ± 0.25µg/ml).
- Both the extract (IC_{50} value of 47.62 ± 8.88µg/ml) and essential oil (IC_{50} value < 0.0001µg/ml) were found to be toxic in the MTT assay.

7. References

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- Goldblatt P. and Manning J. 2000. Cape Plants: A Conspectus of the Cape Flora of South Africa. National Botanical Institute of South Africa, Pretoria.
- Pillans N. 1950. A revision of the genus *Agathosma* (Rutaceae). Journal of South African Botany, **16**: 55.

8. *Agathosma lanata* P.A. Bean

1. Botanical description
A dense, harsh, round shrub that grows to a height of 80cm. It branches profusely at ground level and is herb-scented. The white flowers occur in dense, wooly terminal clusters. The fruits are three chambered and the ovary is usually three lobed.

2. Distribution
It is found growing on the dry rocky upper slopes of the Rooiberg and Outeniqua Mountains (Goldblatt and Manning, 2000).

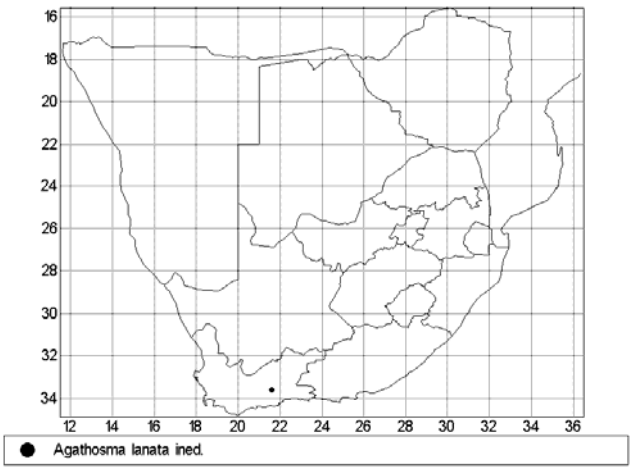


Figure 31: Geographical distribution of *A. lanata*.

3. Origin: Rooiberg (TTS 242).

4. Essential oil composition
4.1. Essential oil yield: 0.19% (dry weight).

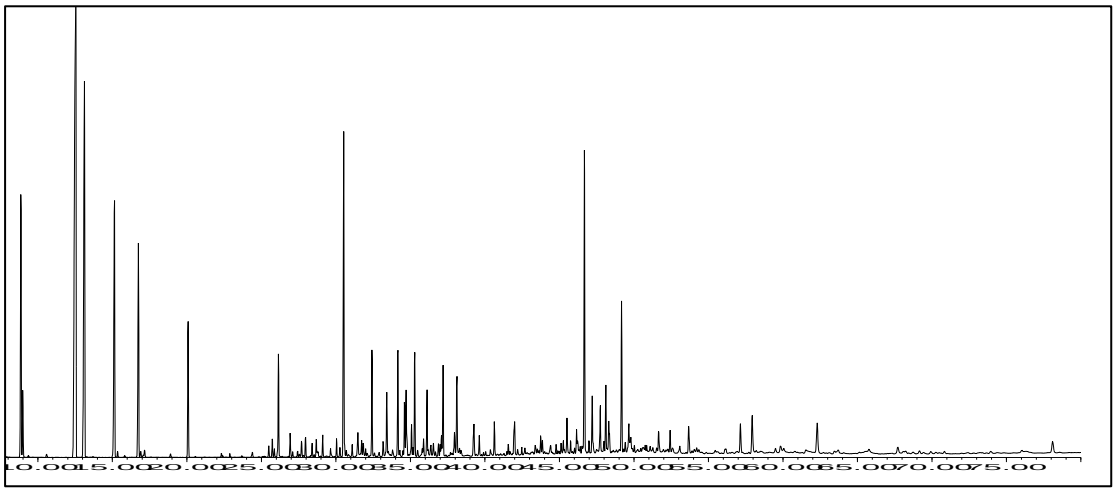


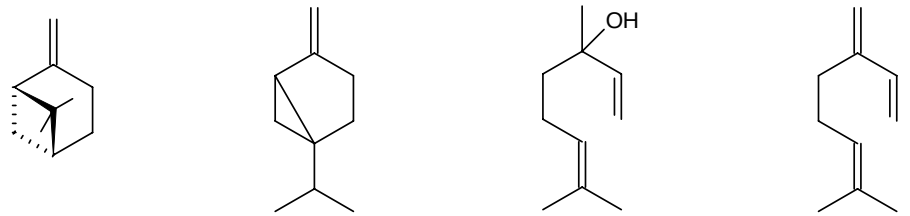
Figure 32: GC-MS chromatogram of *A. lanata*.

Table 16: Compounds identified in the essential oil of *A. lanata*.

RRI	Compound	%
1032	α-pinene	4.7
1035	α-thujene	1.0
1076	camphene	0.1
1118	β-pinene	16.9
1132	sabinene	8.9
1174	myrcene	5.4
1183	pseudolimonene	0.1
1203	limonene	4.0
1213	1,8-cineole	tr
1218	β-phellandrene	0.1
1255	γ-terpinene	0.1
1280	*p*-cymene	2.2
1337	geijerene	0.1
1384	α-pinene oxide	0.1
1398	(*E*)-4,8-dimethyl-1,3,7-nonatriene (tentative identification)	0.2
1429	perillene	1.6
1450	*trans*-linalool oxide (furanoid)	0.4
1458	*cis*-1,2-limonene epoxide	0.1
1466	α-cubebene	0.1
1474	*trans*-sabinene hydrate	0.3
1478	*cis*-linalool oxide (furanoid)	0.3
1479	δ-elemene	0.2
1497	α-copaene	0.3
1535	β-bourbonene	0.2
1553	linalool	5.7
1571	*trans*-*p*-menth-2-en-1-ol	0.2
1580	nopinone	0.3
1586	pinocarvone	0.5
1611	terpinen-4-ol	1.7
1639	*trans*-*p*-mentha-2,8-dien-1-ol	0.3
1648	myrtenal	1.0
1664	*trans*-pinocarveol	1.8
1687	methyl chavicol	0.9
1690	cryptone	1.3
1706	α-terpineol	1.6
1729	*cis*-1,2-epoxyterpinen-4-ol	0.3
1740	α-muurolene	1.0
1751	carvone	0.2
1758	*cis*-piperitol	0.1
1763	naphthalene	0.2
1773	δ-cadinene	0.3
1773	γ-cadinene	1.3
1797	*p*-mentha-1,5-dien-7-ol	0.2

RRI	Compound	%
1802	cuminaldehyde	0.5
1804	myrtenol	1.4
1853	calamenene	0.8
1864	*p*-cymen-8-ol	0.4
1882	*cis*-carveol	0.1
1900	epi-cubebol	0.5
1941	α-calacorene	0.1
1949	(*Z*)-3-hexenyl nonaoate	0.3
1957	cubebol	0.5
1973	dodecanol	0.1
1984	γ-calacorene	0.2
2029	methyl eugenol	0.2
2029	perilla alcohol	0.3
2071	humulene epoxide II	0.2
2080	cubenol	0.2
2088	1-epi-cubenol	0.2
2096	elemol	0.6
2113	cumin alcohol	0.4
2144	spathulenol	4.9
2187	τ-cadinol	0.8
2209	τ-muurolol	1.0
2219	dimyrcene IIa	0.5
2255	α-cadinol	2.4
2343	10-hydroxycalamenene	0.4
2568	oplopanone	0.7
2676	manool	1.0
Total		**85.0**

β-pinene (16.9%) and sabinene (8.9%) are the major compounds present in the essential oil of *A. lanata*. Linalool and myrcene represent 5.7% and 5.4% of the total composition.



β-pinene sabinene linalool myrcene

Figure 33: Structures of the major compounds present in the essential oil of *A. lanata*.

5. Non-volatile compounds

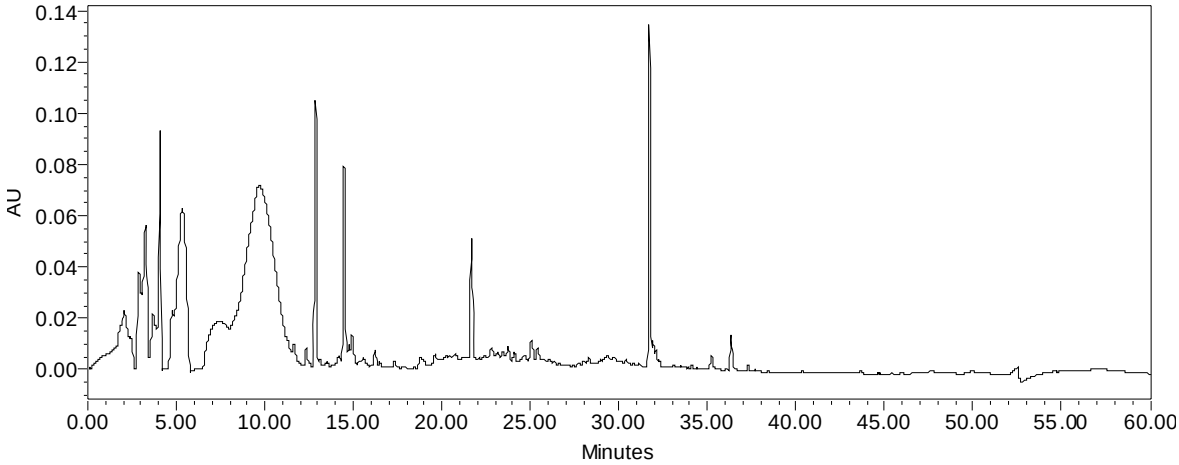


Figure 34: HPLC chromatogram of the dichloromethane and methanol (1:1) extract of *A. lanata*.

Table 17: Compounds detected in the crude extract of *A. lanata*.

R_t	UV max and / tentative identification	%
0.88	203.8, 278.1, 292.4, 315.0, 336.5, 362.9, 382.9 and 397.4	0.76
1.15	203.8, 278.1, 296.0, 315.0, 328.1, 354.4, 370.9, 384.1 and 397.4	0.47
1.83	202.7, 287.6, 382.9 and 397.4	2.06
2.05	202.7, 287.6 and 397.4	3.14
2.89	206.2, 285.3, 348.4, 370.9 and 397.4	2.39
3.26	206.2, 285.3, 341.2 and 376.9	4.33
3.67	202.7, 263.9, 313.8, 348.4, 366.1, 382.9 and 397.4	2.01
4.08	207.3, 370.9 and 399.8	3.40
4.76	205.0, 315.0, 341.2, 366.1 and 382.9	1.49
5.34	206.2, 288.8, 304.3, 319.8, 347.2 and 379.3	10.43
7.37	202.7, 297.1, 319.8, 341.2 and 391.3	4.38
7.56	203.8, 306.7, 319.8, 348.4, 379.3 and 392.6	2.52
9.68	205.0 and 370.9	40.86
11.65	208.5, 267.5, 336.5, 369.7 and 384.1	0.95
12.34	210.9, 258.0 and 391.3	0.52
12.87	220.3 and 266.3	3.90
14.47	205.0, 271.0 and 355.6 (flavonol)	3.36
14.92	230.9 and 317.4	0.75
16.22	233.2, 273.4 and 324.5	0.45
21.67	227.3, 284.1 and 330.5 (flavanone)	2.10
31.72	221.5 and 348.4	4.49
31.91	223.8 and 397.4	0.46
36.34	225.0 and 277.0	0.52
55.50	212.0, 292.4, 343.6, 368.5 and 395.0	4.25

6. Biological activity

- The extract was active in the antimicrobial assay against *Staphylococcus aureus* (MIC value of 1.5mg/ml).
- The essential oil was active in the anti-inflammatory assay (IC_{50} value of 54.81 ± 8.52µg/ml), whilst the extract did not display any activity at 100µg/ml.
- Both the extract and essential oil were inactive at 100µg/ml in the DPPH assay. However, the extract was active in the ABTS assay (IC_{50} value of 26.30 ± 0.25µg/ml).
- Both the extract (IC_{50} value of 26.17 ± 9.58µg/ml) and essential (IC_{50} value < 0.0001µg/ml) were toxic in the MTT assay.

7. References

- Goldblatt P. and Manning J. 2000. Cape Plants: A Conspectus of the Cape Flora of South Africa. National Botanical Institute of South Africa, Pretoria.

9. *Agathosma namaquensis* Pillans

1. Distribution



Figure 35: Geographical distribution of *A. namaquensis*.

2. Origin: Khamiesberg (TTS 289).

3. Essential oil composition

3.1. Essential oil yield: 1.03% (dry weight).

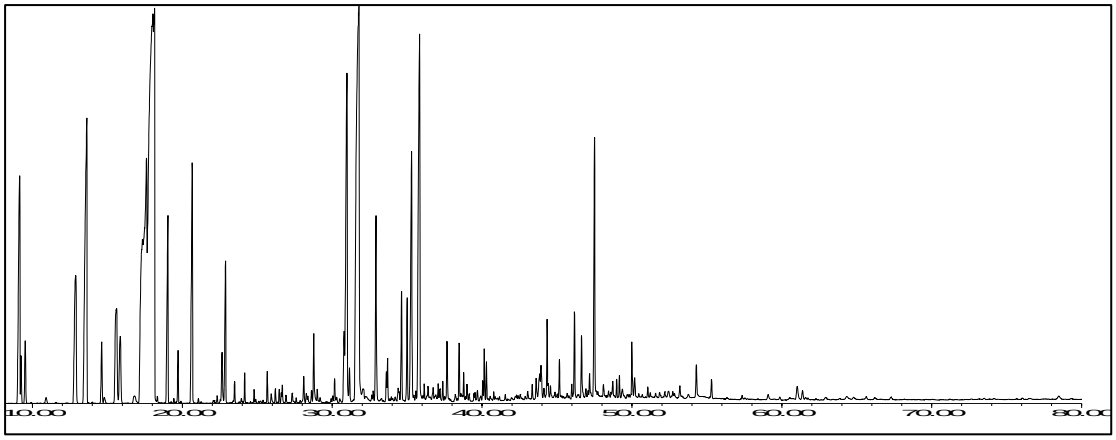


Figure 36: GC-MS chromatogram of *A. namaquensis*.

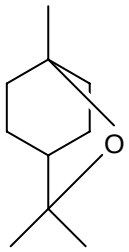
Table 18: Compounds identified in the essential oil of *A. namaquensis*.

RRI	Compound	%
1032	α-pinene	3.9
1035	α-thujene	0.4
1048	2-methyl-3-butenol	0.8
1076	camphene	tr
1118	β-pinene	2.2
1132	sabinene	6.2
1146	methyl 4-methylpentanoate	0.4
1159	δ-3-carene	0.1

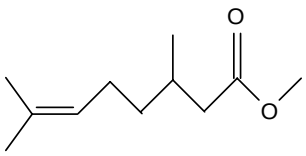
RRI	Compound	%
1174	myrcene	1.6
1183	pseudolimonene	0.9
1195	dehydro-1,8-cineole	0.1
1203	limonene	8.5
1213	1,8-cineole	22.1
1218	β-phellandrene	10.0
1246	(*Z*)-β-ocimene	1.7
1255	γ-terpinene	tr
1266	(*E*)-β-ocimene	0.3
1280	*p*-cymene	2.2
1290	terpinolene	tr
1327	3-methyl-2-butenol	0.1
1337	geijerene	1.4
1382	allo-ocimene	0.1
1384	α-pinene oxide	0.1
1409	rosefuran	tr
1429	perillene	0.1
1450	*trans*-linalool oxide (furanoid)	0.1
1474	*trans*-sabinene hydrate	0.2
1476	(*Z*)-β-ocimene epoxide	tr
1478	*cis*-linalool oxide (furanoid)	tr
1487	citronellal	0.5
1490	iso-geijerene	0.1
1498	(*E*)-β-ocimene epoxide	0.1
1553	linalool	4.6
1571	methyl citronellate	10.0
1580	isopulegol	0.1
1611	terpinen-4-ol	1.0
1638	*cis*-*p*-menth-2-en-1-ol	0.1
1641	methyl benzoate	0.2
1651	citronellyl acetate	0.4
1655	2,6-dimethyl-5-hepten-1-ol	tr
1682	δ-terpineol	0.6
1690	cryptone	1.7
1706	α-terpineol	5.3
1729	*cis*-1,2-epoxyterpinen-4-ol	0.1
1744	phellandral	0.1
1751	carvone	0.1
1758	*cis*-piperitol	0.1
1772	citronellol	0.3
1802	cuminaldehyde	0.2
1815	*p*-mentha-1,3-dien-7-al	0.1
1823	*p*-mentha-1(7),5-dien-2-ol	0.1
1830	2,6-dimethyl-3(*E*),5(*E*),7-octatrien-2-ol	0.1
1845	*trans*-carveol	0.1
1851	traginone	0.1

RRI	Compound	%
1857	geraniol	tr
1864	*p*-cymen-8-ol	0.1
1866	methyl hydrocinnamate	0.2
1882	*cis*-carveol	0.7
1981	(*Z*)-methyl cinnamate	0.1
2001	isocaryophyllene oxide	0.1
2008	caryophyllene oxide	0.1
2030	methyl eugenol	0.3
2088	(*E*)-methyl cinnamate	0.3
2113	cumin alcohol	0.2
2144	spathulenol	0.1
2148	dictamnol	1.5
2198	thymol	0.1
2239	carvacrol	0.2
2241	*p*-isopropylphenol	0.1
2254	citronellic acid	0.1
Total		**93.8**

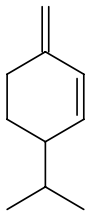
The major compounds present in the essential oil of *A. namaquensis* include 1,8-cineole (22.1%), methyl citronellate (10.0%) and β-phellandrene (10.0%). Limonene, sabinene and α-terpineol represent 8.5%, 6.2% and 5.3% of the total composition.



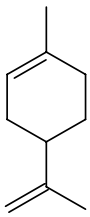
1,8-cineole



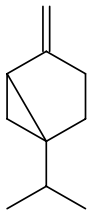
methyl citronellate



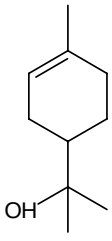
β-phellandrene



limonene



sabinene



α-terpineol

Figure 37: Structures of the major compounds present in the essential oil of *A. namaquensis*.

4. Non-volatile compounds

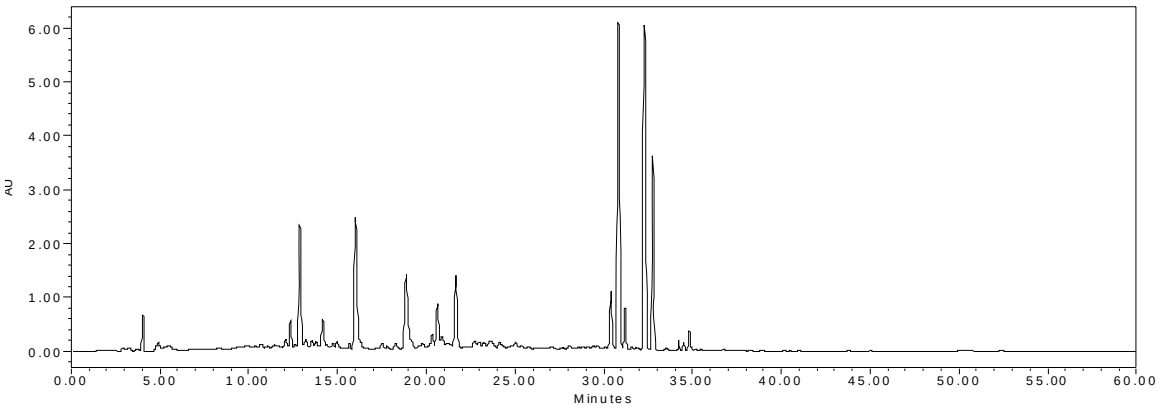


Figure 38: HPLC chromatogram of the dichloromethane and methanol (1:1) extract of *A. namaquensis*.

Table 19: Compounds detected in the crude extract of *A. namaquensis*.

R_t	UV max and / tentative identification	%
4.04	202.7 and 271.0	1.53
5.50	203.8, 279.3 and 379.3	1.00
9.61	207.3 and 312.6	1.21
9.90	208.5 and 292.4	0.95
12.35	212.0 and 258.0	1.58
12.87	221.5 and 269.9	6.42
14.16	212.0, 266.3 and 338.9	1.97
16.01	207.3 and 337.7	9.94
18.86	206.2, 255.7 and 354.4 (flavonol)	6.67
20.34	207.3 and 335.3	1.41
20.62	206.2, 256.8 and 353.2 (flavonol)	2.92
20.88	208.5, 254.5 and 355.6 (flavonol)	0.92
21.65	202.7, 284.1 and 331.7 (flavanone)	4.88
22.71	207.3, 254.5 and 336.5 (flavone)	1.02
23.65	208.5, 269.9, 296.0 and 326.9	1.36
24.12	210.9, 268.7 and 300.7	1.02
30.40	205.0 and 322.1	3.08
30.83	207.3, 235.6, 287.6 and 337.7	20.24
31.19	209.7, 258.0 and 334.1 (flavone)	1.82
32.28	208.5, 234.4, 298.3 and 343.6 (puberulin)	19.64
32.76	202.7, 221.5, 298.3 and 337.7 (flavanone)	9.44
34.82	209.7, 262.7 and 395.0	0.97

Puberulin (6,8-dimethoxy-7-prenyloxycoumarin), a coumarin previously isolated by Finkelstein and Rivett (1976), from an Eastern Cape species of *Agathosma puberula* was detected in the extract at a retention time of 32.28 min. It is one of the major compounds present in this species and was found to have its absorbance maxima at approximately 234.4 nm, 298.3 nm and 343.6 nm.

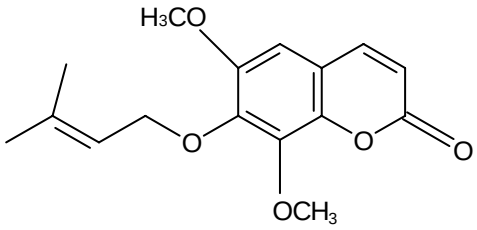


Figure 39: Structure of puberulin as proposed by Finkelstein and Rivett (1976).

5. Biological activity

- The extract displayed good activity in the antimicrobial assay against *Bacillus cereus* (MIC value of 1.25mg/ml), *Klebsiella pneumoniae* (MIC value of 2.5mg/ml) and *Staphylococcus aureus* (MIC value of 0.5mg/ml).
- The essential oil was active in the anti-inflammatory assay (IC_{50} value of 31.54 ± 1.66µg/ml). The extract did not display any activity at 100µg/ml.
- The extract was active in both the anti-oxidant assays (IC_{50} value of 47.25 $\pm$ 7.47µg/ml in the DPPH assay and 15.66 $\pm$ 4.57µg/ml in the ABTS assay). The essential oil was inactive at 100µg/ml.
- The extract was not toxic in the MTT assay at the concentrations tested (IC_{50} value > 100µg/ml), however the essential oil was found to be toxic (IC_{50} value < 0.0001µg/ml).

6. References

- Brown S.A., March R.E., Rivett D.E.A. and Thompson H.J. 1988. Intermediates in the formation of puberulin by *Agathosma puberula*. Phytochemistry, **27**(2): 391.
- Finkelstein N. and Rivett D.E.A. 1976. Puberulin, a new prenyloxy-coumarin from *Agathosma puberula*. Phytochemistry, **15**: 1080.
- Germishuizen G. and Meyer N.L. 2003. Plants of Southern Africa: an annotated checklist. *Strelitzia* **14**. National Botanical Institute, Pretoria, South Africa.
- Pillans N. 1950. A revision of the genus *Agathosma* (Rutaceae). Journal of South African Botany, **16**: 55.

10. *Agathosma ovalifolia* Pillans

1. Botanical description
A single-stemmed, round shrub that grows to a height of 1.5m. It has an acrid- or spice-scent when crushed. The white flowers are red-dotted and are located in lax terminal clusters. The fruits are two chambered and the ovary is usually one or two lobed.

2. Distribution
This species is distributed from the Swartberg Mountains to Willowmore and is generally found on rocky quartzitic upper slopes (Goldblatt and Manning, 2000).

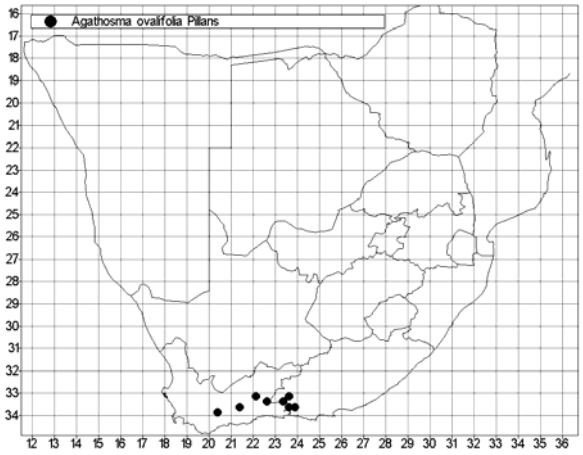


Figure 40: Geographical distribution of *A. ovalifolia*.

3. Origin: Droëkloof Mountains (TTS 240).

4. Essential oil composition
4.1. Essential oil yield: 0.16% (dry weight).

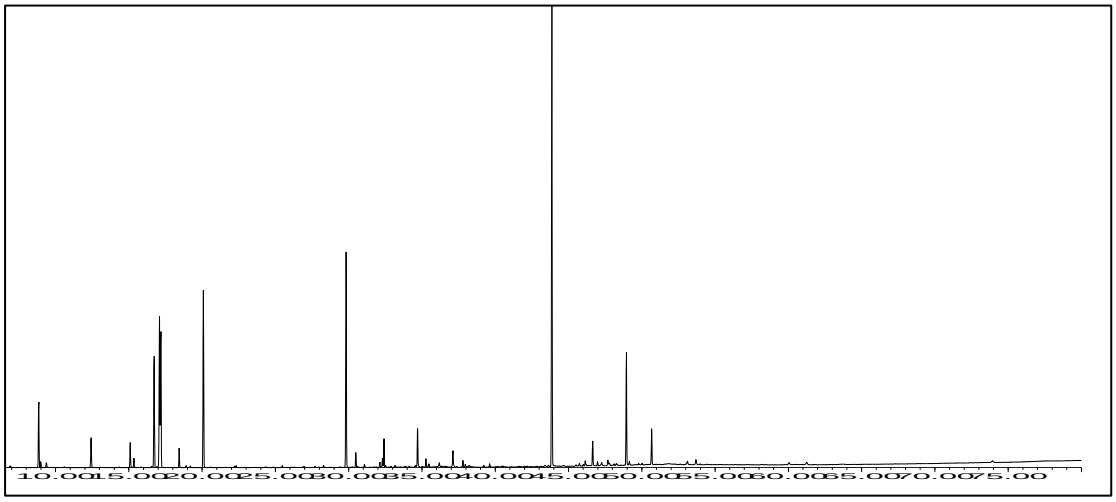
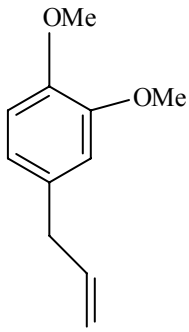


Figure 41: GC-MS chromatogram of *A. ovalifolia*.

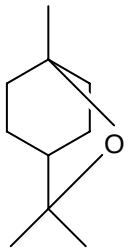
Table 20: Compounds identified in the essential oil of *A. ovalifolia*.

RRI	Compound	%
1032	α-pinene	3.7
1035	α-thujene	0.3
1048	2-methyl-3-buten-2-ol	0.3
1076	camphene	0.1
1118	β-pinene	1.8
1174	myrcene	1.4
1183	pseudolimonene	0.5
1203	limonene	6.5
1213	1,8-cineole	9.7
1218	β-phellandrene	7.5
1246	(*Z*)-β-ocimene	1.0
1255	γ-terpinene	0.1
1266	(*E*)-β-ocimene	0.1
1280	*p*-cymene	9.6
1525	unknown	11.2
1553	linalool	0.7
1611	terpinen-4-ol	1.5
1690	cryptone	2.3
1756	2-oxo-1,4-cineole	1.0
2029	methyl eugenol	23.0
2144	spathulenol	1.3
2245	elemicin	5.8
Total		**89.4**

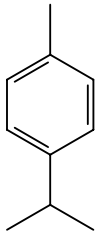
The major compounds present in the essential oil of *A. ovalifolia* include methyl eugenol (23.0%), 1,8-cineole (9.7%) and *p*-cymene (9.6%). β-phellandrene, limonene and elemicin constitute 7.5%, 6.5% and 5.8% of the total composition.



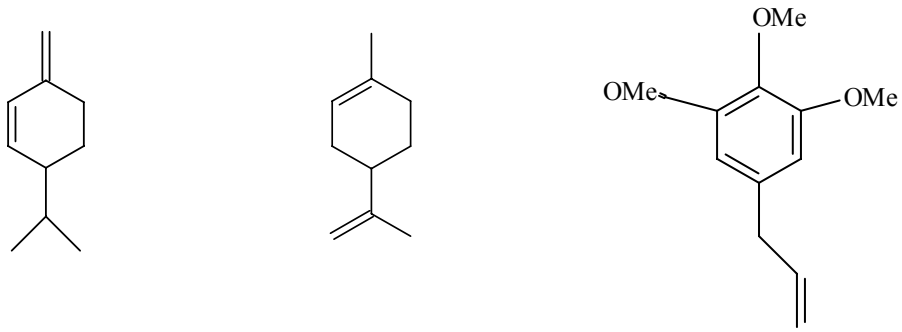
methyl eugenol



1, 8-cineole



p-cymene



β-phellandrene limonene elemicin

Figure 42: Structures of the major compounds present in the essential oil of *A. ovalifolia*.

5. Non-volatile compounds

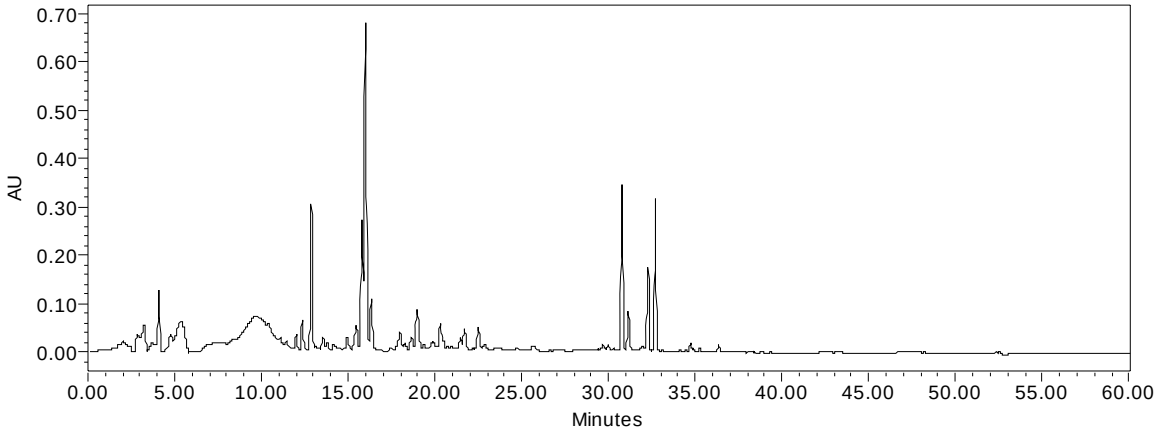


Figure 43: HPLC chromatogram of the dichloromethane and methanol (1:1) extract of *A. ovalifolia*.

Table 21: Compounds detected in the crude extract of *A. ovalifolia*.

R_t	UV max and / tentative identification	%
2.02	208.5, 288.8 and 392.6	1.71
3.24	208.5, 292.4, 344.8, 369.7 and 292.6	2.34
4.09	207.3 and 370.9	2.67
4.76	206.2, 303.1, 344.8, 359.9 and 292.6	1.66
5.36	209.7, 292.4, 328.1, 344.8, 359.9 and 392.6	5.95
7.40	207.3, 301.9, 344.8, 366.1 and 378.1	2.40
7.51	208.5, 301.9, 344.8, 359.9 and 392.6	1.71
8.39	207.3, 301.9, 344.8, 375.7 and 392.6	1.65
9.67	207.3	16.84
10.42	207.3 and 370.9	4.41
12.88	219.1 and 266.3	6.41
15.46	210.9, 269.9 and 347.2 (flavone)	1.81
15.79	210.9, 269.9 and 348.4 (flavone)	5.84
15.99	208.5 and 337.7	16.71
16.32	209.7, 255.7 and 348.4 (flavone)	3.28

R_t	UV max and / tentative identification	%
18.97	207.3, 255.7 and 354.4 (flavonol)	3.07
20.31	208.5 and 343.6	2.06
30.78	208.5 and 310.2	6.13
31.14	209.7, 259.2 and 334.1 (flavone)	1.88
32.29	202.7, 281.7 and 343.6	3.59
32.69	208.5, 297.1 and 337.7 (flavanone)	5.48
55.50	212.0, 343.6, 368.5 and 392.6	2.40

6. Biological activity

- The extract was active in the antimicrobial assay, having an MIC value of 0.5mg/ml against *Bacillus cereus* and 3mg/ml against *Staphylococcus aureus*.
- The essential oil was active in the anti-inflammatory assay (IC_{50} value of 52.84 ± 2.47µg/ml). The extract did not display any activity at 100µg/ml.
- The extract was active in both the anti-oxidant assays (IC_{50} value of 52.84 $\pm$ 2.47µg/ml in the DPPH assay and 26.25 $\pm$ 0.21µg/ml in the ABTS assay). The essential oil was inactive at 100µg/ml.
- Both the extract (IC_{50} value of 74.09 ± 2.18µg/ml) and essential oil (IC_{50} value < 0.0001µg/ml) were toxic in the MTT assay.

7. References

- Germishuizen G. and Meyer N.L. 2003. Plants of Southern Africa: an annotated checklist. *Strelitzia* **14**. National Botanical Institute, Pretoria, South Africa.
- Goldblatt P. and Manning J. 2000. Cape Plants: A Conspectus of the Cape Flora of South Africa. National Botanical Institute of South Africa, Pretoria.
- Pillans N. 1950. A revision of the genus *Agathosma* (Rutaceae). Journal of South African Botany, **16**: 55.

11. *Agathosma ovata* (Thunb.) Pillans

1. Common name
'Basterboegoe', False buchu.

2. Botanical description
A leafy, variable, compact, evergreen shrub, usually single-stemmed that grows to a height of 3m. It is herb-scented when crushed and produces a dense cluster of axillary flowers that are white, pink or purple in colour and cover the shrub between mid Autumn and early Spring (May to September). The five petalled star shaped flowers are 8mm broad and are borne towards the tips of the branches. The leaves are small and typically ovate (10-15mm long). Glands containing volatile oils dot the leaves. The fruits are five chambered and the ovary is usually four or five lobed.

3. Distribution
It requires a well drained, humus rich soil and is generally found on rocky sandstone and silcrete, on open slopes and forest margins. This species is distributed from the Western Cape, Witteberg region up into Kwazulu-Natal and Lesotho (Goldblatt and Manning, 2000). *Agathosma ovata* 'Kluitjieskraal' is found growing near the Kluitjieskraal river at Tulbagh / Wolseley and in the Ceres district. It is also found growing on Table Mountain sandstone (Gould, 1990).

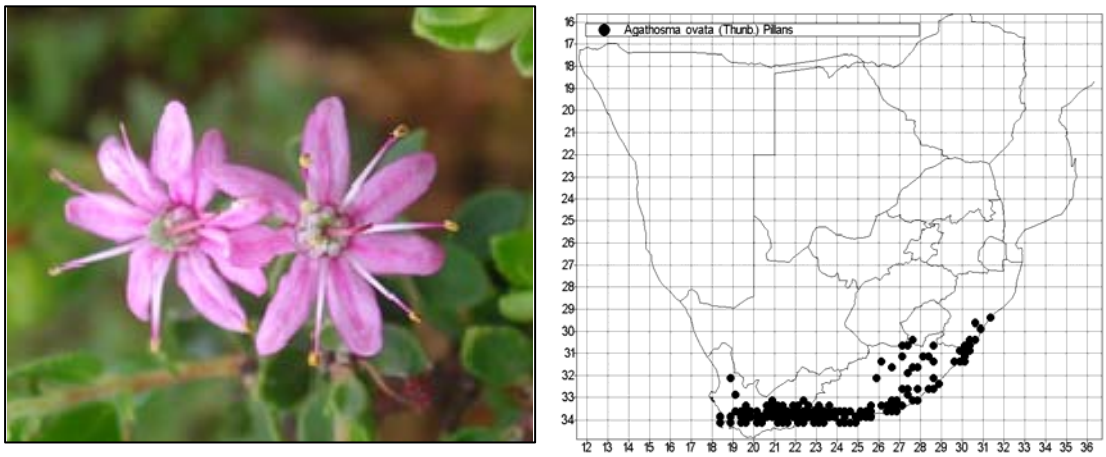


Figure 44: Flower and geographical distribution of *A. ovata*.

4. Origin: Bredasdorp (AV 826).

5. Modern use
Agathosma ovata (Kluitjieskraal) has a neat appearance and grows at a moderate rate. It is an ideal water-wise plant for any home garden and requires minimal water once established. It is used as a groundcover, filler plant, clipped into a hedge, grown in a pot or used in herb gardens (Gould M., 1990).

6. Essential oil composition

6.1. Essential oil yield: 0.85% (dry weight).

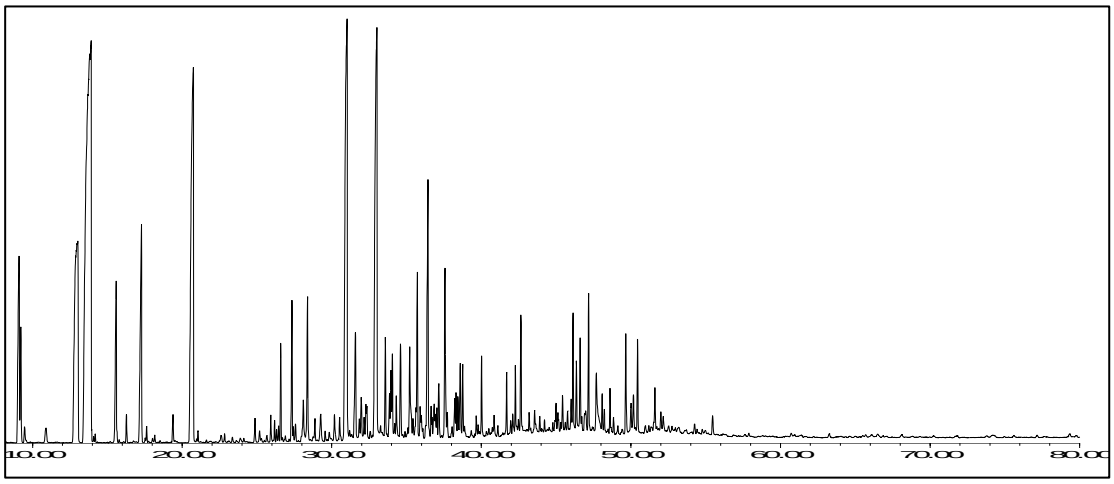


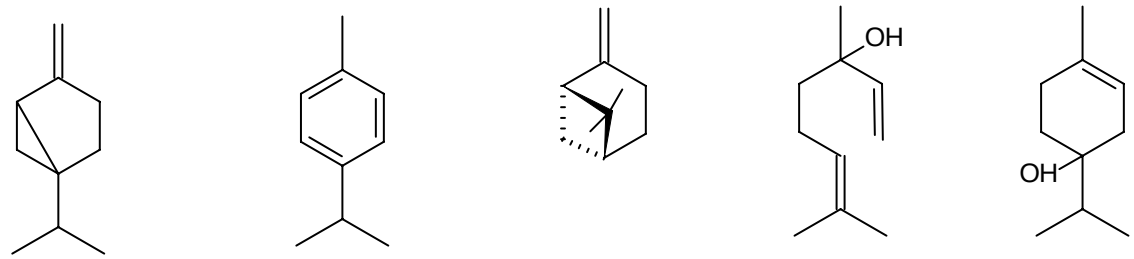
Figure 45: GC-MS chromatogram of *A. ovata*.

Table 22: Compounds identified in the essential oil of *A. ovata*.

RRI	Compound	%
1032	α-pinene	2.6
1035	α-thujene	1.0
1048	2-methyl-3-buten-2-ol	0.2
1076	camphene	0.2
1118	ß-pinene	7.0
1132	sabinene	38.2
1174	myrcene	1.4
1203	limonene	2.3
1218	β-phellandrene	0.1
1246	(*Z*)-β-ocimene	tr
1255	γ-terpinene	0.1
1266	(*E*)-β-ocimene	tr
1280	*p*-cymene	9.1
1290	terpinolene	tr
1327	3-methyl-2-butenol	0.1
1398	(*E*)-4,8-dimethyl-1,3,7-nonatriene (tentative identification)	0.1
1424	*o*-methylanisole	0.1
1429	perillene	0.5
1450	*trans*-linalool oxide (furanoid)	0.7
1474	*trans*-sabinene hydrate	0.3
1478	*cis*-linalool oxide (furanoid)	0.7
1497	α-copaene	0.2
1532	camphor	0.2

RRI	Compound	%
1553	linalool	10.0
1562	isopinocamphone	tr
1571	*trans*-*p*-menth-2-en-1-ol	0.7
1597	bornyl acetate	0.2
1600	β-elemene	tr
1611	terpinen-4-ol	7.8
1626	2-methyl-6-methylene-3,7-octadien-2-ol	0.1
1638	*cis*-*p*-menth-2-en-1-ol	0.5
1651	sabinaketone	0.4
1664	*trans*-pinocarveol	0.6
1689	*trans*-piperitol	0.6
1700	limonen-4-ol	0.1
1706	α-terpineol	0.7
1740	α-muurolene	0.1
1755	bicyclogermacrene	0.3
1758	*cis*-piperitol	0.4
1773	γ-cadinene	0.1
1797	*p*-mentha-1,5-dien-7-ol	0.5
1853	calamenene	tr
1900	epi-cubebol	tr
1957	cubebol	tr
2008	caryophyllene oxide	0.1
2050	(*E*)-nerolidol	tr
2073	*p*-mentha-1,4-dien-7-ol	0.2
2073	1,10-diepicubenol	0.1
2098	globulol	0.2
2100	4-hydroxy-4-methylcyclohex-2-enone	0.3
2113	cumin alcohol	0.4
2144	spathulenol	0.6
2184	*cis*-*p*-menth-3-ene-1,2-diol	0.1
2209	τ-muurolol	0.2
2235	α-muurolol	0.1
2239	τ-cadinol	0.1
2255	α-cadinol	0.5
2256	4-hydroxycryptone	0.2
Total		**91.3**

Some of the major compounds present in the essential oil of *A. ovata* include: sabinene (38.2%), linalool (10.0%), *p*-cymene (9.1%), β-pinene (7.0%) and terpinen-4-ol (7.8%). The oil contains a large number of common monoterpenes.



sabinene *p*-cymene β-pinene linalool terpinen-4-ol

Figure 46: Structures of the major compounds present in the essential oil of *A. ovata*.

7. Non-volatile compounds

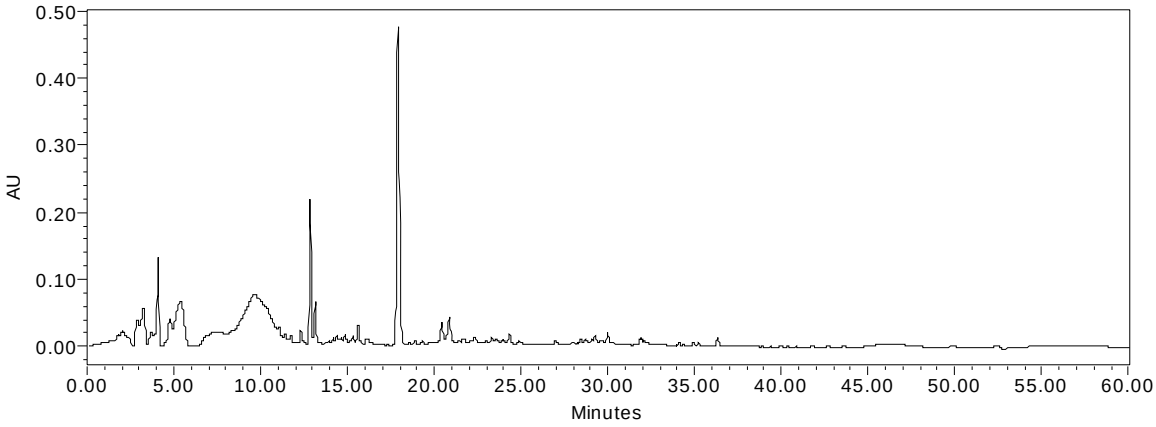


Figure 47: HPLC chromatogram of the dichloromethane and methanol (1:1) extract of *A. ovata*.

Table 23: Compounds detected in the crude extract of *A. ovata*.

R_t	UV max and / tentative identification	%
1.82	207.3, 292.4 and 391.3	2.33
2.04	203.8, 285.3 and 391.3	2.33
2.87	208.5, 291.2, 323.3, 353.2, 368.5 and 391.3	1.98
3.25	208.5, 291.2, 305.5, 340.1, 368.5 and 398.6	3.19
3.68	209.7, 291.2, 346.0, 368.5 and 392.6	1.27
4.08	208.5	3.66
4.76	206.2, 2912, 313.8, 343.6, 368.5, 399.8	2.29
5.36	209.7, 291.2, 313.8, 347.2, 368.5 and 391.3	8.07
7.33	208.5, 299.5, 313.8, 348.4 and 368.5	3.23
7.51	206.2, 313.8, 347.2 and 368.5	2.46
9.61	206.2 and 370.9	30.05
11.07	208.5, 261.6 and 386.5	1.47
11.40	208.5, 274.6 and 380.5	1.00
11.75	205.0, 280.5 and 384.1	0.83
12.32	208.5, 258.0 and 380.5	0.91
12.85	220.3 and 265.1	5.63
13.14	215.6, 278.1 and 268.5	1.85

R_t	UV max and / tentative identification	%
14.87	217.9, 272.2 and 330.5 (flavone)	0.64
15.32	216.7, 271.0 and 335.3 (flavone)	0.69
15.59	209.7, 255.7 and 354.4 (flavonol)	1.21
17.91	203.8, 255.7 and 354.4 (flavonol)	16.91
20.42	208.5, 265.1 and 346.0 (flavone)	1.42
20.88	207.3, 254.5 and 354.4 (flavonol)	2.23
22.31	229.7 and 328.1	0.76
24.34	230.9 and 328.1	0.66
55.00	212.0, 343.6, 368.5 and 392.6	0.78
55.56	212.0, 343.6, 368.5 and 392.6	2.15

8. Biological activity

- The extract was active in the antimicrobial assay, having MIC values of 0.125mg/ml against *Bacillus cereus* and 0.156mg/ml against *Staphylococcus aureus*.
- The essential oil was active in the anti-inflammatory assay (IC_{50} value of 26.54 ± 1.18µg/ml). The extract did not display any activity at 100µg/ml.
- The extract and essential oil were inactive at 100µg/ml in the DPPH assay. However, the extract was active in the ABTS assay (IC_{50} value of 46.81 ± 1.54µg/ml).
- Both the extract (IC_{50} value of 25.20 ± 6.30µg/ml) and essential oil (IC_{50} value < 0.0001µg/ml) were toxic in the MTT assay.

9. References

- Campbell W.E., Finch K.P., Bean P.A. and Finkelstein N. 1987. Alkaloids of the Rutoideae: tribe Diosmeae. Phytochemistry, **26**(2): 433.
- Germishuizen G. and Meyer N.L. 2003. Plants of Southern Africa: an annotated checklist. *Strelitzia* **14**. National Botanical Institute, Pretoria, South Africa.
- Goldblatt P. and Manning J. 2000. Cape Plants: A Conspectus of the Cape Flora of South Africa. National Botanical Institute of South Africa, Pretoria.
- Gould M. 1990. *Agathosma ovata*: designed for living. Veld and Flora, 76:4.
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- Pillans N. 1950. A revision of the genus *Agathosma* (Rutaceae). Journal of South African Botany, **16**: 55.
- Simpson D. 1998. Buchu – South Africa's amazing herbal remedy. Scottish Medical Journal, **43**: 189.
- van Wyk B.E. and Gericke N. 2000. People's Plants. Briza Publications, Pretoria, South Africa.

12. ***Agathosma parva*** P.A. Bean

1. Common name
'Klipspringboegoe'.

2. Botanical description
A round, harsh, glaucous, often bronzed shrublet, coppicing from a woody caudex that grows to a height of 50cm. It is scarcely aromatic. It has bright purple or pink flowers that are found in terminal clusters. The fruits are three chambered.

3. Distribution
This species is found on rocky, shallow sand on arid north facing slopes of the Perdeberg and Riviersonderend Mountains (Goldblatt and Manning, 2000).

4. Origin: Khamiesberg (TTS 298).

5. Essential oil composition
5.1. Essential oil yield: 0.32% (dry weight).

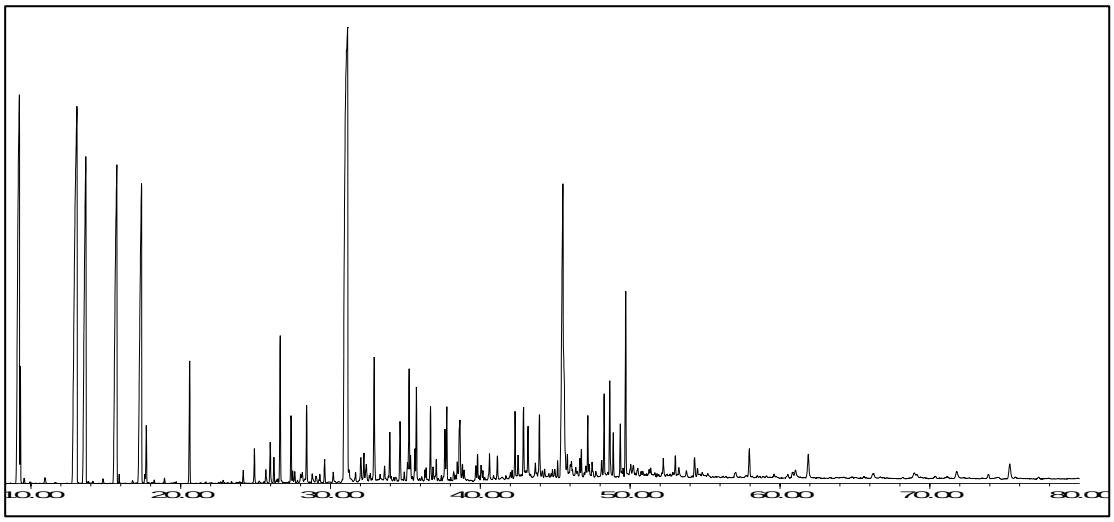


Figure 48: GC-MS chromatogram of *A. parva*.

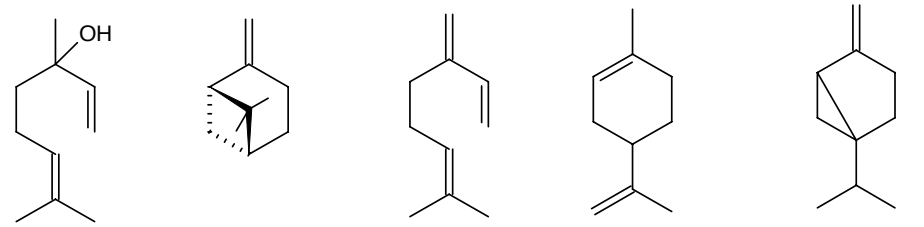
Table 24: Compounds identified in the essential oil of *A. parva*.

RRI	Compound	%
1032	α-pinene	1.4
1035	α-thujene	9.0
1076	camphene	0.1
1118	ß-pinene	14.3
1132	sabinene	7.2
1174	myrcene	8.4
1203	limonene	6.5
1213	1,8-cineole	0.1
1218	β-phellandrene	0.4
1246	(*Z*)-β-ocimene	tr
1280	*p*-cymene	0.9

RRI	Compound	%
1384	α-pinene oxide	0.3
1398	(*E*)-4,8-dimethyl-1,3,7-nonatriene (tentative identification)	0.2
1429	perillene	1.0
1450	*trans*-linalool oxide (furanoid)	0.3
1451	β-thujone	0.1
1458	*cis*-1,2-limonene epoxide	0.1
1466	α-cubebene	0.1
1474	*trans*-sabinene hydrate	0.1
1478	*cis*-linalool oxide (furanoid)	0.4
1487	citronellal	tr
1497	α-copaene	0.1
1498	(*E*)-β-ocimene epoxide	0.1
1553	linalool	28.9
1571	*trans*-*p*-menth-2-en-1-ol	0.1
1586	pinocarvone	0.2
1580	nopinone	0.2
1611	terpinen-4-ol	0.8
1639	*trans*-*p*-mentha-2,8-dien-1-ol	0.1
1648	myrtenal	0.3
1664	*trans*-pinocarveol	0.5
1678	*cis*-*p*-mentha-2,8-dien-1-ol	0.1
1687	α-humulene	0.1
1690	cryptone	0.6
1704	γ-muurolene	0.2
1706	α-terpineol	0.6
1725	verbenone	0.1
1729	*cis*-1,2-epoxyterpinen-4-ol	0.1
1740	α-muurolene	0.4
1751	carvone	0.1
1773	γ-cadinene	0.3
1797	*p*-mentha-1,5-dien-7-ol	0.1
1802	cuminaldehyde	0.1
1804	myrtenol	0.6
1845	*trans*-carveol	0.1
1853	calamenene	0.1
1864	*p*-cymen-8-ol	tr
1868	(*E*)-geranyl acetone	tr
1900	epi-cubebol	0.1
1949	(*Z*)-3-hexenyl nonaoate	0.3
1957	cubebol	0.1
2001	isocaryophyllene oxide	0.1
2008	caryophyllene oxide	0.4
2057	ledol	0.1
2088	1-epi-cubebol	0.1
2104	viridiflorol	tr

RRI	Compound	%
2113	cumin alcohol	0.1
2127	3,7-dimethyl-1,7-octadiene-3,6-diol	0.1
2144	spathulenol	0.3
2148	dictamnol	0.1
2187	τ-cadinol	0.3
2209	τ-muurolol	0.4
2235	α-muurolol	0.2
2255	α-cadinol	0.9
2360	hydroxycalacorene	0.1
2419	4-isopropyl-6-methyl-1,2,3,4-tetrahydronaphthalen-1-one	0.1
2568	oplopanone	tr
Total		**89.6**

The major compound linalool represents 28.9% of the total composition of the essential oil of *A. parva*. β-pinene, α-thujene, myrcene and sabinene represent 14.3%, 9.0%, 8.5% and 7.2% of the total composition.



linalool β-pinene myrcene limonene sabinene

Figure 49: Structures of the major compounds present in the essential oil of *A. parva*.

6. Non-volatile compounds

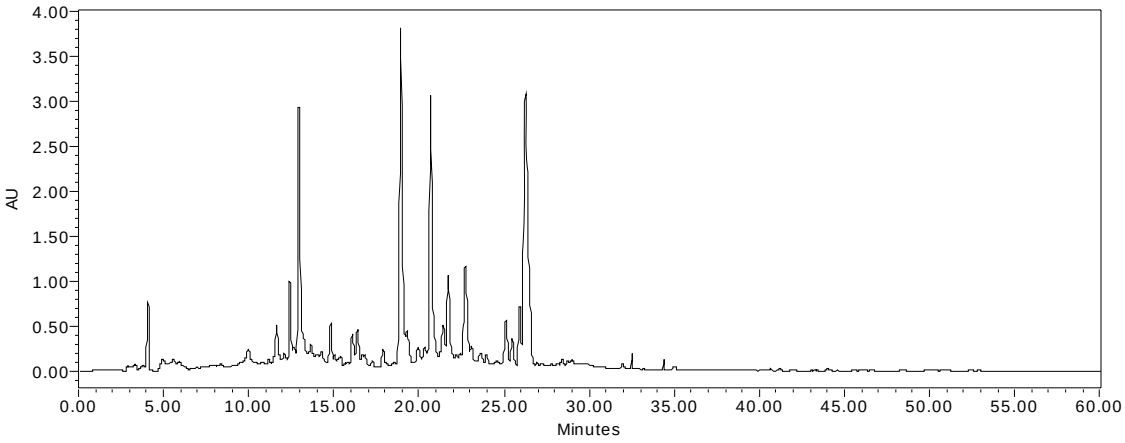


Figure 50: HPLC chromatogram of the dichloromethane and methanol (1:1) extract of *A. parva*.

Table 25: Compounds detected in the crude extract of *A. parva*.

R_t	UV max and / tentative identification	%
4.10	206.2	2.20
9.97	209.7, 253.3 and 291.2	3.75
11.64	206.2, 266.3 and 296.0	2.74
12.42	210.9 and 258.0	3.01
12.94	209.7, 225.0 and 310.2	11.24
13.66	209.7 and 324.5	2.03
14.82	206.2, 261.6 and 290.0	2.02
16.09	208.5, 261.6 and 354.4	1.61
16.40	205.0 and 258.7	1.84
17.89	208.5 and 356.8	1.31
18.93	209.7, 255.7 and 354.4 (flavonol)	14.31
19.31	206.2, 254.5 and 352.0 (flavonol)	2.46
19.96	206.2 and 291.2	1.47
20.68	209.7, 255.7 and 354.4 (flavonol)	12.07
21.43	203.8, 256.8 and 348.4 (flavone)	2.78
21.73	202.7, 284.1 and 331.7 (flavanone)	4.71
22.72	203.8, 256.8, 297.1 and 352.0	5.85
25.10	207.3 and 313.8	2.57
25.47	207.3 and 331.7	1.58
25.92	206.2 and 269.9	2.59
26.26	209.7, 282.9 and 331.7 (flavanone)	17.86

7. Biological activity

- The extract displayed excellent activity against all pathogens in the antimicrobial assay, having MIC values of 2mg/ml against *Bacillus cereus*, 1.5mg/ml against *Candida albicans* and *Klebsiella pneumoniae*, and 1mg/ml against *Staphylococcus aureus*.
- The essential oil was active in the anti-inflammatory assay (IC_{50} value of 37.03 ± 2.32µg/ml). The extract did not display any activity at 100µg/ml.
- The extract was active in both the anti-oxidant assays (IC_{50} value of 72.37 $\pm$ 3.06µg/ml in the DPPH assay and 25.45 $\pm$ 0.33µg/ml in the ABTS assay). The essential oil was inactive at 100µg/ml.
- Both the extract (IC_{50} value of 68.83 ± 9.31µg/ml) and essential oil (IC_{50} value < 0.0001µg/ml) were toxic in the MTT assay.

8. References

- Goldblatt P. and Manning J. 2000. Cape Plants: A Conspectus of the Cape Flora of South Africa. National Botanical Institute of South Africa, Pretoria.

13. *Agathosma pubigera* Sond.

1. Botanical description
A resprouting, slightly aromatic, glaucous, much branched shrublet that grows to a height of 60cm. The flowers are white in colour and are found in terminal clusters. The fruits are one chambered.

2. Distribution
Found growing on the lower slopes of the Cederberg Mountains (Goldblatt and Manning, 2000).

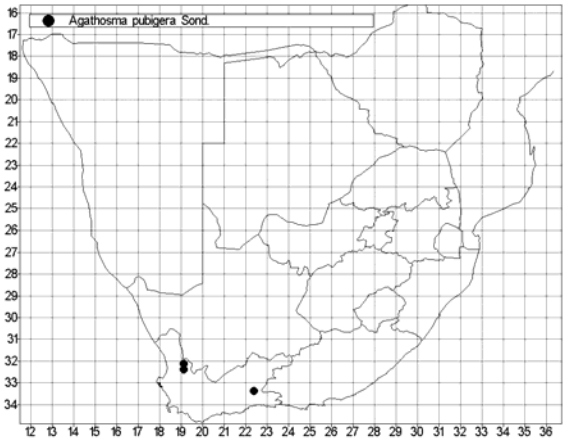


Figure 51: Geographical distribution of *A. pubigera*.

3. Origin: Pakhuis (TTS 357).

4. Essential oil composition
4.1. Essential oil yield: 0.52% (dry weight).

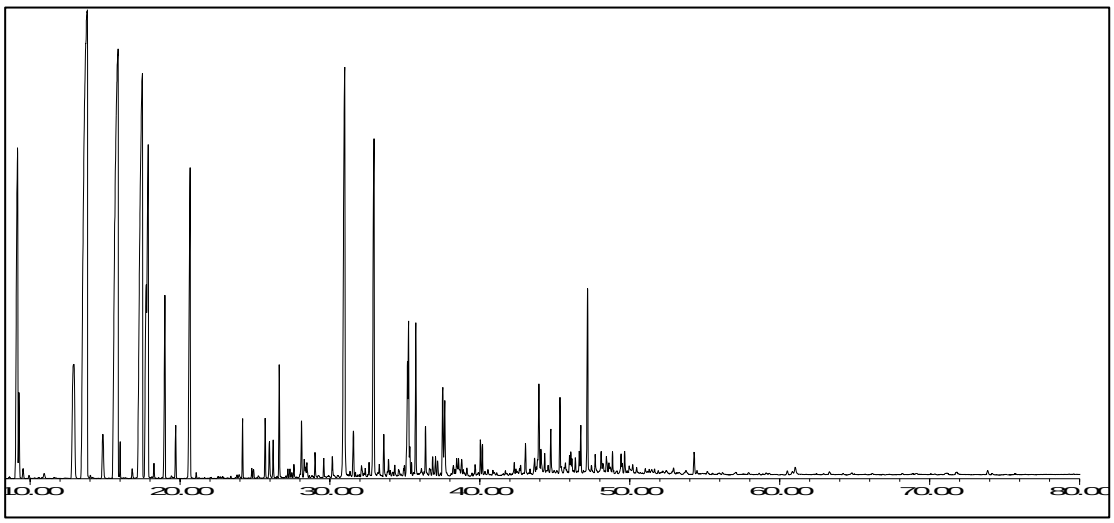


Figure 52: GC-MS chromatogram of *A. pubigera*.

Table 26: Compounds identified in the essential oil of *A. pubigera*.

RRI	Compound	%
1020	methyl 2-methylbutyrate	tr
1032	α-pinene	4.9
1035	α-thujene	0.1
1048	2-methyl-3-buten-2-ol	tr
1076	camphene	0.1
1118	β-pinene	2.2
1132	sabinene	22.9
1159	δ-3-carene	0.5
1174	myrcene	18.1
1183	pseudolimonene	0.1
1195	dehydro-1,8-cineole	0.1
1203	limonene	12.1
1213	1,8-cineole	tr
1218	β-phellandrene	6.0
1225	(*Z*)-3-hexenal	0.1
1246	(*Z*)-β-ocimene	1.4
1266	(*E*)-β-ocimene	0.3
1280	*p*-cymene	3.4
1290	terpinolene	tr
1382	allo-ocimene	0.1
1384	α-pinene oxide	tr
1398	(*E*)-4,8-dimethyl-1,3,7-nonatriene (tentative identification)	0.2
1429	perillene	0.6
1450	*trans*-linalool oxide (furanoid)	0.1
1451	β-thujone	tr
1458	*cis*-1,2-limonene epoxide	0.1
1474	*trans*-sabinene hydrate	0.4
1476	(*Z*)-β-ocimene epoxide	0.1
1498	(*E*)-β ocimene epoxide	0.1
1553	linalool	6.9
1571	*trans*-*p*-menth-2-en-1-ol	0.3
1576	*cis*-isopulegone	0.1
1586	myrcenone	0.1
1602	6-methyl-3,5-heptadien-2-one	0.1
1611	terpinen-4-ol	3.8
1626	2-methyl-6-methylene-3,7-octadien-2-ol	0.1
1638	*cis*-*p*-menth-2-en-1-ol	0.2
1665	citronellyl isobutyrate	tr
1683	δ terpineol	0.6
1687	α-humulene	tr
1690	cryptone	1.1
1700	limonen-4-ol	0.1
1706	α-terpineol	0.8
1729	*cis*-1,2-epoxyterpinen-4-ol	0.3

RRI	Compound	%
1740	α-muurolene	0.1
1744	phellandral	0.1
1748	piperitone	0.1
1751	carvone	0.1
1758	*cis*-piperitol	0.1
1766	decanol	0.6
1772	citronellol	0.5
1797	*p*-methylacetophenone	0.1
1797	*p*-mentha-1,5-dien-7-ol	0.1
1802	cuminaldehyde	0.1
1830	2,6-dimethyl-3(*E*),5(*E*),7-octatrien-2-ol	0.1
1845	*trans*-carveol	0.1
1864	*p*-cymen-8-ol	0.2
1868	(*E*)-geranyl acetone	0.1
1949	(*Z*)-3-hexenyl nonaoate	0.1
1973	dodecanol	0.2
2000	tridecenyl acetate	0.2
2001	isocaryophyllene oxide	0.1
2008	caryophyllene oxide	0.5
2030	methyl eugenol	0.1
2045	humulene epoxide I	0.2
2071	humulene epoxide II	0.4
2096	elemol	0.1
2113	cumin alcohol	0.1
2144	spathulenol	1.1
2158	eremoligenol	0.1
2200	(*E*)-methyl isoeugenol	0.1
2209	τ-muurolol	0.1
2219	dimyrcene IIa	0.2
2245	elemicin	0.1
2247	*trans*-α-bergamotol	0.1
2255	α-cadinol	0.1
2269	dimyrcene IIb	0.1
2384	farnesyl acetone	tr
Total		**94.9**

Sabinene (22.9%), myrcene (18.1%) and limonene (12.1%) are the major compounds present in the essential oil of *A. pubigera*. Linalool (6.9%), β-phellandrene (6.0%) and α-pinene (4.9%) are also present in large concentrations.

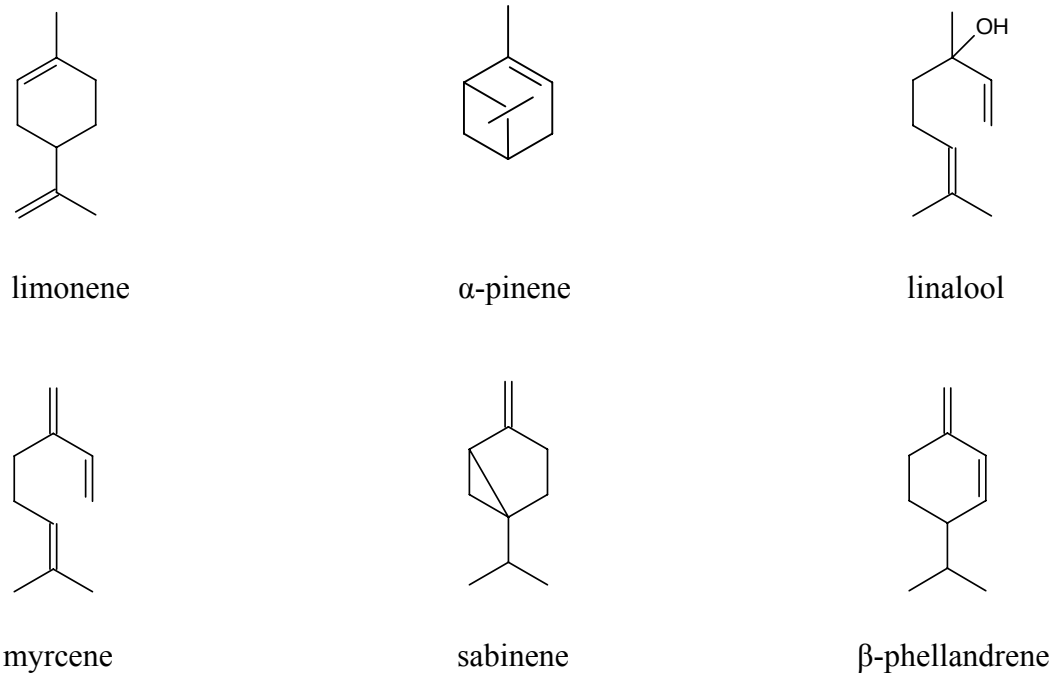


Figure 53: Structures of the major compounds present in the essential oil of *A. pubigera*.

5. Non-volatile compounds

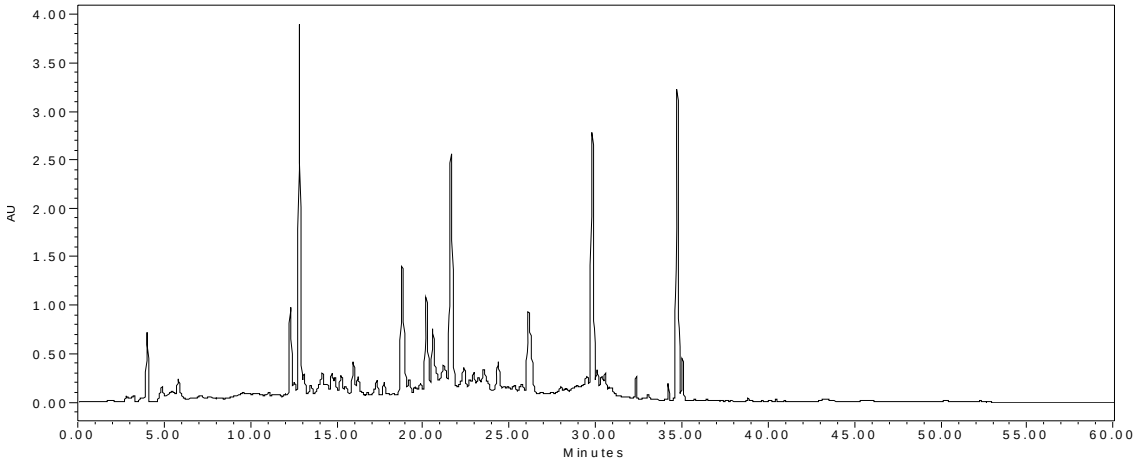


Figure 54: HPLC chromatogram of the dichloromethane and methanol (1:1) extract of *A. pubigera*.

Table 27: Compounds detected in the crude extract of *A. pubigera*.

R_t	UV max and / tentative identification	%
4.02	207.3	2.10
9.55	207.3	1.77
12.32	212.0 and 258.0	3.13
12.84	207.3, 221.5 and 266.3	11.36
14.17	206.2 and 326.9	2.42
15.96	208.5 and 348.4	1.96

R_t	UV max and / tentative identification	%
16.23	201.5 and 273.4	1.73
17.33	208.5 and 352.0	1.84
18.80	207.3, 255.7 and 352.0 (flavonol)	7.65
20.19	207.3, 255.7 and 352.0 (flavonol)	4.99
20.57	207.3, 255.7 and 352.0 (flavonol)	4.60
21.19	205.0, 273.4 and 321.0 (flavone)	3.44
21.62	207.3, 284.1 and 328.1 (flavanone)	12.06
22.37	207.3 and 331.7	3.09
23.53	207.3 and 326.9	2.49
24.36	207.3, 269.9 and 334.1 (flavone)	2.79
26.12	284.1 and 328.1 (flavanone)	6.63
28.90	207.3 and 323.3	1.87
29.49	207.3 and 323.3	2.67
29.81	208.5, 249.7 and 319.8	10.73
34.74	201.5, 230.9 and 338.9	10.70

6. Biological activity

- The extract displayed good activity against all the pathogens in the antimicrobial assay, having MIC values of 2mg/ml against *Bacillus cereus*, 3mg/ml against *Candida albicans*, 2.5mg/ml against *Klebsiella pneumoniae* and 0.8mg/ml against *Staphylococcus aureus*.
- The essential oil was active in the anti-inflammatory assay (IC_{50} value of 35.15 ± 2.00µg/ml). The extract did not display any activity at 100µg/ml.
- The extract was active in both the anti-oxidant assays (IC_{50} value of 35.61 $\pm$ 0.86µg/ml in the DPPH assay and 29.94 $\pm$ 0.39µg/ml in the ABTS assay). The essential oil was inactive at 100µg/ml.
- Both the extract (IC_{50} value of 54.68 ± 4.95µg/ml) and essential oil (IC_{50} value < 0.0001µg/ml) were toxic in the MTT assay.

7. References

- Germishuizen G. and Meyer N.L. 2003. Plants of Southern Africa: an annotated checklist. *Strelitzia* **14**. National Botanical Institute, Pretoria, South Africa.
- Goldblatt P. and Manning J. 2000. Cape Plants: A Conspectus of the Cape Flora of South Africa. National Botanical Institute of South Africa, Pretoria.
- Pillans N. 1950. A revision of the genus *Agathosma* (Rutaceae). Journal of South African Botany, **16**: 55.

14. ***Agathosma pungens*** (E. Mey. ex Sond.) Pillans

1. Botanical description

A single-stemmed, much branched, leafy shrub that grows to a height of 80cm. The leaves are spine-tipped and pleasantly aromatic. The flowers are axillary, usually solitary, and white, pink to purple in colour. The fruits are two chambered.

2. Distribution

Found growing on the upper slopes of the Swartberg and Khamanassie Mountains, up to the Uniondale region (Goldblatt and Manning, 2000).

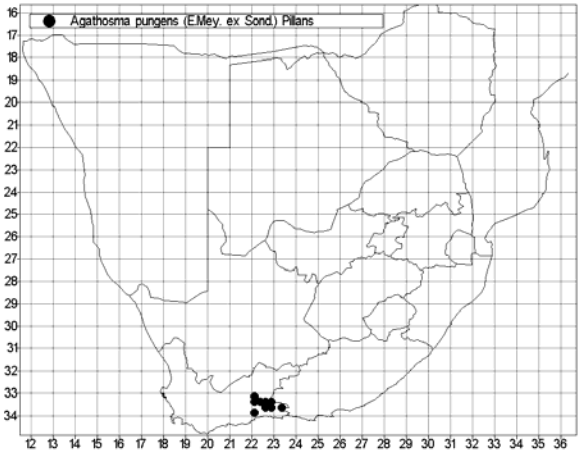


Figure 55: Geographical distribution of *A. pungens*.

3. Origin: Khamanassie (TTS 253).

4. Essential oil composition

4.1. Essential oil yield: 0.43% (dry weight).

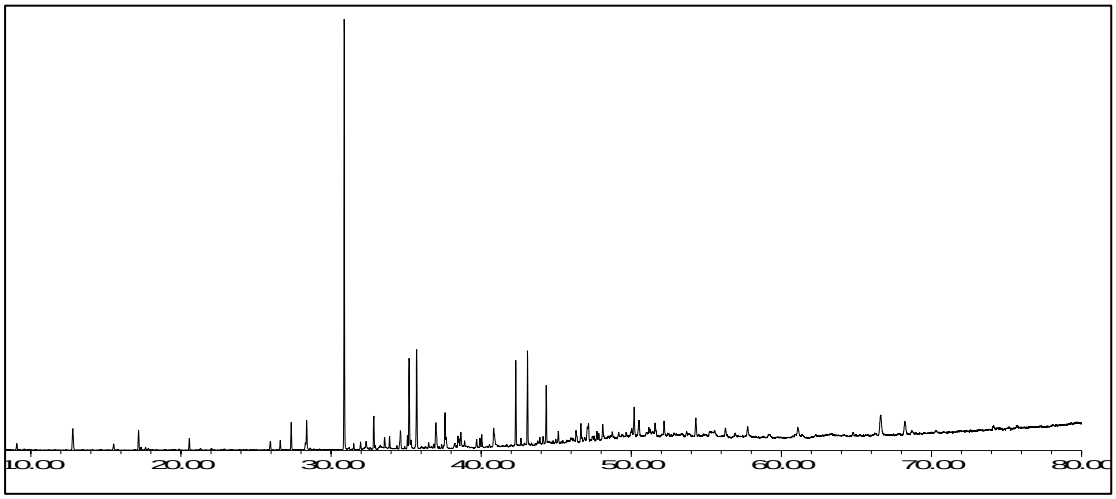
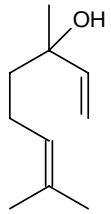


Figure 56: GC-MS chromatogram of *A. pungens*.

Table 28: Compounds identified in the essential oil of *A. pungens*.

RRI	Compound	%
1032	α-pinene	0.1
1035	α-thujene	tr
1118	β-pinene	tr
1174	myrcene	tr
1203	limonene	0.2
1210	2-methyl-2-butenal	tr
1218	β-phellandrene	0.4
1270	3-hydroxy-2-butanone	tr
1280	*p*-cymene	0.1
1327	3-methyl-2-butenol	0.1
1398	(*E*)-4,8-dimethyl-1,3,7-nonatriene (tentative identification)	tr
1429	perillene	0.1
1450	*trans*-linalool oxide (furanoid)	3.2
1475	acetic acid	tr
1478	*cis*-linalool oxide (furanoid)	3.4
1553	linalool	15.4
1562	isopinocamphone	0.1
1563	pinocarvone	0.3
1570	methyl citronellate	0.2
1602	6-methyl-3,5-heptadien-2-one	0.1
1611	terpinen-4-ol	1.3
1616	hotrienol	0.2
1628	4,4-dimethylbut-2-enolide	0.4
1648	myrtenal	0.8
1664	*trans*-pinocarveol	0.8
1687	methyl chavicol	0.4
1690	cryptone	1.9
1704	myrtenal acetate	0.2
1719	borneol	0.2
1726	α-terpineol	3.1
1733	neryl acetate	0.2
1748	piperitone	0.3
1750	*cis*-linalool oxide (pyranoid)	2.3
1751	carvone	0.3
1758	*cis*-piperitol	0.3
1770	*trans*-linalool oxide (pyranoid)	2.8
1772	citronellol	tr
1798	methyl salicylate	0.2
1804	myrtenol	0.2
1804	*trans*-carveol	0.3
1857	geraniol	0.3
1864	*p*-cymen-8-ol	0.4
1874	(*Z*)-3-hexenyl octanoate (tentative identification)	0.6

RRI	Compound	%
1882	*cis*-carveol	0.7
1949	(*Z*)-3-hexenyl nonaoate	5.2
1969	3,7-dimethyl-1-octene-3,7-diol	4.9
2008	caryophyllene oxide	0.1
2030	methyl eugenol	1.4
2050	anisaldehyde	0.9
2113	cumin alcohol	0.3
2127	3,7-dimethyl-1,7-octadiene-3,6-diol	tr
2144	spathulenol	1.9
2184	*cis*-*p*-menth-3-ene-1,2-diol	0.7
2256	4-hydroxycryptone	0.5
2312	(*Z*)-2,6-dimethyl-2,7-octadiene-1,6-diol	1.5
2776	*p*-menthane-1,2,8-triol (tentative identification)	3.6
Total		**62.9**



linalool

The major compound linalool represents 15.4% of the total composition of the essential oil of *A. pungens*. (*Z*)-3-hexenyl nonaoate and 3,7-dimethyl-1-octene-3,7-diol represent 5.2% and 4.9%.

Figure 57: Structure of the major compound present in the essential oil of *A. pungens*.

5. Non-volatile compounds

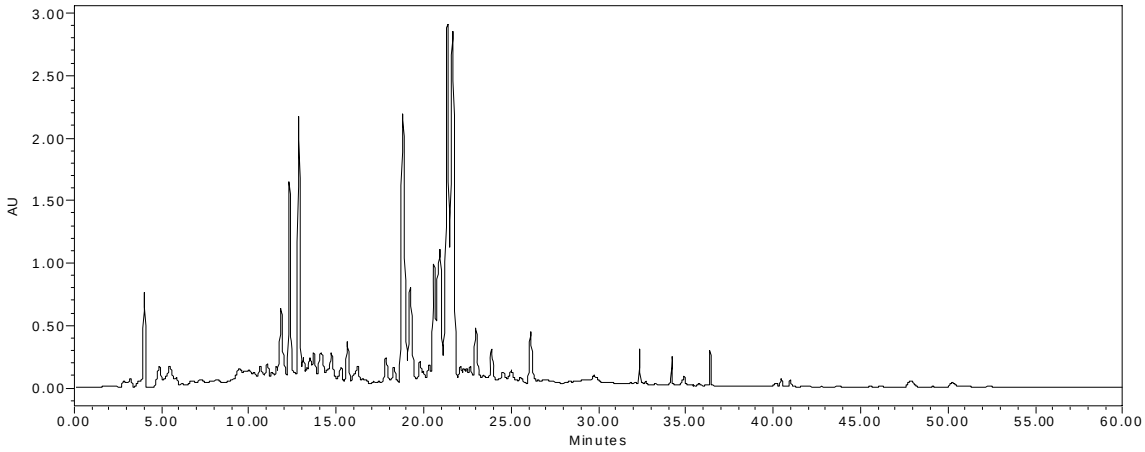


Figure 58: HPLC chromatogram of the dichloromethane and methanol (1:1) extract of *A. pungens*.

Table 29: Compounds detected in the crude extract of *A. pungens*.

R_t	UV max and / tentative identification	%
4.00	203.8 and 268.7	2.45
5.47	281.7	1.72

R_t	UV max and / tentative identification	%
9.44	207.3 and 316.2	2.25
11.84	201.5 and 282.9	3.85
12.32	213.2 and 258.0	6.11
12.84	217.9 and 265.1	7.54
13.71	206.2 and 273.4	1.41
14.11	208.5, 287.6 and 329.3 (flavanone)	2.27
14.71	205.0 and 285.3	2.44
15.63	212.0 and 335.3	1.68
16.22	208.5, 267.5 and 344.8 (flavone)	1.45
17.82	207.3, 255.7 and 353.2 (flavonol)	1.43
18.80	205.0, 255.7 and 353.2 (flavonol)	11.77
19.23	206.2, 255.7 and 353.2 (flavonol)	4.54
20.58	206.2, 256.8 and 353.2 (flavonol)	4.10
20.92	206.2, 255.7 and 353.2 (flavonol)	7.45
21.35	201.5, 254.5 and 353.2 (flavonol)	13.86
21.64	201.6, 213.2, 285.3 and 338.9 (flavanone)	16.01
22.98	206.2, 254.5 and 352.0 (flavonol)	2.53
23.88	207.3, 254.5 and 350.8 (flavonol)	1.62
25.00	208.5, 255.7 and 317.4	1.23
26.11	212.0, 282.9 and 326.9 (flavanone)	2.29

6. Biological activity

- The extract displayed excellent activity against two pathogens in the antimicrobial assay, having MIC values of 1mg/ml against *Bacillus cereus* and 0.75mg/ml against *Staphylococcus aureus*.
- The essential oil was active in the anti-inflammatory assay (IC_{50} value of 41.10 ± 3.31µg/ml). The extract did not display any activity at 100µg/ml.
- The extract was active in both the anti-oxidant assays (IC_{50} value of 94.65 $\pm$ 1.65µg/ml in the DPPH assay and 31.57 $\pm$ 0.82µg/ml in the ABTS assay). The essential oil was inactive at 100µg/ml.
- Both the extract (IC_{50} value of 66.07 ± 6.78µg/ml) and essential oil (IC_{50} value < 0.0001µg/ml) were toxic in the MTT assay.

7. References

- Campbell W.E., Finch K.P., Bean P.A. and Finkelstein N. 1987. Alkaloids of the Rutoideae: tribe Diosmeae. Phytochemistry, **26**(2): 433.
- Germishuizen G. and Meyer N.L. 2003. Plants of Southern Africa: an annotated checklist. *Strelitzia* **14**. National Botanical Institute, Pretoria, South Africa.
- Goldblatt P. and Manning J. 2000. Cape Plants: A Conspectus of the Cape Flora of South Africa. National Botanical Institute of South Africa, Pretoria.
- Pillans N. 1950. A revision of the genus *Agathosma* (Rutaceae). Journal of South African Botany, **16**: 55.

15. ***Agathosma roodebergensis*** Compton

1. Botanical description
A round, single-stemmed shrub that grows to a height of 1m. The white flowers are found in axillary clusters below the branch tips. The fruits are three chambered.

2. Distribution
Found on the middle to upper sandstone slopes, from the Rooiberg to Outeniqua Mountains (Goldblatt and Manning, 2000).

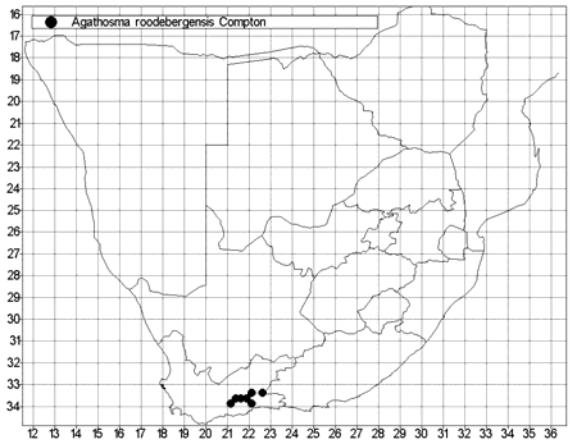


Figure 59: Geographical distribution of *A. roodebergensis*.

4. Origin: Khamiesberg (TTS 237).

4. Essential oil composition
4.1. Essential oil yield: 0.36% (dry weight).

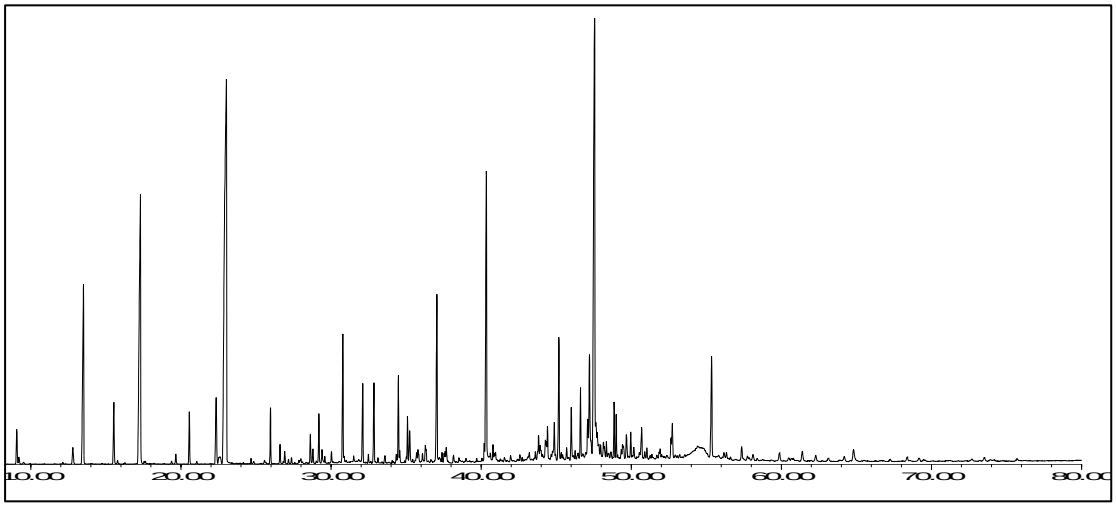


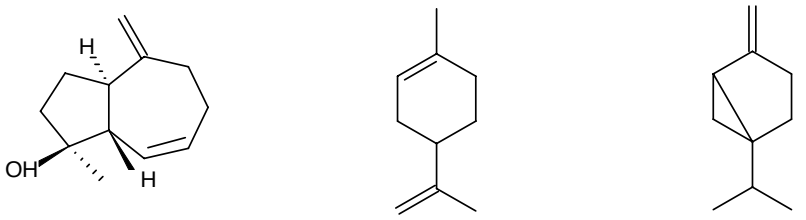
Figure 60: GC-MS chromatogram of *A. roodebergensis*.

Table 30: Compounds identified in the essential oil of *A. roodebergensis*.

RRI	Compound	%
1032	α-pinene	1.1
1035	α-thujene	0.2
1118	β-pinene	0.5
1132	sabinene	5.8
1174	myrcene	1.5
1203	limonene	11.6
1266	(*E*)-β-ocimene	0.2
1280	*p*-cymene	0.9
1284	isogeijerene C	0.7
1290	terpinolene	0.1
1332	geijerene isomer	tr
1337	geijerene	27.9
1398	(*E*)-4,8-dimethyl-1,3,7-nonatriene (tentative identification)	0.3
1429	perillene	0.3
1490	isogeijerene	1.7
1495	bicycloelemene	0.3
1506	decanal	0.1
1571	*trans*-*p*-menth-2-en-1-ol	0.1
1591	pregeijerene	0.3
1600	β-elemene	0.1
1611	terpinen-4-ol	1.0
1639	*trans*-*p*-mentha-2,8-dien-1-ol	0.1
1661	allo-aromadendrene	0.1
1665	citronellyl isobutyrate	tr
1668	2-decyl acetate	0.9
1687	methyl chavicol	0.4
1722	dodecanal	0.2
1726	germacrene D	0.2
1755	bicyclogermacrene	2.6
1763	naphthalene	0.1
1766	decanol	tr
1772	citronellol	0.3
1776	γ-cadinene	tr
1845	2-dodecyl acetate	tr
1851	traginone	5.2
2008	caryophyllene oxide	0.3
2030	methyl eugenol	0.3
2096	elemol	0.6
2104	viridiflorol	0.1
2131	8-epi-dictamnol	0.7
2144	spathulenol	1.3
2148	dictamnol	14.2
2200	(*E*)-3,7-dimethyl-5-octene-1,7-diol	0.2

RRI	Compound	%
2219	dimyrcene IIa	0.4
2257	β-eudesmol	0.3
2269	dimyrcene IIb	0.3
2500	pentacosane	tr
2654	benzyl benzoate	0.2
Total		**83.7**

Geijerene (27.0%), dictamnol (14.2%) and limonene (11.6%) are the major compounds present in the essential oil of *A. roodebergensis*. Sabinene and traginone represent 5.8% and 5.2% of the total composition.



dictamnol limonene sabinene

Figure 61: Structures of the major compounds present in the essential oil of *A. roodebergensis*.

5. Non-volatile compounds

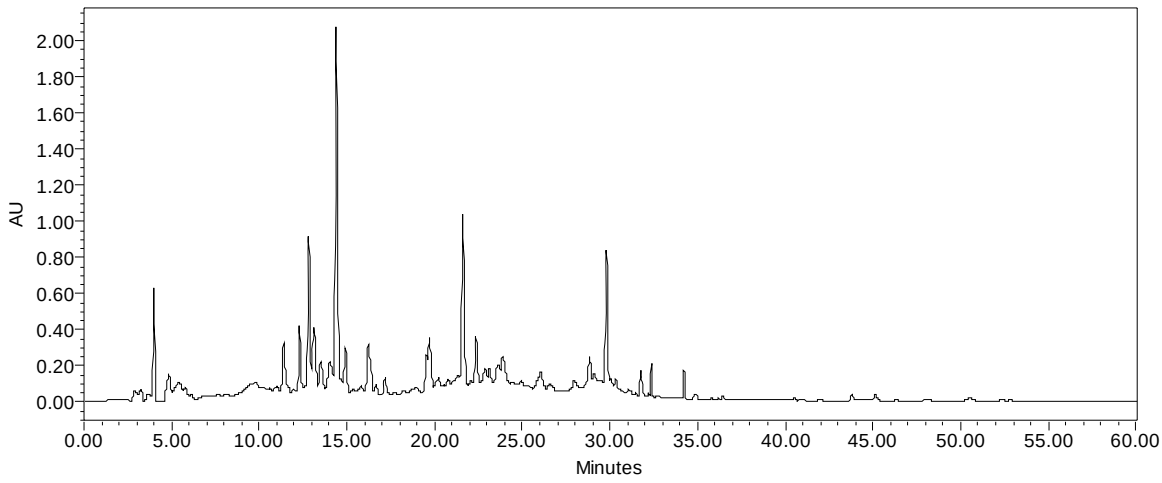


Figure 62: HPLC chromatogram of the dichloromethane and methanol (1:1) extract of *A. roodebergensis*.

Table 31: Compounds detected in the crude extract of *A. roodebergensis*.

R_t	UV max and / tentative identification	%
3.99	207.3	3.04
5.42	205.0 and 277.0	2.20
9.48	207.3 and 319.8	2.78
9.77	208.5 and 290.0	3.12
11.39	214.4 and 325.7	3.49
12.29	210.9 and 258.0	3.20
12.82	220.3 and 267.5	5.97
13.12	205.0 and 288.8	4.01
13.51	207.3, 297.1 and 323.3 (flavanone)	2.45
14.04	207.3 and 325.7	3.01
14.39	207.3, 271.0 and 353.2 (flavone)	14.99
14.97	205.0, 290.0 and 317.4	2.60
16.22	284.1 and 324.5 (flavanone)	3.88
19.70	205.0, 279.3 and 329.1 (flavanone)	3.15
20.22	207.3, 299.5 and 322.1	2.09
21.61	201.5, 285.3 and 328.1 (flavanone)	8.48
22.36	203.8, 278.1, 306.7 and 325.7	3.62
22.88	208.5 and 324.5	2.36
23.65	207.3 and 323.3	2.60
23.89	207.3 and 325.7	3.92
26.04	208.5 and 325.7	3.34
26.57	208.5 and 324.5	2.02
27.96	208.5 and 324.5	2.44
28.82	207.3 and 324.5	3.34
29.81	208.5, 249.7 and 321.0	5.85
30.33	208.5 and 323.3	2.05

6. Biological activity

- The extract showed good activity against two pathogens in the antimicrobial assay, having MIC values of 0.5mg/ml against *Bacillus cereus* and 1mg/ml against *Staphylococcus aureus*.
- The essential oil was active in the anti-inflammatory assay (IC_{50} value of 40.40 ± 2.96µg/ml). The extract did not display any activity at 100µg/ml.
- The extract was active in both the anti-oxidant assays (IC_{50} value of 56.71 $\pm$ 4.76µg/ml in the DPPH assay and 29.63 $\pm$ 0.32µg/ml in the ABTS assay). The essential oil was inactive at 100µg/ml.
- Both the extract (IC_{50} value of 38.05 ± 7.29µg/ml) and essential oil (IC_{50} value < 0.0001µg/ml) were toxic in the MTT assay.

7. References

- Campbell W.E., Finch K.P., Bean P.A. and Finkelstein N. 1987. Alkaloids of the Rutoideae: tribe Diosmeae. Phytochemistry, **26**(2): 433.

- Germishuizen G. and Meyer N.L. 2003. Plants of Southern Africa: an annotated checklist. *Strelitzia* **14**. National Botanical Institute, Pretoria, South Africa.
- Goldblatt P. and Manning J. 2000. Cape Plants: A Conspectus of the Cape Flora of South Africa. National Botanical Institute of South Africa, Pretoria.
- Pillans N. 1950. A revision of the genus *Agathosma* (Rutaceae). Journal of South African Botany, **16**: 55.

16. *Agathosma stipitata* Pillans

1. Botanical description
A single stemmed, much branched, stiff shrub that grows to a height of 80cm. It has a lemon-scent when crushed. The flowers are white and are axillary. The fruits are five chambered and stalked.

2. Distribution
It is found on dry, rocky sandstone plateaus at middle altitude. This species is distributed in the Perdeberg and Riviersonderend Mountains (Goldblatt and Manning, 2000).

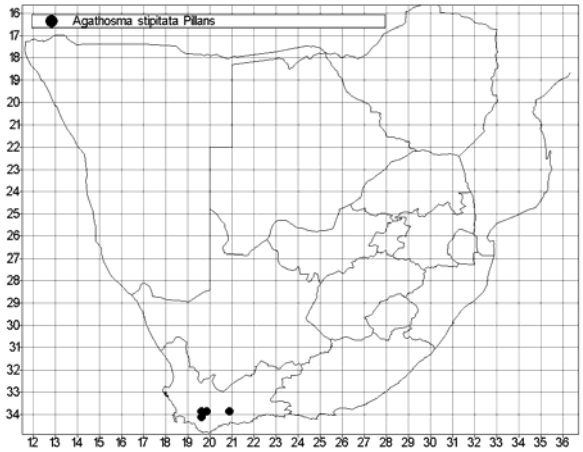


Figure 63: Geographical distribution of *A. stipitata*.

3. Origin: Rooiberg (TTS 301).

4. Essential oil composition
4.1. Essential oil yield: 0.44% (dry weight).

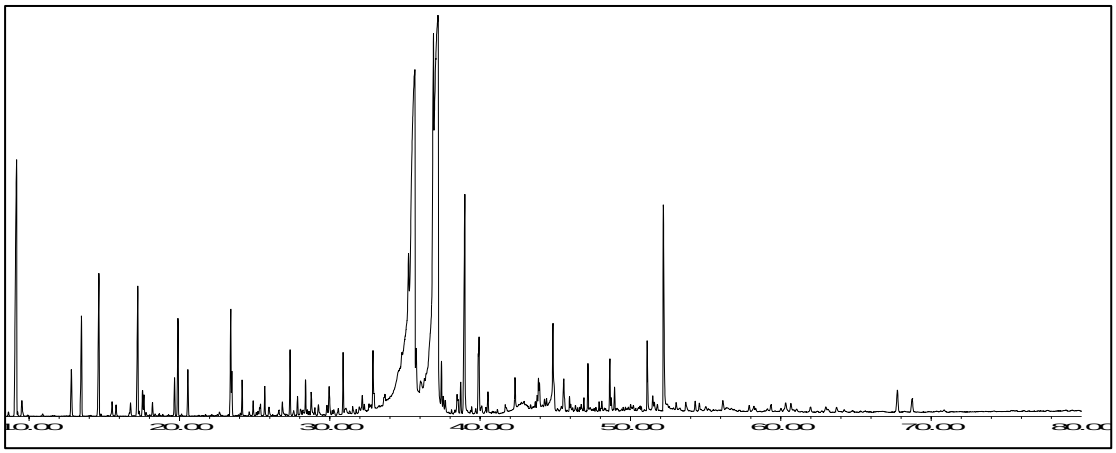


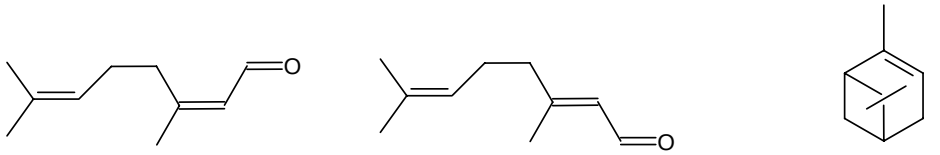
Figure 64: GC-MS chromatogram of *A. stipitata*.

Table 32: Compounds identified in the essential oil of *A. stipitata*.

RRI	Compound	%
1032	α-pinene	8.9
1048	2-methyl-3-buten-2-ol	0.3
1118	ß-pinene	0.7
1132	sabinene	1.6
1146	methyl 4-methylpentanoate	1.9
1174	myrcene	0.2
1183	pseudolimonene	tr
1195	dehydro-1,8-cineole	0.2
1203	limonene	2.0
1213	1,8-cineole	0.5
1218	β-phellandrene	tr
1225	(*Z*)-3-hexenal	tr
1258	methyl 4-methylhexanoate	0.9
1266	(*E*)-β-ocimene	0.5
1280	*p*-cymene	0.5
1348	6-methyl-5-hepten-2-one	1.6
1384	α-pinene oxide	0.2
1390	(*E*)-2-hexenyl isobutyrate	0.1
1391	(*Z*)-3-hexenol	0.3
1399	methyl octanoate	0.2
1413	rosefuran	tr
1418	(*Z*)-2-hexen-1-ol	tr
1429	perillene	0.1
1450	*trans*-linalool oxide (furanoid)	0.5
1458	*cis*-1,2-limonene epoxide	0.1
1468	*trans*-1,2-limonene epoxide	0.1
1475	acetic acid	tr
1478	*cis*-linalool oxide (furanoid)	0.3
1487	citronellal	0.2
1498	(*E*)-β-ocimene epoxide	tr
1499	α-campholene aldehyde	0.2
1500	methyl nonaoate	0.2
1518	karanaenone (tentative identification)	0.1
1522	2-nonanol	0.4
1553	linalool	0.6
1571	*trans*-*p*-menth-2-en-1-ol	0.1
1611	terpinen-4-ol	0.5
1690	cryptone	0.8
1694	neral	34.8
1733	neryl acetate	tr
1740	geranial	16.1
1765	geranyl acetate	0.2
1772	citronellol	0.1
1808	nerol	0.3

RRI	Compound	%
1815	2-tridecanone	3.3
1845	*trans*-carveol	0.1
1850	isopiperitenone	0.1
1857	geraniol	1.2
1878	guaiacol	0.2
2000	tridecenyl acetate	1.0
2008	caryophyllene oxide	0.4
2019	2-pentadecanone	tr
2094	*p*-cresol	0.1
2098	globulol	0.2
2144	spathulenol	0.4
2254	citronellic acid	0.1
2308	neric acid	0.8
2337	geranic acid	3.4
Total		**87.6**

The major compounds neral and geranial represent 34.8% and 16.1% of the total composition of the essential oil of *A. stipitata*. α-Pinene represents 8.9% .



neral geranial α-pinene

Figure 65: Structures of the major compounds present in the essential oil of *A. stipitata*.

5. Non-volatile compounds

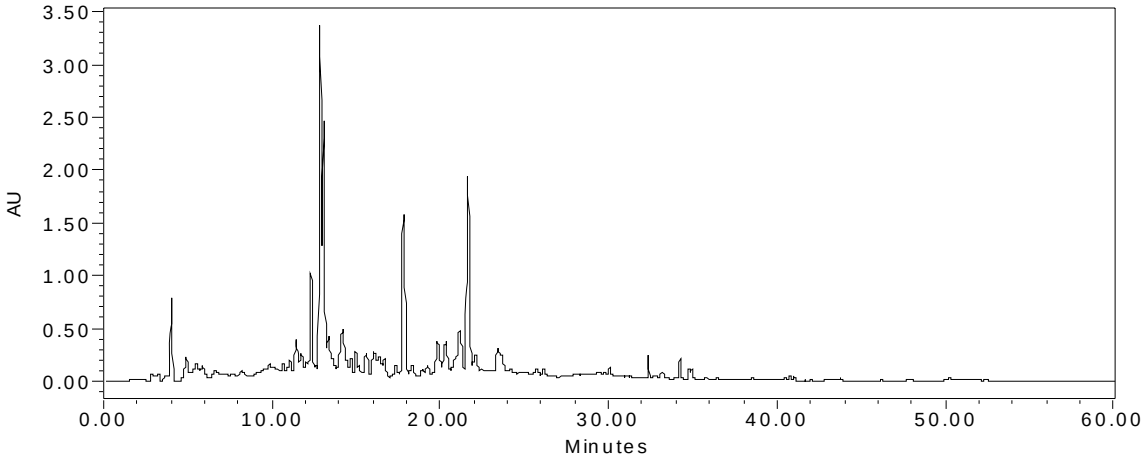


Figure 66: HPLC chromatogram of the dichloromethane and methanol (1:1) extract of *A. stipitata*.

Table 33: Compounds detected in the crude extract of *A. stipitata*.

R_t	UV max and / tentative identification	%
4.03	202.7 and 271.0	3.15
5.52	202.7 and 277.0	2.15
9.55	207.3, 280.5 and 310.2	2.28
11.43	214.4 and 325.7	2.97
12.33	212.0 and 258.0	5.25
12.86	225.0 and 265.1	15.27
13.06	219.1 and 328.1	11.74
13.35	203.8 and 317.4	3.08
14.19	203.8, 273.4 and 330.5 (flavanone)	6.26
15.60	207.3, 265.1, 301.9 and 344.8	2.14
16.07	206.2 and 325.7	2.43
16.37	207.3 and 273.4	2.17
17.81	206.2, 255.7 and 353.2 (flavonol)	10.24
19.83	207.3 and 271.0	3.71
20.33	206.2, 263.9 and 335.3 (flavone)	3.73
21.14	207.3 and 269.9	4.51
21.63	284.1 and 326.9 (flavanone)	11.77
22.08	207.3 and 284.1	2.36
23.42	207.3 and 269.9	4.80

6. Biological activity

- The extract was active against the pathogens in the antimicrobial assay, having MIC values of 2mg/ml against *Staphylococcus aureus* and *Bacillus cereus*, and 3mg/ml against *Klebsiella pneumoniae* and *Candida albicans*.
- The IC_{50} value of the essential oil in the anti-inflammatory assay could not be determined due to UV interference by its major compounds neral (34.8%) and geranial (16.1%). These compounds showed strong absorption at 234 nm which rendered the spectrophotometric measurement impossible. The extract did not display any activity at 100µg/ml.
- Both the extract and essential oil were inactive in the DPPH assay at 100µg/ml. However, the extract displayed activity in the ABTS assay (IC_{50} value of 28.20 ± 0.34µg/ml).
- Both the extract (IC_{50} value of 40.96 ± 8.24µg/ml) and essential oil (IC_{50} value < 0.0001µg/ml) were toxic in the MTT assay.

7. References

- Germishuizen G. and Meyer N.L. 2003. Plants of Southern Africa: an annotated checklist. *Strelitzia* **14**. National Botanical Institute, Pretoria, South Africa.
- Goldblatt P. and Manning J. 2000. Cape Plants: A Conspectus of the Cape Flora of South Africa. National Botanical Institute of South Africa, Pretoria.
- Pillans N. 1950. A revision of the genus *Agathosma* (Rutaceae). Journal of South African Botany, **16**: 55.

17. *Agathosma zwartbergense* Pillans

1. Botanical description
A single-stemmed, tangled, dwarf shrublet that grows to a height of 20cm. It has a lemon-scent when crushed. Two to four pink flowers are present in terminal clusters. The fruits are five chambered and the ovary is usually four or five lobed.

2. Distribution
Found on the upper sandstone slopes of the Swartberg and Khamanassie Mountains (Goldblatt and Manning, 2000).

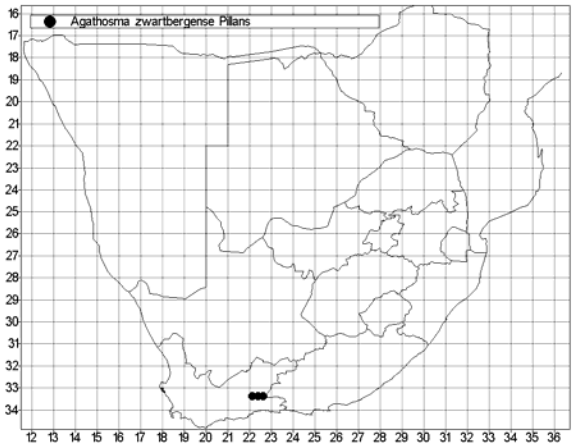


Figure 67: Geographical distribution of *A. zwartbergense*.

3. Origin: Swartberg Range (TTS 257).

4. Essential oil composition
4.1. Essential oil yield: 0.56% (dry weight).

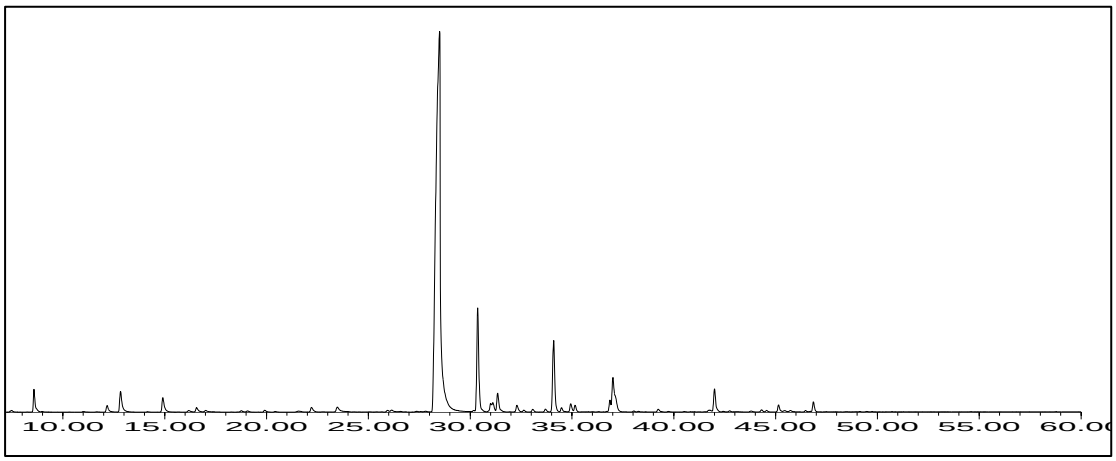
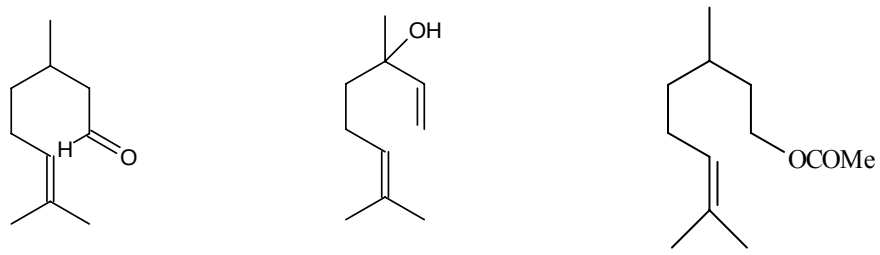


Figure 67: GC-MS chromatogram of *A. zwartbergense*.

Table 34: Compounds identified in the essential oil of *A. zwartbergense*.

RRI	Compound	%
1032	α-pinene	1.2
1035	α-thujene	0.3
1118	β-pinene	0.5
1132	sabinene	1.8
1159	δ-3-carene	tr
1174	myrcene	1.3
1203	limonene	0.4
1218	β-phellandrene	0.1
1255	γ-terpinene	0.1
1266	(*E*)-β-ocimene	0.1
1280	*p*-cymene	0.2
1290	terpinolene	0.1
1337	geijerene	0.4
1365	melonal	0.6
1450	*trans*-linalool oxide (furanoid)	tr
1487	citronellal	64.7
1553	linalool	8.0
1571	methyl citronellate	0.5
1583	isopulegol	1.5
1611	terpinen-4-ol	0.6
1641	methyl benzoate	0.2
1655	2,6-dimethyl-5-hepten-1-ol	0.2
1668	citronellyl acetate	5.7
1687	methyl chavicol	0.3
1706	α-terpineol	0.5
1740	geranial	tr
1765	geranyl acetate	0.7
1772	citronellol	3.8
1860	8-epi-dictamnol	0.1
1973	dodecanol	0.1
2050	(*E*)-nerolidol	0.2
2148	dictamnol	0.7
Total		**94.9**

The major compound citronellal represents 64.7% of the total composition of the essential oil of *A. zwartbergense*. Linalool and citronellyl acetate represent 8.0% and 5.7%.



citronellal linalool citronellyl acetate

Figure 69: Structures of the major compounds present in the essential oil of *A. zwartbergense*.

5. Non-volatile compounds

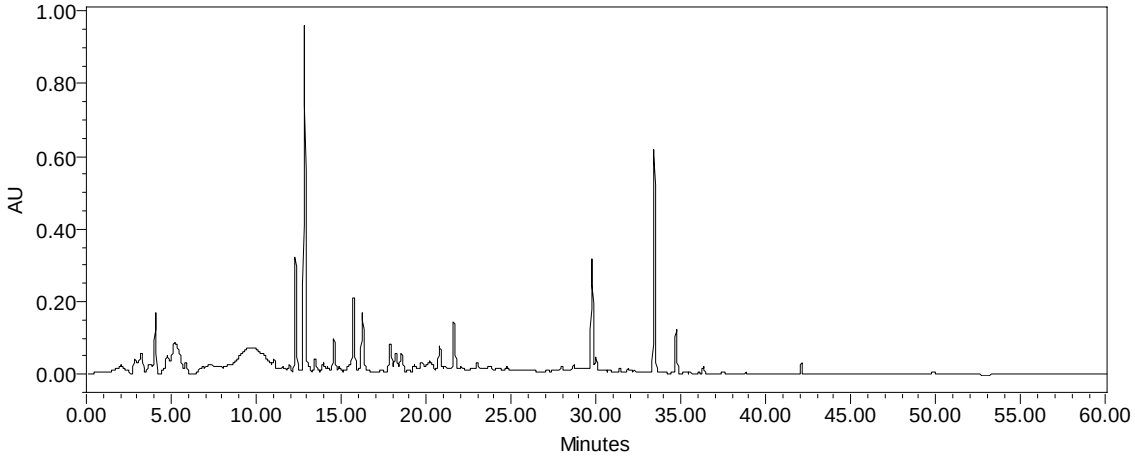


Figure 70: HPLC chromatogram of the dichloromethane and methanol (1:1) extract of *A. zwartbergense*.

Table 35: Compounds detected in the crude extract of *A. zwartbergense*.

R_t	UV max and / tentative identification	%
1.81	203.8, 284.1 and 393.8	1.46
2.03	202.7 and 284.1	1.38
3.24	207.3, 292.4, 321.0 and 376.9	1.98
4.07	205.0, 278.1 and 349.6	2.67
4.76	206.2, 265.1 and 376.9	1.99
5.21	206.2	5.98
7.30	208.5	2.16
9.63	208.5	10.35
9.84	208.5	5.67
10.47	208.5 and 277.0	2.27
12.31	210.9 and 258.0	5.29
12.85	220.3 and 266.3	16.80
14.57	212.0 and 294.8	2.18
15.73	209.7, 269.9 and 349.6 (flavone)	4.90
16.25	209.7, 268.7 and 349.9 (flavone)	4.31

R_t	UV max and / tentative identification	%
17.90	208.5, 255.7 and 354.4 (flavonol)	2.12
18.21	209.7, 268.7 and 341.2 (flavone)	1.77
18.55	209.7, 269.9 and 335.3 (flavone)	1.58
20.20	209.7, 271.0 and 316.2	1.90
20.78	207.3, 255.7 and 354.4 (flavonol)	2.32
21.64	205.0 and 284.1	3.12
29.76	210.9, 249.7 and 319.8	6.22
33.42	225.0 and 349.6	9.55
34.72	259.2, 356.8 and 396.2	2.03

6. Biological activity

- The extract was active against *Staphylococcus aureus* in the antimicrobial assay, having an MIC value 1.5mg/ml.
- The essential oil was active in the anti-inflammatory assay (IC_{50} value of the 29.93 ± 1.99µg/ml). The extract was inactive at 100µg/ml.
- Both the extract and essential oil were inactive in the DPPH assay at 100µg/ml. However, the extract was active in the ABTS assay (IC_{50} value of the 31.73 ± 0.36µg/ml).
- Both the extract (IC_{50} value of 38.12 ± 3.08µg/ml) and essential oil (IC_{50} value < 0.0001µg/ml) were toxic in the MTT assay.

7. References

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APPENDIX II

THE BIOLOGICAL ACTIVITY AND ESSENTIAL OIL COMPOSITION OF INDIGENOUS *AGATHOSMA* (RUTACEAE) SPECIES

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Abstract

The essential oil composition, antimicrobial, anti-inflammatory and cytotoxic activities of 17 indigenous *Agathosma* species (18 samples) were investigated in order to validate their use in traditional healing. The results were related to the chemical composition of the essential oils as determined by GC and GC/MS. The antimicrobial activity was evaluated using the minimum inhibitory concentration (MIC) method on four pathogens, i.e. *Staphylococcus aureus* (ATCC 12600), *Bacillus cereus* (ATCC 11778), *Klebsiella pneumoniae* (NCTC 9633) and *Candida albicans* (ATCC 10231). The anti-inflammatory activity was evaluated using the 5-lipoxygenase assay while the cytotoxic activity was determined using the MTT (3-[4,5-dimethyl-2-thiazol-yl]-2,5-diphenyl-2H-tetrazolium bromide) cellular viability assay. The antimicrobial assay revealed that the most active essential oil against *Candida albicans* was *A. collina* (MIC value of 3 mg/ml) whilst the most active essential oils against *Bacillus cereus* were *A. crenulata* and *A. pungens* (MIC values of 3 mg/ml). Nine of the

species had MIC values of 4 mg/ml against the Gram-positive pathogen *Staphylococcus aureus.* The essential oils showed less activity against the Gram-negative pathogen, *Klebsiella pneumoniae*. All the essential oils exhibited good *in vitro* anti-inflammatory activity with *A. collina* being the most potent (IC_{50} value of 25.98 ± 1.83 μg/ml). The results show that the essential oils are strong inhibitors of the enzyme 5-lipoxygenase. The essential oils proved to be toxic in the MTT assay displaying IC_{50} values of < 0.0001 μg/ml which were relatively toxic when compared to a plant-derived compound such as quinine (IC_{50} value of 136.06 ± 4.06 μg/ml). The results revealed some relationships between the major components, some bioactivities and toxicities. The essential oils were found to differ qualitatively and quantitatively in compositions and their analysis resulted in the identification of a total of 335 compounds in 18 of the samples.

Key Word Index

Agathosma arida, *Agathosma bathii*, *Agathosma betulina*, *Agathosma capensis, Agathosma collina*, *Agathosma crenulata*, *Agathosma hirsuta*, *Agathosma lanata*, *Agathosma namaquensis*, *Agathosma ovalifolia*, *Agathosma ovata*, *Agathosma parva*, *Agathosma pubigera*, *Agathosma pungens*, *Agathosma roodebergensis*, *Agathosma stipitata*, *Agathosma zwartbergense,* Rutaceae, buchu, essential oil composition, sabinene, linalool, β-pinene, menthone, isomenthone, pulegone, myrcene, limonene, β-phellandrene, neral, geranial, α-pinene, 1,8-cineole, citronellal, linalool, methyl citronellate, antimicrobial, anti-inflammatory, toxicity.