THE ANTIMICROBIAL ACTIVITY AND PHYTOCHEMISTRY OF LEAF ESSENTIAL OILS OF SELECTED RUTACEAE SPECIES

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Declaration

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

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.....day of .....July, 2002

Abstract

The Rutaceae is an aromatic family of plants confined to the Fynbos biome in South Africa. This family is represented by a number of genera e.g. *Agathosma, Adenandra, Coleonema, Vepris* etc. all containing a number of species which have been used for centuries in traditional healing.

Although many of these species have been used for centuries in local healing rites the biological activity and phytochemistry are poorly recorded.

Guided by both traditional use and chemotaxonomic criteria fourteen species were selected and collected from the wild. The leaves were hydrodistilled to extract the essential oils. The chemotypic variation was recorded by gas chromatography analysis (GC) and GC coupled to mass spectrometry. The oils were then subjected to antimircrobial activity against 8 bacteria and 4 fungi.

The essential oil composition varied quantitatively and qualitatively within and between populations. The results from the disc diffusion assay revealed that the essential oil from all the plants were active against all organisms except *Pseudomonas aeruginosa* and *Aspergillus niger. Coleonema pulchellum* (A) was the most active in terms of the zones of inhibition observed, particularly for that of *Enterococcus faecalis.* Minimum inhibitory concentrations (MIC) were performed on the seven most active sample specimens. None of the essential oils showed a clear MIC for *Escherichia coli.* MIC values for *Staphylococcus aereus* ranged from 8 mg/ml for *Coleonema pulchellum* (A) to 32 mg/ml for *Acmadenia sheilae.* The results for *Enterococcus faecalis* were more promising across the seven samples tested. All except *Adenandra villosa* and *Coleonema calicynum* had MICs of 8 mg/ml. It was decided to explore whether there were any differences in the MIC values with different solvents as a base to explore solubility. In this case acetone and dimethylsulfoxide acid (DMSO) were used separately. The MICs for the acetone solvent method compared to the DMSO solvent method, differed substantially with the exception of *Escherichia coli.*

In conclusion, the chemical profiles varied substantially within and between the species and the oils had a moderate to poor level of activity.

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Study Objectives

1. To determine the antimicrobial properties of the essential oils distilled from indigenous species belonging to the Rutaceae.
2. To chromatographically record, for the first time, the essential oil composition of the selected species.
3. To provide a scientific rationale for the traditional use of Rutaceous species in herbal preparations.

Introduction

The Traditional Healer's Position Within the Scope of Modern Medicine

Traditional medicine is the oldest most tried and tested form of medicine. In a sense it is degrading to refer to it as an "alternative", since it forms the basis of all medicine; conventional drugs, homeopathic medicine, Chinese medicines, etc. If we accept the fact that Africa is the cradle of mankind and the use of herbs is as old as mankind itself, then, it stands to reason that African traditional medicines and practices are indeed the oldest most tried and tested form of medicine.

Traditional medicine has been used by all cultures for centuries and is still the main form of medical treatment among 80 % of the world's population. It is disappointing to hear some doctors describing traditional medicine as quackery since many of today's drugs (e.g. quinine, reserpine, ephedrine, ipecac) come directly from plants while most synthetic drugs are based on chemicals extracted from herbs.

Essential oils from aromatic and medicinal plants have been known since antiquity to possess biological activity, notably antibacterial, antifungal and antioxidant properties. With the growing interest in the use of essential oils in both food and the pharmaceutical industries, a systematic examination of plant extracts for these properties has become increasingly important (Baratta *et al.* 1998). Natural products of botanical origin represent almost 50 % of all drugs in clinical use. Phytochemicals used in modern medicine today include quinine, morphine, codeine, aspirin and more recently anticancer drugs have been isolated from *Taxus* spp. and *Catharanthus roseus* (periwinkle). It is estimated that 70 % of South Africans consult traditional healers. Many of the traditional medicines used in South Africa are still derived from plants. Large quantities of plants and their derivatives (extracts) are sold in the informal sector.

Traditional healers use plants in a variety of ways. Parts of plants can be applied directly to wounds and cuts or prepared as powders that are used like snuff, or in the form of smoke or fumes, or as infusions. Knowledge of healing is passed on from generation to generation by healers or may be received from the ancestors through dreams. (http://www.bth.co.uk)

The harvesting of plants, their drying and analysis in laboratories mimics what traditional healers previously did. Laboratories use a variety of solvents to extract molecules of the active compounds, which are made into medicines in the form of liquids, ointments or pills. Traditional preparation of medicines involve the reduction of plant parts into powders, infusions and smoke or fumes. (http://www.museums.org.za/sam/ulwazi/plants.htm)

Developed and developing countries show a great interest in indigenous medicine, and many developing countries use traditional medicines at the primary health care

level. Many currently used drugs are expensive or not readily available, and a major setback to their continued usage is the development of resistance. There is thus an urgent need for new, inexpensive drugs that will be able to act for longer periods before resistance sets in (McGaw *et al.* 2000). The convention of using roots, bulbs, leaves, and other plant matter to cure the ailing is steeped in mystery. Traditional healers claim good results for diseases from a vast array of plants. Diagnoses are often subjective and are based upon 'feelings', visions and are wrought with superstition. Scientists however, demand an analytical explanation based on theory and observation rather than assumptions. Most men of science forswear them due to lack of scientific proof of its efficacy, incorrect diagnoses, imprecise dosing, unhygienic conditions, occult imprecision and superstitious aspects. In their favour nonetheless, is the fact that herbal remedies are cheaper, more accessible and acceptable to people in developing countries, are sources of potential new drugs and have a lesser level of bodily resistance to them as compared to synthetically engineered medication. To disclaim the traditional healer's medicine would therefore negate an expansive source of potentially beneficial cures. Thus it has become important to reconcile the two.

Meet The Family

The Rutaceae (the citrus family) is a large family comprising about one hundred and fifty genera and sixteen hundred species which are widely distributed throughout the tropical and temperate regions of the world (Buyukbingol *et al.* 1991). The Rutaceae is represented in South Africa by many indigenous genera which form part of South Africa's unique floristic biome, the Fynbos. Although many Rutaceae species have been used in traditional healing, their biological activity and phytochemical properties remain unknown. Members of the genus *Coleonema*, a close relative of the 'buchus" (*Agathosma*) have an established historic use in herbal remedies. The pungently aromatic *Coleonema juniperina* for example, is extensively used in the Nieuwoudtville area to treat fevers while *Coleonema pulchellum* is used in a mixture with *Artemisia afra* to treat colds and chest pains (van Wyk *et al.* 1997). Traditional uses of decoctions containing *Vepris lanceolata* include the treatment of gonorrhoea, bronchitis and amenorrhoea. The Rutaceae in general produce copious amounts of essential oils. Essential oils are known to have antimicrobial properties (e.g. tea tree oil) but as the composition of the oil is highly complex it is the combination of active molecules which makes some oils more potent than others.

Understanding the Essential Ingredient

According to the 8th edition of the French Pharmacopoeia (1965), essential oils (= essences = volatile oils = oils) are: "products, generally of rather complex composition, comprising the volatile principles contained in the plants, and more or less modified during the preparation process.

Essential oils occur virtually only in higher plants. They accumulate in all of the types of vegetable organs: flowers of course (bergamot tree, tuberose), but also leaves (citronella, eucalyptus, laurel), and, although this is less common, barks (cinnamon), woods (rosewood, sandalwood), roots (vetiver), rhizomes (turmeric, ginger), fruits (allspice, anise, star anise), and seed (nutmeg). Essential oils are complex and highly variable mixtures of constituents which belong, virtually exclusively, to two groups characterised by distinct biogenetic origins: the group of terpenoids, and the group - far less common- of aromatic compounds derived from phenylpropane. In most case the biological function of the terpenoids of essential oils remain obscure. It is conceivable, however, that they have an ecological role. This hypothesis is supported by experiments which prove the role of some terpenoids in plant interactions (allelopathic agents, particularly germination inhibitors), as well as plant-animal interactions: protection against predators (insects, fungi), and attraction of pollinating species. For some authors, the terpenoids might support some sort of 'communication', especially since their structural variety allows for the transfer of selective "biological messages" (Bruneton, 1993). Because the chemistry of essential oils is very complex, essential oils are diverse in their effects. As stipulated in 'The Real Essentials Newsletter' this also supports their antimicrobial effects, because the wide variety of antiseptic compounds in essential oils makes the mutation of microorganisms extremely difficult. In 1985, Dr. Jean C. Lapraz stated that no microbe could survive in the presence of the essential oils of cinnamon or oregano.

Uses of Drugs Containing Essential Oils

-Pharmacy. The vast majority of drugs are used crude, especially for the preparations of infusions (mint, balm, European vervain, orange blossoms), and under the form of simple galenicals. They are also used to prepare essential oils, a small number of which may have a therapeutic interest (particularly as external antiseptics), but the major part of which are designed for the aromatization of medications for the oral route. Essential oils are also the underpinning of a specific therapy: "aromatherapy".

-Perfumery. Perfumery is the chief outlet for essential oils, concretes, absolutes, and other resinoids. Cosmetology and the hygiene product industry are also consumers, even though the cost of natural products is often high and leads to the selection, for mass production, of synthetic alternatives. On the border between pharmacy and hygiene products, note the presence of essential oils in bath products ("soothing" or "relaxing" baths): they present the risk of percutaneous absorption of terpenoid constituents.

-Food Technology. Some drugs are used raw (herbs and spices), others are used as essential oils, resinoids, or oleoresins, dispersed, encapsulated, or complexed. Although refrigeration has replaced spices to ensure the conservation of foods, the development of new culinary practices (prepared foods, frozen dinners), a taste for the exotic (and for marketing), the flavour (or lack thereof) of the products of an intensive agriculture, and other factors have led to a rapid increase in the consumption of these types of products. All of the sectors of food technology are consumers: non-alcoholic beverages, confectionery, dairy products, meat products, soups, sauces, snacks, bakery products, without forgetting animal foods, and without forgetting ongoing research on the aromatization of meat is still afoot, and other work, just as entertaining, in hopes of conditioning the consumer (Bruneton, 1993).

-Miscellaneous Industries. Mostly chemical isolates are used i.e. pure substances isolated from essential oils, e.g. α- and β-pinenes, sclareol, (+) and (–) linalool, (+) citronellol, citral, eugenol, safrole, and more as starting materials for the synthesis of active drug principles, vitamins, fragrances, and so forth. Isolates are also used in perfumery (Bruneton, 1993).

Materials and Methods



Figure 1.



Figure 2.



Figure 3.

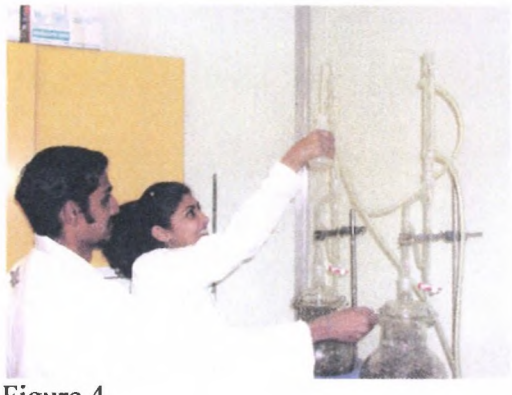


Figure 4.

Figure 1 - 4: Sequential events depicting the hydrodistillation process of plant material using the Clevenger apparatus.

Collection and Distillation

Samples of 14 plants representing four genera of the Rutaceae (Table 1) were collected. Plant material was harvested from various areas in southern Africa. Plant material was steam distilled to extract the essential oils. The plant material was placed in a Clevenger apparatus filled with enough water to cover the plant material (Fig. 1 – 4). This was then brought to the boil for 3 hours to allow the oils to condense out sufficiently. The heterogeneous vapours were condensed on the cold surface of the condenser, and the essential oil separated from the water, based on the difference in density and immiscibility.

Table 1: Yield (essential oil) and locality of the various Rutaceae specimens investigated.

SPECIES	LOCALITY	YIELD
Acmadenia alternifolia	Stevens Bank, Harkerville State Forest	0.15 %
Acmadenia obtusata	Coega	0.18 %
Acmadenia sheilae	Rooiberg, Ladismith	0.12 %
Adenandra obtusata	Arniston	0.63 %
Adenandra villosa	Table Mountain, Cape Town	0.03 %
Coleonema album	Cape St Blaize, Mossel Bay	0.27 %
Coleonema aspalathoides	Heuningberg Nature Reserve, Bredasdorp	0.15 %
Coleonema calicynum	Kirstenbosch, Cape Town	0.41 %
Coleonema pulchellum (A)	Wiwatersrand Botanical Garden	0.24 %
Coleonema pulchellum (B)	Schoenmakerskop, Port Elizabeth	0.78 %
Coleonema pulchellum (C)	Domestic garden in Johannesburg	0.27 %
Coleonema virgatum	Garcias Pass	0.71 %
Diosma prama (A)	Rooiberg, Ladismith	0.27 %
Diosma prama (B)	Rooiberg, Ladismith	0.52 %

Preparation of Agar

Thirty-eight grams of Muller-Hinton agar was added to 1 litre of distilled water. The mixture was then autoclaved (Butterworth) for 15 min at 121°C. This agar was used to support bacterial growth.



Figure 5: The loop is heated to ensure sterility.



Figure 6: The discs are saturated with essential oils.

Sixty-five grams of Tryptone Soya agar was added to 1 litre of distilled water. The mixture was then autoclaved for 15 min in a Butterworth autoclave at 121°C. This agar was used to support fungal growth.

Disc Diffusion Assay

Antimicrobial assays were performed on the hydrodistilled oils using the following bacterial test organisms: *Escherichia coli* (ATCC 8739.), *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 9027), *Serratia odorifera* (ATCC 33132), *Bacillus cereus* (ATCC 11778), *Staphylococcus epidermidis* (ATCC 2223) and *Bacillus subtilis* (ATCC 15244). A series of fungal test organisms were also included in the study i.e. *Candida albicans* (ATCC 10231), *Cryptococcus neoformans* (ATCC 90112), *Aspergillus niger* (clinical strain) and *Candida tropicalus* (clinical strain). Overnight bacterial spore suspensions were inoculated (Fig 5) and made up to yield an inoculum size of 1 x 10^6 and 100 ml of Muller-Hinton agar was poured into a square plate to allow all the samples to have the same standard conditions and left to set. One millilitre of the inoculum was then mixed with an additional 100 ml of Muller-Hinton agar and poured into the culture plate thus providing us with a suitable seeded agar plate for growth. Using aseptic manipulation, 6 mm discs were saturated with 20 µl of the hydrodistilled oil. These were then transferred onto the seeded agar plates. Neomycin (30 µg, Oxoid) was used as a positive control. The discs were left to pre-diffuse for approximately an hour before being incubated at 37 °C and left overnight (Fig. 6). Zones of inhibition were observed after 24 hrs. The exact same method was employed for fungal disc diffusion except that the medium was Tryptone Soya agar and the control was Nystatin (100 iu, Oxoid). The plates were incubated for 2 – 5 days depending on the test organism.

MIC/Microplate Method

Once antimicrobial activity was identified, a minimum inhibitory concentration was determined using the INT microplate method. The following extracts were used: *Acmadenia sheilae, Adenandra villosa, Coleonema calicyna, Coleonema pulchellum* (A), *Coleonema pulchellum* (B), *Coleonema pulchellum* (C) and *Diosma prama* (A). There was no inhibition of *Pseudomonas aeruginosa* on any of the discs for the diffusion assay. It was therefore excluded as a test organism in the MIC/microplate method. *Escherichia coli, Staphylococcus aureus, Enterococcus faecalis* cultures



Figure 7: Preparing serial dilutions for MIC assay.

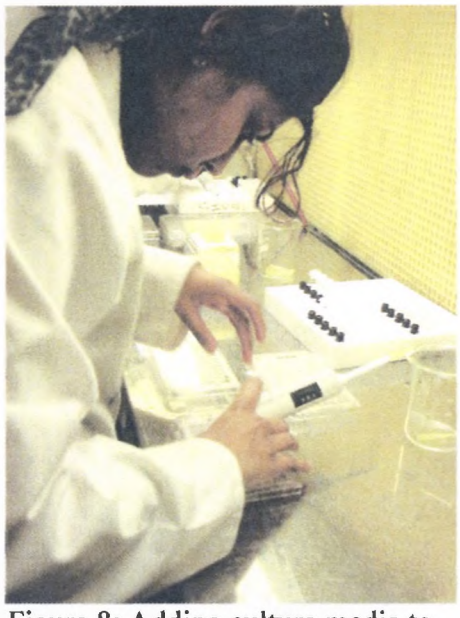


Figure 8: Adding culture media to microtitre plates.



Figure 9: Adding essential oils to microtitre wells.

were initially inoculated in a Muller-Hinton broth for bacterial growth and *Candida albicans* and *Cryptococcus neoformans* were grown in Tryptone Soya broth. They were incubated for 24 hrs at 37°C. The inoculum was transferred to 5 ml sterile broth and incubated at 37°C for 4 hrs (Mc Farlands standard). The plant extracts were weighed out (51.2 mg) and dissolved in 400 µl of acetone to yield a concentration of 64 mg/ml once the culture was added. It was decided to compare the solvent's influence on activity so a parallel test was performed using Dimethylsulfoxide (DMSO) as an alternate solvent. Hydro-distilled plant extracts at starting concentrations of 64 mg/ml are transferred into the first well of a micro titre plate. Serial dilutions are performed so that essential oil concentrations of 32, 16, 8, 4, 2, 1, 0.5 and 0.25 µg/ml are obtained (Fig. 7). The micro titre wells were then transferred to the microbiology lab where culture was then added. A fixed bacterial culture yielding an inoculum size of 1×10^6 was added to all wells and incubated at 37°C for 24 hrs. A 2 mg/ml *p*-iodonitrotetrazolium violet (INT) solution was used to enhance visualisation for the bacterial growth. Each inoculation well then received 40 µl of INT. The plates were observed at 1 hr, 2 hrs and 24 hrs. The minimum inhibitory percent was defined as the lowest percentage of oil which resulted in complete inhibition of visible growth.

Gas Chromatography (GC)

Shimadzu 17A gas chromatograph was used with the following parameters; Column: J&W-DB1 (30 m x 0.25 mm id, 0.25 μm film thickness), temperatures: injection port 230 °C, column 60 °C for 1 min., 5 °C/min to 180 °C for 2 min., (total = 25 min.). This method was used to determine any qualitative and quantitative variation within and between populations.

GC/MS Method

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

automatically from peak areas of the total ion chromatogram. Library search was carried out using Wiley GC/MS Library and TBAM Library of essential oil Constituents.

Species Monographs

Acmadenia alternifolia Cham.

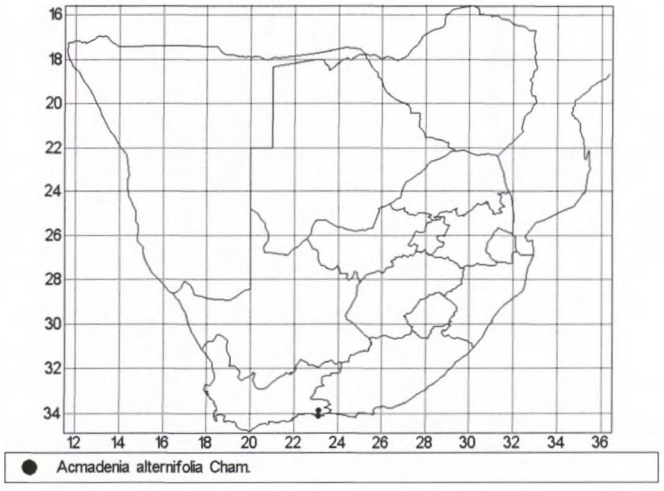
Botanical Description:

Shrubs: fine leafy, single-stemmed, aromatic shrublet to 1 m.

Leaves: ascending, incurved above, linear to lanceolate, acute, sharply mucronate, margins scrabid. Flowers: several, crowded at branch tips, pink. Fruits with long horns. Mainly June-Aug. (Goldblatt and Manning, 2000).

Distribution:

Rocky outcrops and cliff, Knysna to Keursboom River.



Essential oil composition:

Essential oil obtained by hydrodistillation (3 hrs) from plants collected at Stevens Bank, Harkerville. The oil yield is 0.15 % (wet wt).

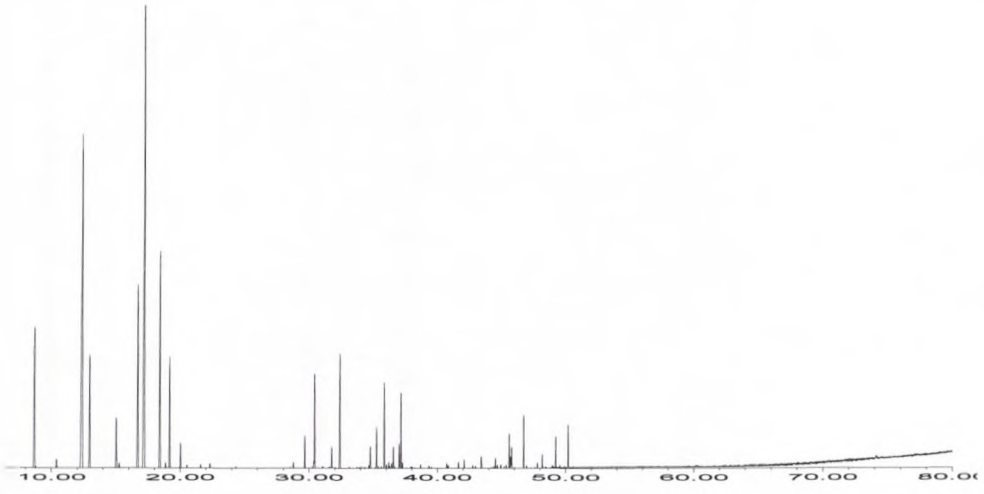
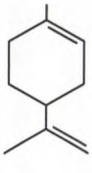


Figure 10: GC chromatogram displaying the partition of the essential oil components of *Acmadenia alternifolia*.

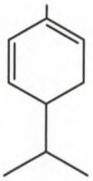
Table 2: GC/MS data and integration percentages for *Acmadenia alternifolia* essential oil.

RRI (Relative Retention Index)	Component	Area %
1032	**α-pinene**	**14.54**
1076	camphene	1.50
1118	**β-pinene**	**30.60**
1132	**sabinene**	**6.63**
1174	myrcene	2.78
1203	**limonene**	**6.88**
1218	**β-phellandrene**	**17.82**
1246	(Z)-β-ocimene	5.97
1255	γ-terpinene	0.24
1266	(E)-β-ocimene	2.81
1280	p-cymene	0.76
1553	linalool	0.84
1611	terpinen-4-ol	0.89
1690	cryptone	0.20
1706	α-terpineol	0.32
1726	germacrene-D	0.50
1755	bicyclogermacrene	0.22
1773	δ-cadinene	0.55
2098	globulol	0.16
2144	spathulenol	0.22
2255	α-cadinol	0.12
	TOTAL	**94.55**

Twenty-one compounds are identified in the leaf essential oils, which represent 94.55 % of the total composition. The bicyclic monoterpenoid, β-pinene, is the major compound in this species of *Acmadenia* (30 % of the total). Other monoterpenes; β-phellandrene (17.82 %), α-pinene (14.54 %), sabinene (6.63 %) and limonene (6.88 %) are the other major components detected in the hydro-distilled oil.



limonene



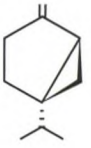
phellandrene



α-pinene



β-pinene



sabinene

Acmadenia obtusata (Thunb.) Batl. & H.L. Wendl.

Botanical Description:

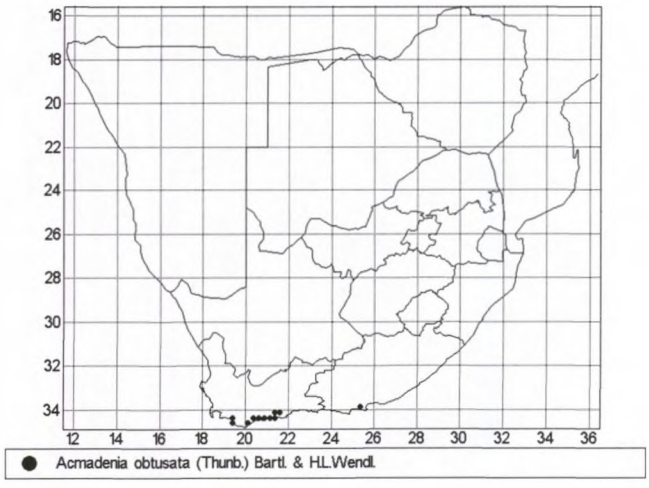
Shrubs: closely leafy, single-stemmed, aromatic shrublet to 30 cm.

Leaves: sometimes opposite, adpressed, imbricate, linear-lanceolate, erect, margins narrow, scabrid-serrulate.

Flowers: solitary, terminal, often crowded on short branchlets near stem apices, bright pink. Mainly Sept.-Nov. (Goldblatt and Manning, 2000).

Distribution:

Limestone and calcareous sands, Stanford to Alexandria.



Essential oil composition:

Essential oil obtained by hydrodistillation (3 hrs) from plants collected from the Coega area. The oil yield is 0.18 % (wet wt).

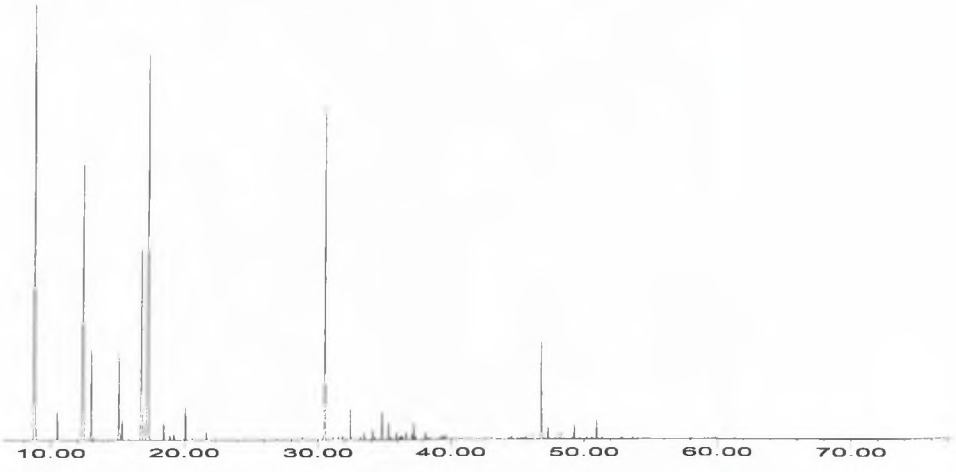


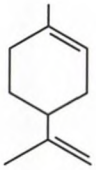
Figure 11: GC chromatogram displaying the partition of the essential oil components of *Acmadenia obtusata*.

Table 3: GC/MS data and integration percentages for *Acmadenia obtusata* essential oil.

RRI	Component	Area %
1032	**α-pinene**	**27.44**
1035	α-thujene	0.65
1076	camphene	1.16
1118	**β-pinene**	**15.09**
1132	sabinene	2.96
1138	butyl benzene	0.13
1174	myrcene	2.83
1183	p-mentha-1(7),8-diene (pseudolimonene)	0.62
1203	**limonene**	**7.54**
1218	**β-phellandrene**	**23.20**
1246	(Z)-β-ocimene	0.45
1255	γ-terpinene	0.12
1266	(E)-β-ocimene	0.16
1280	p-cymene	0.87
1290	terpinolene	0.06
1319	(E)-2,6-dimethyl-1,3,7-nonatriene	0.16
1497	α-copaene	0.04
1553	**linalool**	**7.61**
1562	isopinocamphone	0.07
1571	trans-p-menth-2-en-1-ol	0.07
1586	pinocarvone	0.08
1604	isobornyl acetate	0.04
1611	terpinen-4-ol	0.45
1638	cis-p-menth-2-en-1-ol	0.05
1648	myrtenal	0.13
1664	trans-pinocarveol	0.22
1674	p-mentha-1,5-dien-8-ol	0.06
1690	cryptone	0.49
1706	α-terpineol	0.25
1719	borneol	0.06
1725	verbanone	0.14
1743	α-cadinene	0.11
1744	phellendral	0.11
1755	bicyclogermacrene	0.05
1760	isobornyl benzoate	0.04
1773	δ-cadinene	0.28
1776	γ-cadinene	0.08
1802	cumin aldehyde	0.08
1804	myrtenol	0.19
1811	p-mentha-1,3-dien-7-al	0.06
1845	trans-carveol	0.08
1857	geraniol	0.07

RRI	Component	Area %
1864	p-cymen-8-ol	0.09
2202	germacrene-D-4-ol	0.02
2098	globulol	0.03
2113	cumin alcohol	0.09
2144	spathulenol	1.55
2187	t-cadinol	0.10
2209	t-muurolol	0.10
2219	δ-cadinol	0.04
2255	α-cadinol	0.08
	TOTAL	**96.53**

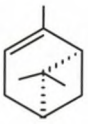
Fifty-two compounds are identified in the leaf essential oils, which represents 96.53 % of the total composition. It is observed that *Acmadenia obtusata's* dominant components closely resemble *Acmadenia alternifolia.* The quantities of these vary however with α-pinene being predominant at 27.44 % of the total. β-Phellandrene also represents a large proportion of the total i.e. 23.20 %. β-Pinene, linalool and limonene appear respectively in the quantities of 15.09 %, 7.61 % and 7.54 %.



limonene



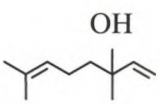
phellandrene



α-pinene



β-pinene



linalool



Figure 12: Flowering shrub of *Acmadenia sheilae* (Rooiberg).

Acmadenia sheilae I. Williams

Botanical Description:

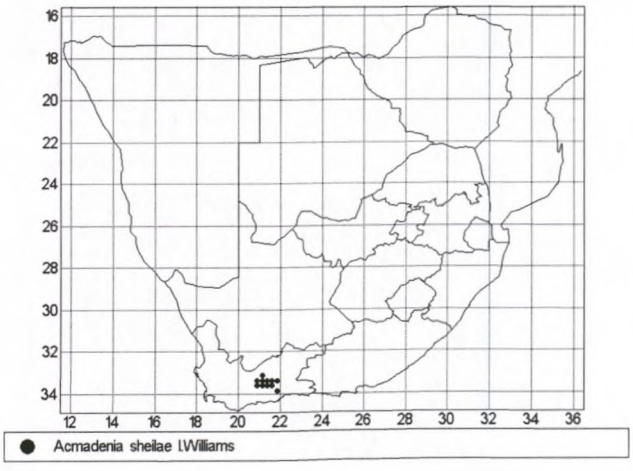
Shrubs: closely leafy, single-stemmed, aromatic shrublet to 40 cm.

Leaves: short, petiolate, opposite and decussate, oblong, obtuse.

Flowers: solitary, terminal, pink to red, anthers with a large, pointed apical gland. Mainly July-Nov. (Goldblatt and Manning, 2000).

Distribution:

Sandstone slopes, abundant on Klein Swartberg, Touwsberg and Rooiberg.



Essential oil composition:

Essential oil obtained by hydrodistillation (3 hrs) from plants collected in the Rooiberg, Ladismith area. The oil yield is 0.12 % (wet wt).

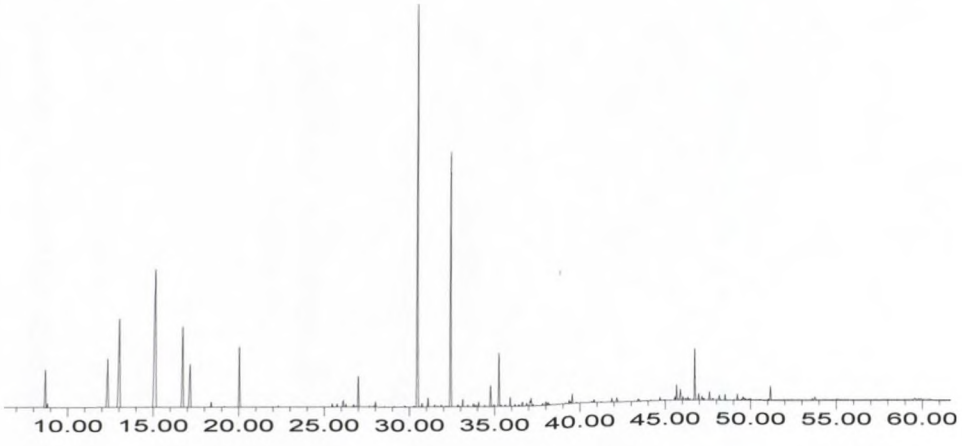


Figure 13: GC chromatogram displaying the partition of the essential oil components of *Acmadenia sheilae*.

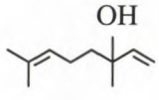
Table 4: GC/MS data and integration percentages for *Acmadenia sheilae* essential oil.

RRI	Component	Area %
1032	**α-pinene**	**9.30**
1118	**β-pinene**	**9.33**
1132	**sabinene**	**16.12**
1174	**myrcene**	**25.09**
1203	limonene	7.45
1213	1,8-cineole	2.14
1218	β-phellandrene	2.20
1280	p-cymene	2.95
1429	perillene	0.32
1430	α-thujone	0.14
1451	β-thujone	0.97
1553	**linalool**	**8.95**
1571	trans-p-menth-2-en-1-ol	0.24
1611	terpinen-4-ol	4.48
1683	cis-p-menth-2-en-1-ol	0.16
1690	cryptone	0.33
1682	α-terpineol	0.79
1729	cis-1,2-epoxy-terpin-4-ol	0.22
1772	citronellol	0.26
1773	δ-cadinene	0.09
1864	p-cymen-8-ol	0.17
2100	heneicosane	0.15
2144	spathulenol	0.48
2184	cis-p-menth-3-en-1,2-diol	0.13
2255	α-cadinol	0.08
	TOTAL	**92.54**

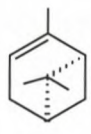
In this leaf essential oil we are able to positively identify twenty-five of the different compounds thereby making up a total of 92.54% known volume. The acyclic monoterpene myrcene, contributes the largest volume at 25.09 %. β-Pinene and α-pinene each represent 9.3 % of the total. Linalool, a linear monoterpenic alcohol constitutes 8.95 %. Thus the five major constituents combined make up *ca.* 70 %.



myrcene



linalool



α-pinene



β-pinene



sabinene



Figure 14: Flowers of *Adenandra uniflora*.

Adenandra obtusata Sond.

Botanical Description:

Shrubs: densely leafy, aromatic shrublet to 50 cm, glutinous on young parts.

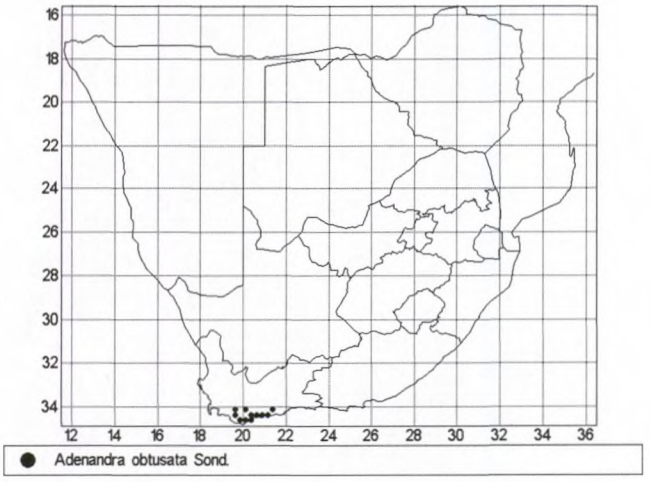
Leaves: erect to spreading, imbricate, oblong, margins thickened, revolute.

Flowers: sessile or subsessile, in 1-4 flowered, glutinous heads, white, pink outside.

Mainly Sept.-Nov. (Goldblatt and Manning, 2000).

Distribution:

Limestone hills and flats, Agulhas to Cape Infanta.



Essential oil composition:

Essential oil obtained by hydrodistillation (3 hrs) from plants collected from Arniston.

The oil yield is 0.63 % (wet wt).

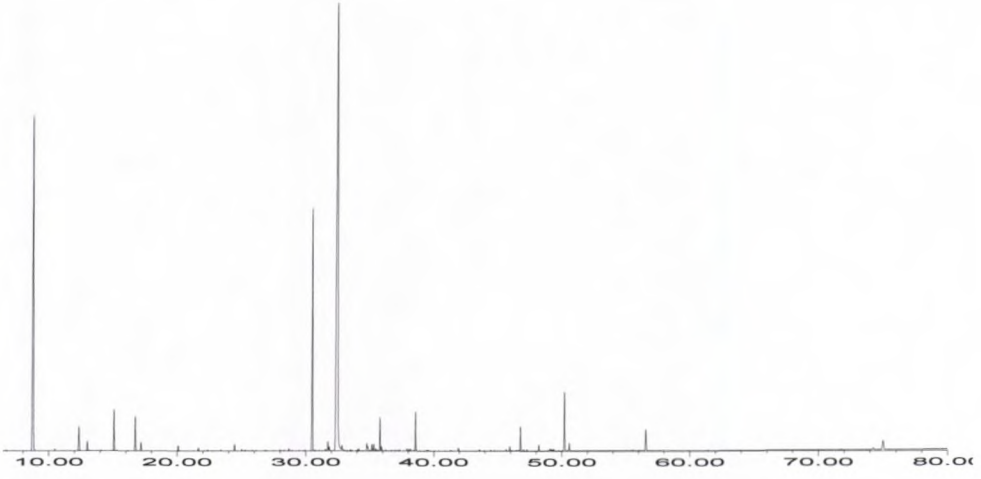


Figure 15: GC chromatogram displaying the partition of the essential oil components of *Adenandra obtusata.*

Table 5: GC/MS data and integration percentages for *Adenandra obtusata* essential oil.

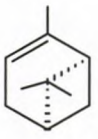
RRI	Component	Area %
1032	**α-pinene**	**33.16**
1076	camphene	0.15
1118	β-pinene	1.65
1132	sabinene	0.62
1174	**myrcene**	**2.39**
1203	limonene	1.89
1218	β-phellandrene	0.48
1280	p-cymene	0.31
1319	(E)-2,6-dimethyl-1,3,7-nonatriene	0.15
1337	geijerene	0.06
1360	hexanol	0.07
1384	α-pinene oxide	0.38
1398	nonanone	0.07
1478	cis-linalool oxide (furanoid)	0.06
1553	**linalool**	**10.60**
1586	pinocarvone	0.06
1604	isobornyl acetate	0.10
1604	**2-undecanone**	**33.34**
1628	aromadendrene	0.61
1648	myrtenal	0.15
1664	trans-pinocarveol	0.14
1683	trans-verbenol	0.35
1690	cryptone	0.12
1698	myrtenyl acetate	0.31
1706	α-terpineol	0.41
1604	2-undecanone	0.80
1725	verbanone	0.18
1798	methyl salicylate	0.15
1804	myrtenol	0.18
1815	2-tridecanone	0.98
1949	(Z)-3-hexenyl nonoate	0.14
2008	caryophyllene oxide	0.08
2098	globulol	0.18
2144	spathulenol	0.95
2255	α-cadinol	0.15
2300	tricosane	0.14
2524	abietatriene	1.02
	TOTAL	**94.74**

Of the thirty-eight compounds identified in this plant essential oil, interestingly 2-undecanone is the main constituent (33.34 %). This is the first time that this

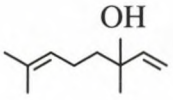
monoterpene ketone has been observed in such large quantities. The percentage of α-pinene (33.16 %) is almost the same as 2-undecanone. It is found that the following monoterpenes exist in the quantities mentioned: the acyclic linalool at 10.60 % and sandracopimaradiene and myrcene together represent about 4 %. Their combined total is therefore approximately 80 % of the 94.74 %.



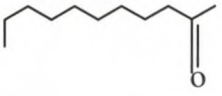
myrcene



α-pinene



linalool



2-undecanone

Adenandra villosa (P.J. Bergius) Licht. ex Roem. & Schult.

Botanical Description:

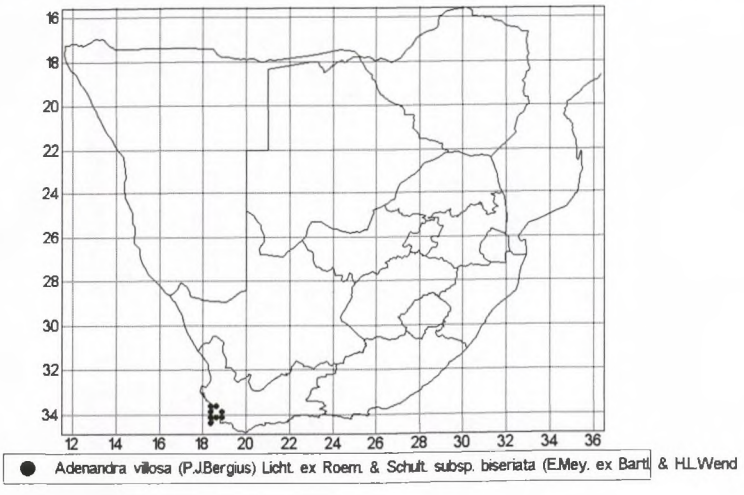
Shrubs: closely leafy, aromatic shrub to 1 m.

Leaves: ascending, sometimes loosely imbricate, elliptic to oblong, often hairy beneath.

Flowers: sub-sessile or on short pedicles, 2-6 in condensed corymbs, white, pink or red outside, calyx usually hairy. Mainly Aug.-Nov. (Goldblatt and Manning, 2000).

Distribution:

Sandstone slopes, Cold Bokkeveld and Piketberg to Langeberg and Kleinrivier Mts.



Essential oil composition:

Essential oil obtained by hydrodistillation (3 hrs) from plants collected from Table Mountain (Cape Town). The oil yield is 0.03 % (wet wt).

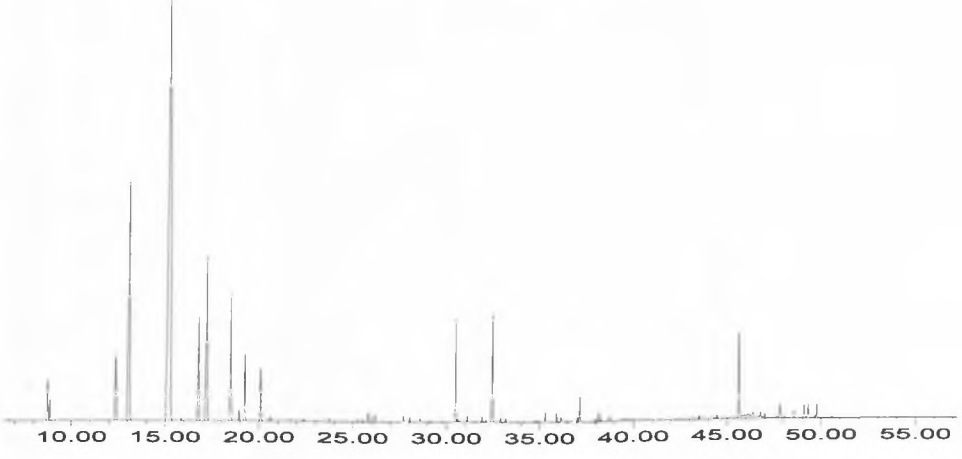


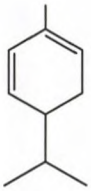
Figure 16: GC chromatogram displaying the partition of the essential oil components of *Adenandra villosa*.

Table 6: GC/MS data and integration percentages for *Adenandra villosa* essential oil.

RRI	Component	Area %
1032	α-pinene	1.63
1035	α-thujene	0.92
1118	β-pinene	2.92
1132	**sabinene**	**13.79**
1174	**myrcene**	**49.55**
1188	α-terpinene	0.08
1203	**limonene**	**3.70**
1213	**1,8-cineole + β-phellandrene**	**6.61**
1246	**(Z)-β-ocimene**	**4.15**
1255	γ-terpinene	0.31
1266	(E)-β-ocimene	1.94
1280	p-cymene	1.55
1290	terpinolene	0.12
1337	geijerene	0.06
1424	0-methyl anisol	0.09
1398	6,7-epoxy myrcene	0.30
1474	trans-sabinene hydrate	0.15
1476	(Z)-β-ocimene epoxide	0.09
1498	(E)-β-ocimene epoxide	0.12
1553	linalool	1.86
1556	cis-sabinene hydrate	0.16
1571	trans-p-menth-2-en-1-ol	0.14
1586	myrcenone	0.12
1600	β-elemene + terpinen-4-ol	1.85
1626	2-methyl-6-methylene-3,7-octadien-2-ol	0.08
1638	cis-p-menth-2-en-1-ol	0.09
1689	trans-piperitol	0.06
1706	α-terpineol	0.21
1726	germacrene-D	0.17
1733	neryl acetate	0.08
1748	piperitone	0.07
1755	bicyclogermacrene	0.05
1758	cis-piperitol + geranyl acetate	0.09
1772	citronellol	0.44
1808	nerol	0.17
1830	2,6-dimethyl-3(E),5(E),7-octatriene-2-ol	0.08
1857	geraniol	0.04
2096	elemol	2.25
2144	spathulenol	0.14
2185	γ-eudesmol	0.38
2219	dimyrcene-II-a	0.12

RRI	Component	Area %
2250	α-eudesmol	0.32
2257	β-eudesmol	0.33
	TOTAL	**97.38**

Forty-three known compounds provided 97.38 % of the total essential oil component. Of these, myrcene dominated at 49.55 %. Bicyclic sabinene constituted 13.79 %. β-Phellandrene and 1,8-cineole together constitute 6.61 %. Ocimene, an acyclic monoterpene, is present at 4.15 % of the total.



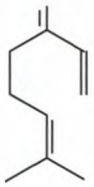
phellandrene



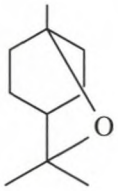
sabinene



ocimene



myrcene



1,8 cineole



Figure 17: Habitat of *Coleonema album* (Mossel Bay). Large dome-shaped shrub.



Figure 18: Flowers of *Coleonema album*.

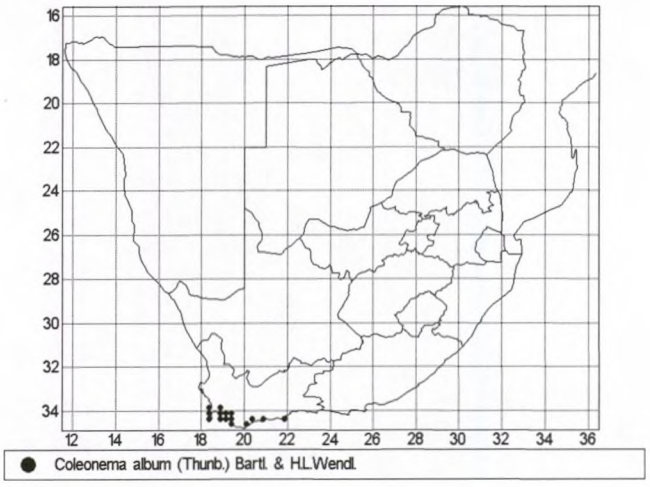
Coleonema album (Thunb.) Bartl. & H.L. Wendl.

Botanical Description:

Shrubs to 2 m. Leaves- linear-oblong, sweet-smelling. Flowers- solitary, crowded at branch tips, white, 6-7 mm diam. Aug.-Nov. (Goldblatt and Manning, 2000).

Distribution:

Coastal sandstone or granite outcrops, Saldanha to Cape Infanta.



Essential oil composition:

Essential oil obtained by hydrodistillation (3hrs) from plants collected from Cape St Blaize, Mossel Bay. The oil yield is 0.27 % (wet wt).

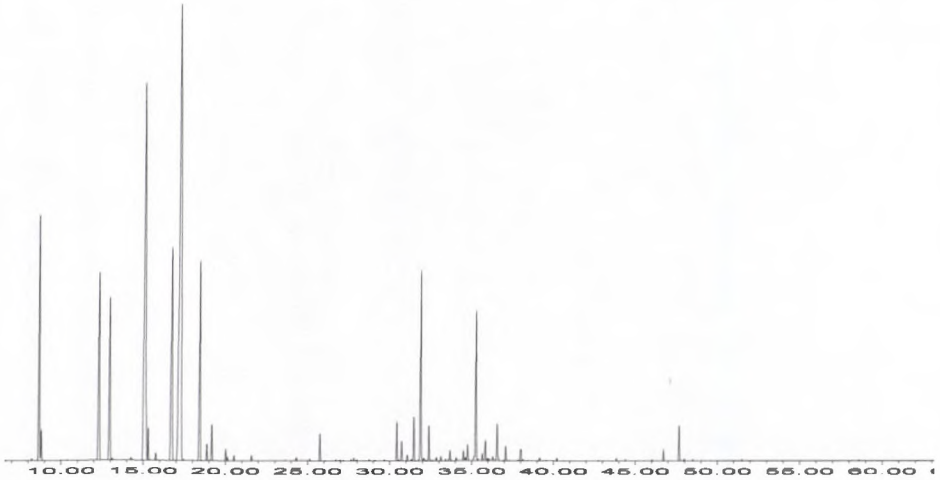


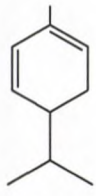
Figure 19: GC chromatogram displaying the partition of the essential oil components of *Coleonema album*.

Table 7: GC/MS data and integration percentages for *Coleonema album* essential oil.

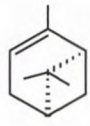
RRI	Component	Area %
1018	methyl-2-methyl butyrate	0.06
1032	**α-pinene**	**8.18**
1035	α-thujene	0.71
1118	**β-pinene**	**7.02**
1132	sabinene	5.32
1138	butyl benzene	0.06
1159	δ-3-canene	0.09
1174	**myrcene** (20.5%) + **α-phellandrene** (1%)	**21.55**
1183	p-mentha-1(7),8-diene (pseudolimonene)	0.68
1188	α-terpinene	0.19
1203	**imonene**	**7.92**
1213	**1,8-cineole (1%) + β-phellandrene (29,1%)**	**30.39**
1246	(Z)-β-ocimene	5.63
1255	γ-terpinene	0.35
1266	(E)-β-ocimene	0.80
1280	p-cymene	0.27
1290	terpinolene	0.11
1319	(E)-2,6-dimethyl-1,3,7-nonatriene	0.10
1382	cis-allo ocimene	0.10
1400	nonanal	0.03
1398	6,7-epoxy myrcene	0.47
1476	(Z)-β-ocimene epoxide	0.06
1499	α-campholene aldehyde	0.04
1553	linalool	0.52
1562	isopinocamphone	0.33
1571	trans-p-menth-2-en-1-ol	0.10
1586	pinocarvone	0.77
1586	myrcenone	2.48
1611	terpinen-4-ol	0.42
1626	2-methyl-6-methylene-3,7-octadiene-2-ol	0.06
1638	cis-p-menth-2-en-1-ol	0.08
1648	myrtenal	0.05
1664	trans-pinocarveol	0.07
1682	δ-terpineol	0.13
1690	cryptone	0.25
1706	α-terpineol	1.93
1705	zizanene	0.03
1725	verbenone	0.22
1726	germacrene-D	0.03
1744	phellendral	0.28
1755	bicyclogermacrene	0.07
1772	citronellol	0.16

RRI	Component	Area %
1804	myrtenol	0.11
1805	α-campholene alcohol	0.15
1845	trans-carveol	0.06
1804	myrtenol	0.05
2030	methyl eugenol	0.04
2144	spathulenol	0.08
2186	eugenol	0.35
2200	trans-methyl isoeugenol	0.03
	TOTAL	**98.98**

Fifty compounds are identified in this species. 1,8-Cineole (1 %) and β-phellandrene (29.1 %) constituted 30.39 % while myrcene (20.5 %) and α-phellandrene (1 %) combined total was 21.55 %. α-Pinene and β-pinene totalled *ca.* 15 %.



phellandrene



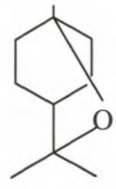
α-pinene



β-pinene



myrcene



1,8 cineole

Coleonema aspalathoides Juss. ex Don

Botanical Description:

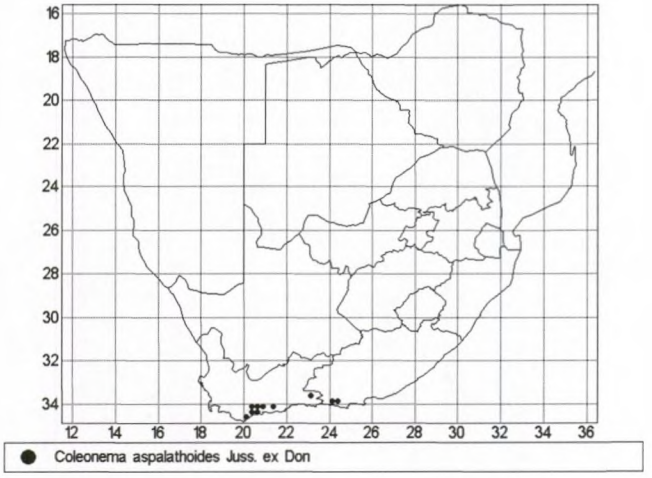
Dense shrub to 1.5 m.

Leaves: linear-oblong, scarcely scented.

Flowers: solitary, crowded pink, petals not obviously clawed, 8-10 diameter. Apr.-Nov. (Goldblatt and Manning, 2000).

Distribution:

Sandstone slopes, Potberg to Zuurberg.



Essential Oil Composition:

Essential oil obtained by hydrodistillation (3 hrs) from plants collected from Heuningvlei Nature Reserve, Bredasdorp. The oil yield is 0.15 % (wet wt).

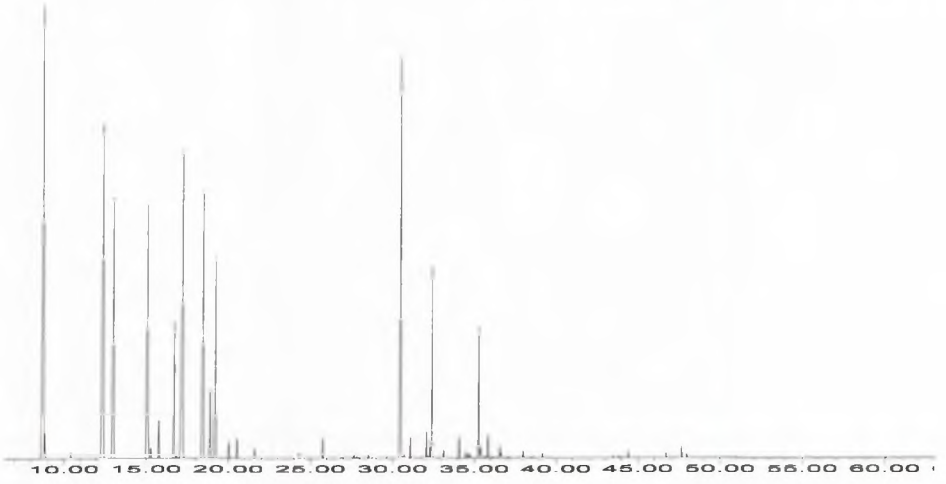
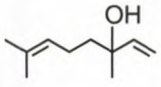


Figure 20: GC chromatogram displaying the partition of the essential oil components of *Coleonema aspalathoides*.

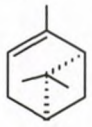
Table 8: GC/MS data and integration percentages for *Coleonema aspalathoides* essential oil.

RRI	Component	Area %
1032	**α-pinene**	**9.05**
1035	α-thujene	0.58
1076	camphene	0.21
1118	β-pinene	6.49
1132	**sabinene**	**24.00**
1174	myrcene	2.62
1203	limonene	3.81
1213	1,8-cineole	2.14
1280	**p-cymene**	**12.07**
1450	trans-linalool oxide (furanoid)	0.27
1474	trans-sabinene hydrate	0.16
1478	cis-linalool oxide(furanoid)	0.17
1553	**linalool**	**11.25**
1562	isopinocamphone	0.27
1571	trans-p-menth-2-en-1-ol	0.76
1586	pinocarvone	0.08
1600	β-elemene	0.74
1611	**terpinen-4-ol**	**12.16**
1638	cis-p-menth-2-en-1-ol	0.50
1664	trans-pinocarveol	0.41
1687	α-humulene	0.13
1689	trans-piperitol + cryptone	0.48
1700	p-mentha-1,8-dien-4-ol	0.15
1706	α-terpineol	1.27
1719	borneol	0.18
1729	cis-1,2-epoxy-terpin-4-ol	0.70
1742	β-selinene	0.08
1748	piperitone	0.12
1758	cis-piperitol	0.31
1797	p-menthylacetophenone	0.10
1805	α-campholene alcohol	0.22
1857	geraniol	0.07
1864	p-cymen-8-ol	0.34
1949	(Z)-3-hexenyl nonanoate	0.13
2001	isocaryophllene oxide	0.12
2008	caryophyllene oxide	0.51
2050	(E)-nerolidol	0.12
2071	humulene epoxide-II	0.08
2100	heneicosane	0.25
2113	cumin alcohol	0.22
2144	spathulenol	0.59
2273	selin-11-en-4∝-ol	0.16
	TOTAL	**94.07**

Forty-two compounds are identified in the leaf essential oils, which represent 94.07 % of the total composition. Sabinene, a bicyclic compound makes up 24 % of the total. Two alcohols linalool and terpinen-4-ol constitute *ca.* 23 %. Bicyclic α-pinene and monocyclic p-cymene further add to the total by *ca.* 21 %.



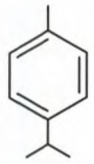
linalool



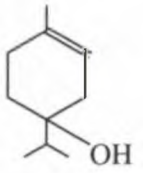
α-pinene



sabinene



p-cymene



terpinen-4-ol

Coleonema calycinum (Steud) I. Williams

Botanical Description:

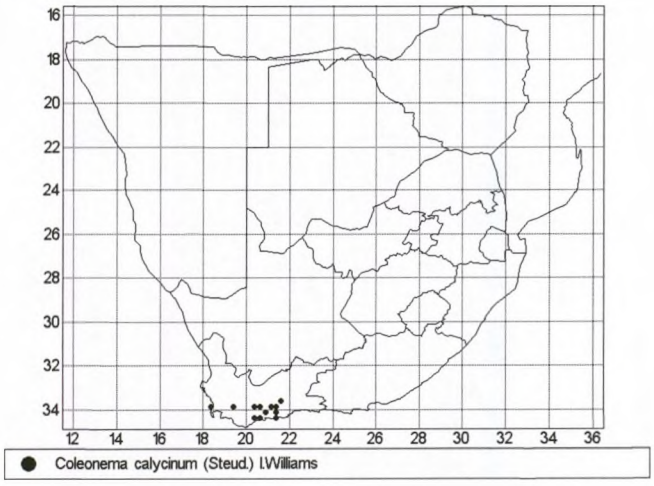
Shrubs to 3 m.

Leaves: linear, 15 mm long, rum-scented.

Flowers: solitary, white, 5.5-6.5 mm diameter. Sept.-Oct. (Goldblatt and Manning, 2000).

Distribution:

Sandstone and limestone slopes, Houwhoek to Riversdale.



Essential oil composition:

Essential oil obtained by hydrodistillation (3 hrs) from plants collected from Kirstenbosch (NBG), Cape Town. The oil yield is 0.41 % (wet wt).

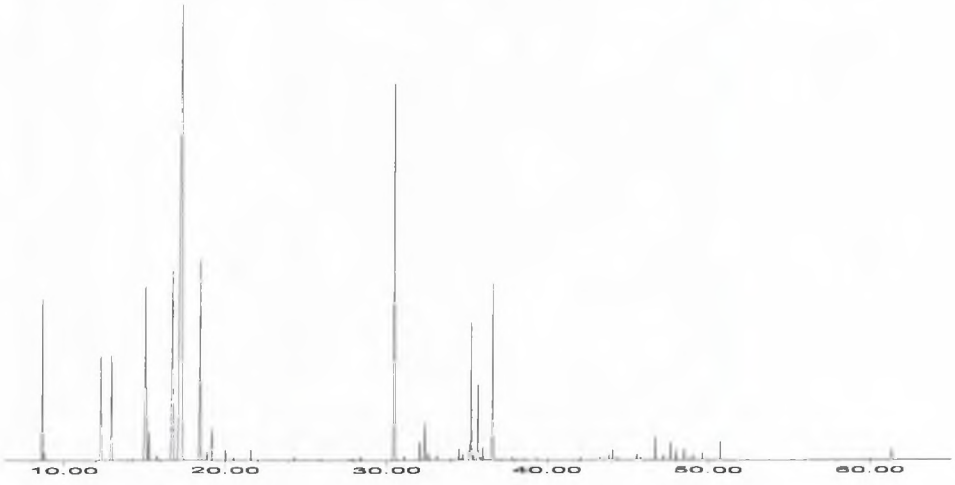


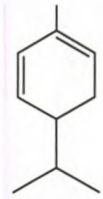
Figure 21: GC chromatogram displaying the partition of the essential oil components of *Coleonema calycinum*.

Table 9: GC/MS data and integration percentages for *Coleonema calycinum* essential oil.

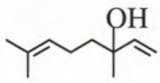
RRI	Component	Area %
1032	α-pinene	5.96
1035	α-thujene	0.23
1118	β-pinene	4.19
1132	sabinene	3.79
1159	δ-3-carene	0.07
1174	**myrcene + α-phellandrene**	**8.24**
1183	p-mentha-1(7),8-dien (pseudolimonene)	0.84
1188	α-terpinene	0.16
1203	**limonene**	**9.23**
1213	**1,8-cineole + β-phellandrene**	**42.04**
1246	**(Z)-β-ocimene**	**7.01**
1255	γ-terpinene	0.23
1266	(E)-β-ocimene	0.81
1280	p-cymene	0.30
1290	terpinolene	0.11
1319	(E)-2,6-dimethyl-1,3,7-nonatriene	0.22
1382	cis-allo-ocimene	0.14
1479	δ-elemene	0.07
1495	bicycloelemene	0.10
1553	**linalool**	**11.03**
1638	trans-p-menth-2-en-1-ol	0.09
1596	α-guaiene	0.12
1611	terpinen-4-ol	0.07
1612	β-caryophyllene	0.34
1638	cis-p-menth-2-en-1-ol	0.06
1682	δ-terpineol	0.18
1690	cryptone	0.09
1705	zizanene	0.10
1706	α-terpineol	1.89
1726	germacrene-D	0.08
1755	bicyclogermacrene	1.26
2030	methyl eugenol	0.09
2144	spathulenol	0.09
2186	eugenol	0.16
	TOTAL	**99.39**

Thirty-four compounds are identified in the leaf essential oils, which represent 99.39 % of the total composition. 1,8-Cineole and β-phellandrene are the major compounds. Their combination totals 42.04 % of the total composition. Limonene (9.23 %) is a cyclic unsubstituted monoterpene. It occurs as the isomers d-limonene

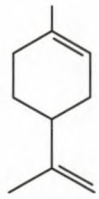
and l-limonene. l-Limonene has a citrus odour, but d-limonene smells like dill and is in fact the major constituent of dill oil. Linalool makes up *ca.* 11 %. α-Phellandrene and acyclic monoterpene, myrcene, are collectively integrated to 8.24 %.



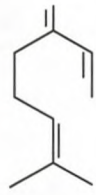
phellandrene



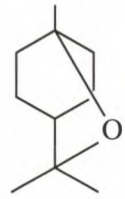
linalool



limonene



myrcene



1,8 cineole

Coleonema pulchellum I. Williams

Botanical Description:

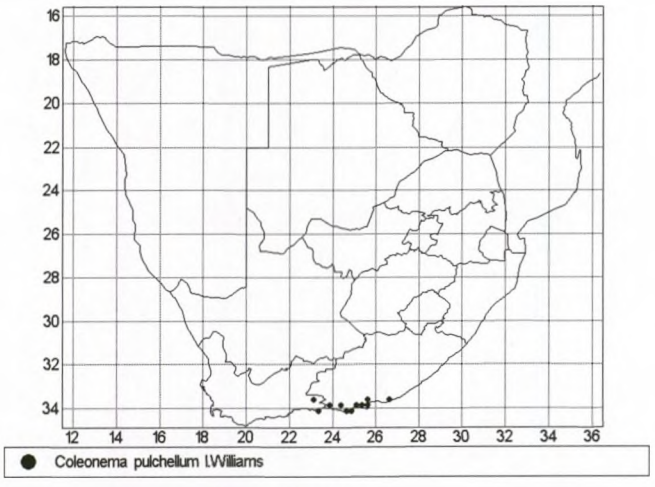
Dense shrub to 1 m.

Leaves: linear, pungent, sweet-smelling.

Flowers: solitary, often crowded, pink, 7-8 mm diameter, flowering time from March to October. (Goldblatt and Manning, 2000).

Distribution:

Abundant on coastal sands between Plettenberg Bay to Port Elizabeth.



Essential oil composition of Sample A:

Essential oil obtained by hydrodistillation (3 hrs) from plants collected at the Witwatersrand Botanical Garden. The oil yield is 0.24 % (wet wt).

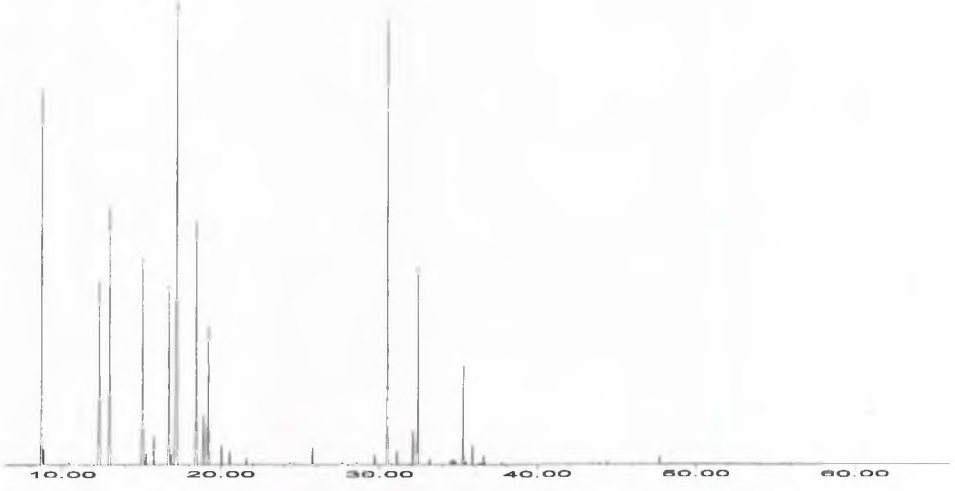


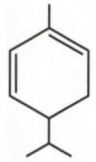
Figure 25: GC chromatogram displaying the partition of the essential oil components of *Coleonema pulchellum* (sample A).

Table 10: GC/MS data and integration percentages for *Coleonema pulchellum* (sample A) essential oil.

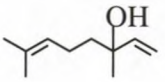
RRI	Component	Area %
1032	**α-pinene**	**9.74**
1035	α-thujene	0.38
1076	camphene	0.06
1118	**β-pinene**	**11.75**
1132	**sabinene**	**8.62**
1174	myrcene + α-phellandrene	6.72
1183	p-mentha-1(7),8-dien (pseudolimonene)	0.36
1188	α-terpinene	0.82
1203	limonene	5.13
1218	**β-phellandrene**	**17.70**
1246	(Z)-β-ocimene	6.77
1255	γ-terpinene	1.30
1266	(E)-β-ocimene	3.80
1280	p-cymene	0.53
1290	terpinolene	0.39
1398	6,7-epoxymyrcene	0.45
1553	**linalool**	**11.75**
1571	trans-p-menth-2-en-1-ol	0.33
1600	β-elemene	0.62
1612	β-caryophyllene	1.04
1611	terpinen-4-ol	2.94
1628	cis-p-menth-2-en-1-ol	0.16
1682	δ-terpineol	0.20
1687	α-humulene	0.10
1689	trans-piperitol	0.04
1706	α-terpineol	1.97
1719	borneol	0.12
1726	germacrene-D	0.38
1755	bicyclogermacrene	0.24
1758	cis-piperitol	0.07
2050	(E)-nerolidol	0.05
2186	eugenol	0.22
	TOTAL	**94.75**

Thirty-two compounds are identified in the leaf essential oils, which represent 94.75 % of the total composition. The monoterpene β-phellandrene (17.70 %) is the major compound in the oil which is probably responsible for the herbaceous character of the oil. The acyclic monoterpene alcohol linalool represent 11.75 % of the total composition while the bicyclic monoterpines, α-pinene and β-pinene, accumulatively

represent *ca.* 21 %. These compounds together with sabinene (8.62 %) represent *ca.* 60 % of the essential oil profile of *Coleonema pulchellum.*



phellandrene



linalool



α-pinene



β-pinene



sabinene



Figure 22: *Coleonema pulchellum*, a shrub of about 1 m high.



Figure 23: Flowers of *Coleonema pulchellum*.



Figure 24: The green shoot of *Coleonema pulchellum* contains copious amounts of essential oils.

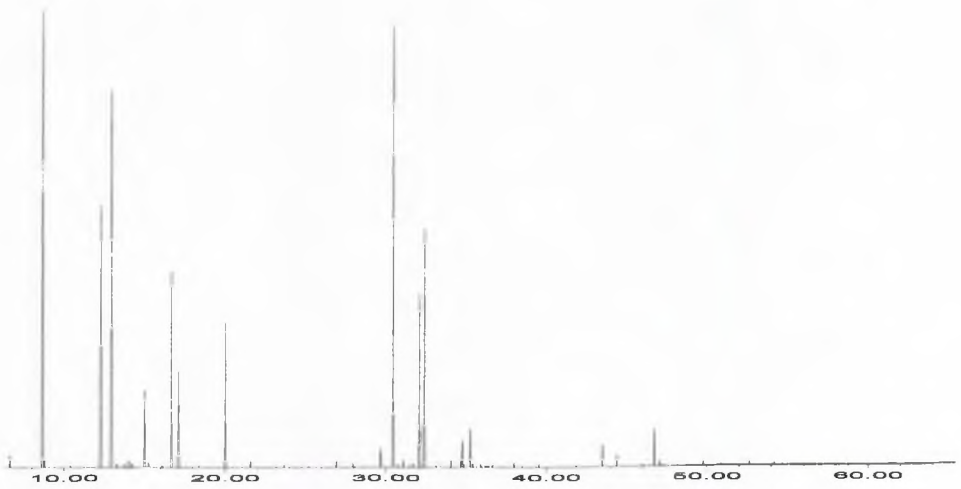


Figure 26: GC chromatogram displaying the partition of the essential oil components of *Coleonema pulchellum* (sample B).

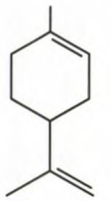
Essential Oil Composition of Sample B:

Essential oil obtained by hydrodistillation (3 hrs) from plants collected from Schoenmakerskop, Port Elizabeth. The oil yield is 0.78 % (wet wt).

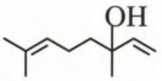
Table 11: GC/MS data and integration percentages for *Coleonema pulchellum* (sample B) essential oil.

RRI	Component	Area %
1032	**α-pinene**	**16.76**
1035	α-thujene	0.35
1076	camphene	0.36
1118	**β-pinene**	**11.57**
1132	**sabinene**	**15.14**
1174	myrcene	3.19
1203	**cimonene**	**7.24**
1213	1,8-cineole (0.3) + α-phellandrene (3.9%)	4.17
1280	p-cymene	5.12
1553	**linalool**	**10.54**
1600	β-elemene	3.75
1611	terpinen-4-ol	5.04
1690	cryptone	1.0
1706	α-terpineol	0.94
2008	caryophyllene oxide	0.54
2144	spathulene	0.76
	TOTAL	**86.47**

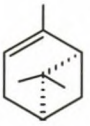
Sixteen compounds are identified in the leaf essential oils, which represent 86.47 % of the total composition. The bicyclic monoterpines, α-pinene and β-pinene, accumulatively represent 28.33 %. The bicyclic monoterpene sabinene represents 15.14 % while the acyclic alcohol linalool is 10.54 % of the total. Limonene makes up 7.24 %. Together, the collective yield of these compounds are *ca* 60 % of the total composition.



limonene



linalool



α-pinene



β-pinene



sabinene

Coleonema pulchellum I. Williams

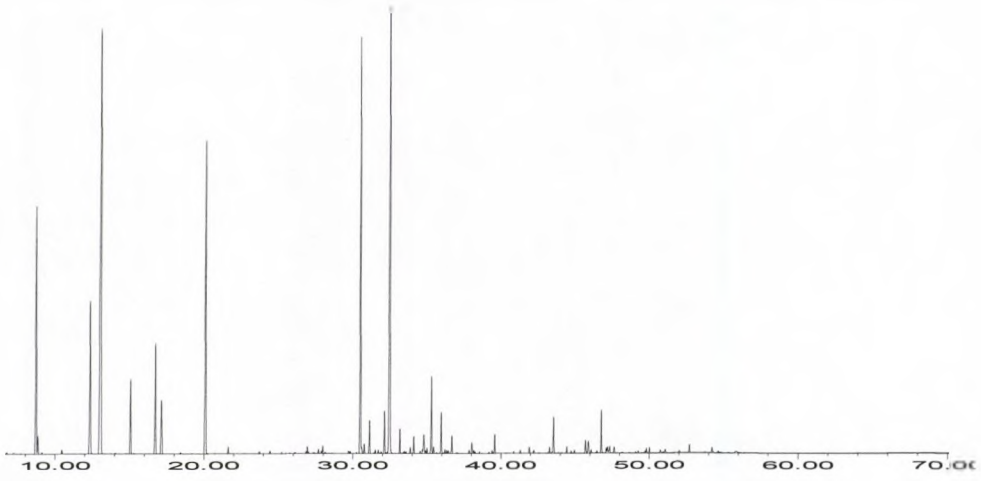


Figure 27: GC chromatogram displaying the partition of the essential oil components of *Coleonema pulchellum* (sample C).

Essential oil composition of Sample C:

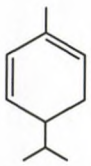
Essential oil obtained by hydrodistillation (3 hrs) from plants collected from a domestic garden in Johannesburg. The oil yield is 0.27 % (wet wt).

Table 12: GC/MS data and integration percentages for *Coleonema pulchellum* (sample C) essential oil.

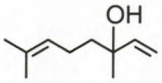
RRI	Component	Area %
1032	**α-pinene**	**17.43**
1035	α-thujene	0.36
1076	camphene	0.17
1118	**β-pinene**	**13.55**
1132	sabinene	7.98
1174	myrcene + α-phellandrene	7.91
1183	p-mentha-1(7),8-dien (pseudolimonene)	0.21
1188	α-terpinene	0.78
1203	limonene	3.40
1213	**1,8-cineole + β-phellandrene**	**11.25**
1246	**(Z)-β-ocimene**	**8.21**
1255	γ-terpinene	1.34
1266	(E)-β-ocimene	5.24
1280	p-cymene	0.33
1290	terpinolene	0.40
1319	(E)-2,6-dimethyl-1,3,7-nonatriene	0.18
1382	cis-allo ocimene	0.14

RRI	Component	Area %
1398	6,7-epoxymyrcene	0.37
1474	trans-sabinene hydrate	0.03
1498	(E)-β-ocimene epoxide	0.09
1553	**linalool**	**10.00**
1562	isopinocamphone	0.10
1570	methyl citronellate	0.38
1600	β-elemene	0.40
1604	2-undecanone	0.32
1611	terpinen-4-ol	3.12
1628	cis-p-menth-2-en-1-ol	0.17
1664	trans-pinocarveol	0.43
1682	δ-terpineol	0.21
1687	α-humulene	0.10
1689	trans-piperitol	0.02
1706	α-terpineol	2.12
1719	borneol	0.29
1726	germacrene-D	0.06
1755	bicyclogermacrene	0.18
1758	cis-piperitol	0.09
1805	α-campholene alcohol	0.17
1845	trans-carveol	0.13
2008	caryophellene oxide	0.04
2030	methyl eugenol	0.05
2050	(E)-nerolidol	0.07
2144	spathulenol	0.07
2186	eugenol	0.20
2200	trans-methyl isoeugenol	0.07
	TOTAL	**98.16**

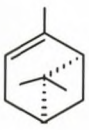
This distilled oil provided thirty-seven identifiable compounds totalling 98.16 % of the composition. The two isomers, α- and β- pinene, represented the largest volume with a combined value of *ca.* 30 %. 1,8-Cineole and β-phellandrene represent 11.25 % while the acyclic alcohol linalool was present in 10 %.



phellandrene



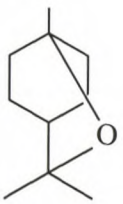
linalool



α-pinene



β-pinene



1,8 cineole

The three *Coleonema pulchellum* specimens were collected from different areas in South Africa. α-Pinene and β-pinene appeared in all three specimens as one of the five most predominating compounds. They represented between 20-30 % of the total composition. Linalool was also common amongst all three samples. β-Phellandrene was present in *Coleonema pulchellum* A and C but could not be detected in the specimen collected in Schoenmakerskop, Port Elizabeth. *Coleonema pulchellum* (B) had the least amount of identifiable compounds. This sample has sabinene and limonene as the major compounds as opposed to the two other samples. The differences clearly show within species variation.

Coleonema virgatum (Schltdl.) Eckl. & Zeyh.

Botanical Description:

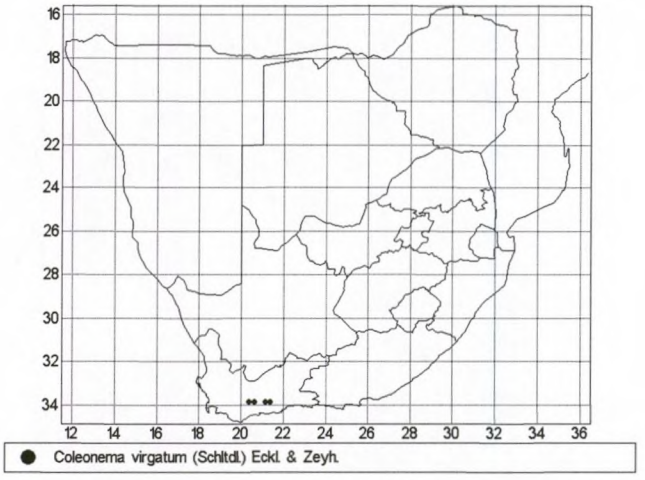
Erect shrubs to 1 m.

Leaves: linear, c. 30 mm.

Flowers: solitary, pink or white, 12 mm diameter. June-Sept. (Goldblatt and Manning, 2000).

Distribution:

Sandstone slopes, Langeberg Mts: Swellendam to Riversdale.



Essential oil composition:

Essential oil obtained by hydrodistillation (3 hrs) from plants collected from Garcias Pass. The oil yield is 0.71 % (wet wt).

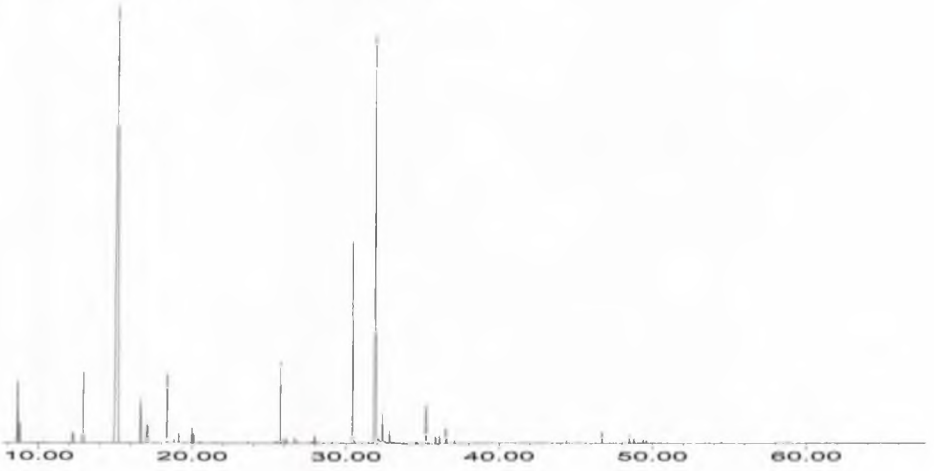


Figure 28: GC chromatogram displaying the partition of the essential oil components of *Coleonema virgatum.*

Table 13: GC/MS data and integration percentages for *Coleonema virgatum* essential oil.

RRI	Component	Area %
1032	**α-pinene**	**4.22**
1035	α-thujene	1.92
1118	β-pinene	0.76
1132	**sabinene**	**4.33**
1174	**myrcene**	**62.98**
1203	limonene	1.75
1213	1,8-cineole (0.5) + β-phellandrene (0.5)	1.03
1246	(Z)-β-ocimene	2.28
1266	(E)-β-ocimene	0.37
1280	p-cymene	0.49
	isomyrcenol (tentative)	0.46
1398	6,7-epoxymyrcene	1.76
1476	(Z)-β-ocimene epoxide	0.16
1553	**linalool**	**3.57**
1583	**myrcenone**	**9.62**
1611	terpinen-4-ol	0.62
1626	2-menthyl-6-methylene-3,7-octadien-2-ol	0.33
1678	ipsdienol	0.06
1706	α-terpineol	0.77
1726	germacrene-D	0.11
1755	bicyclogermacrene	0.15
1772	citronellol	0.07
2144	spathulenol	0.20
2219	dimyrcene II-a	0.11
	TOTAL	**98.12**

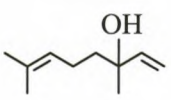
Twenty-four compounds are identified in the leaf essential oil, which represent 98.12 % of the total composition. Myrcene represents approximately 63 % of the total. It is interesting to note that *Agathosma gnidiodes* Schltr. also contained *ca.* 60 % myrcene. The Xhosa used this particular leaf decoction as a stomachic, antispasmodic and febrifuge (van Wyk *et al.* 1997). Myrcenone, a derivative of myrcene was the next largest constituent comprising 9.62 %.



myrcene



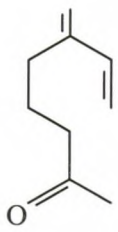
α-pinene



linalool



sabinene



myrcenone

Diosma prama I. Williams

Botanical Description:

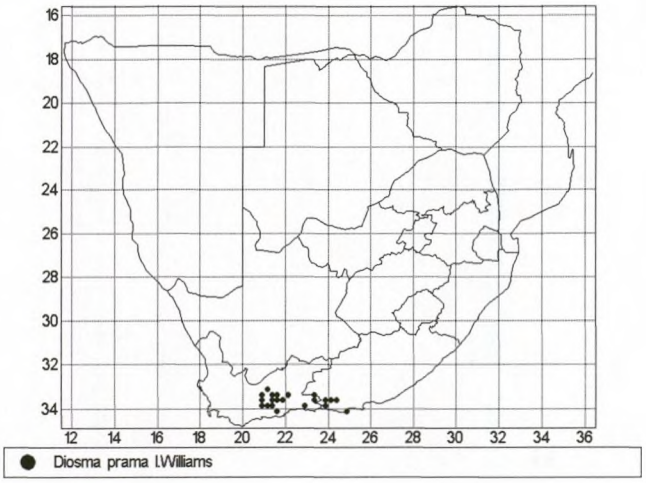
Shrubs: finely leafy, aromatic shrublet to 40 cm. Single-stemmed, branchlets glabrous.

Leaves: sessile, elliptic to lanceolate, obtuse, minutely pubescent. Leaves with postulate gland dots mainly near midrib.

Flowers: solitary, terminal, white. Sept.-Dec. (Goldblatt and Manning, 2000).

Distribution:

Dry sandstone slopes, Touwsberg and Klein Swartberg to Baviaanskloof Mts.



Essential oil composition of Sample A:

Essential oil obtained by hydrodistillation (3 hrs) from plants collected from Rooiberg Ladysmith. The oil yield is 0.27 % (wet wt).

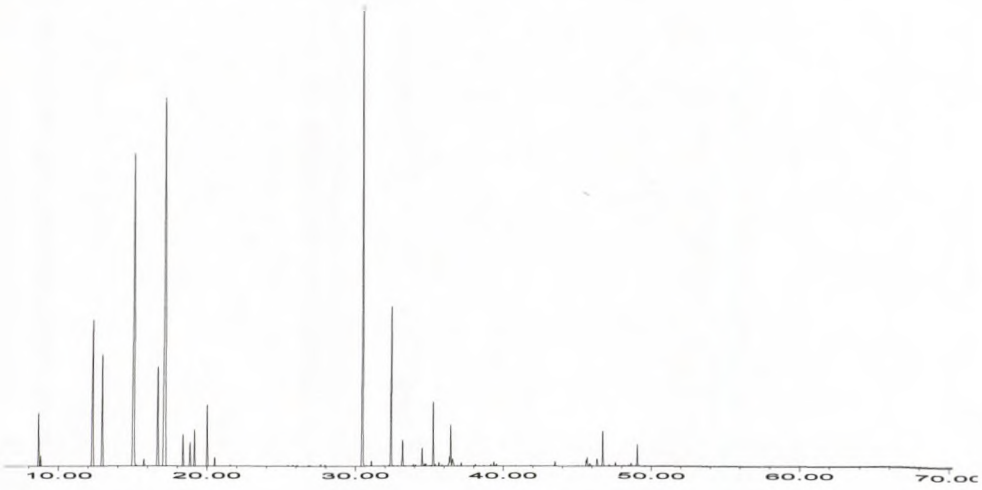
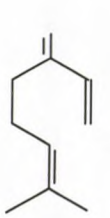


Figure 29: GC chromatogram displaying the partition of the essential oil components of *Diosma prama* (sample A).

Table 14: GC/MS data and integration percentages for *Diosma prama* (sample A) essential oil.

RRI	Component	Area %
1032	α-pinene	3.70
1035	α-thujene	0.83
1118	**β-pinene**	**10.37**
1132	**sabinene**	**6.63**
1174	**myrcene**	**23.52**
1188	α-terpinene	0.07
1203	limonene	4.71
1213	**1,8-cineole + β-phellandrene**	**25.64**
1246	(Z)-β-ocimene	1.11
1255	γ-terpinene	0.79
1266	(E)-β-ocimene	1.24
1280	p-cymene	2.11
1290	terpinolene	0.28
1553	**linalool**	**9.92**
1571	trans-p-menth-2-en-1-ol	0.11
1611	terpinen-4-ol	2.45
1682	δ-terpineol	0.37
1706	α-terpineol	0.94
1747	trans-carvyl acetate	0.54
1755	bicyclogermacrene	0.10
1773	δ-cadinene	0.11
1857	geraniol	0.07
2008	caryophyllene oxide	0.08
2098	globulol	0.11
2100	heneicosane	0.15
2144	spathulenol	0.43
2247	trans-α-bergamotol	0.28
	TOTAL	**96.66**

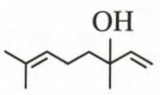
Twenty-seven compounds yielded a 96.66 % identification percentage. As with other Rutaceae sp. the abundant compounds were β-pinene (10.37 %), myrcene (23.52 %), linalool (9.92 %) and 1,8-cineole in combination with β-phellandrene (25.64 %). Their combined yield equalled *ca.* 69 %.



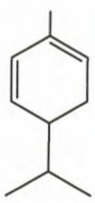
myrcene



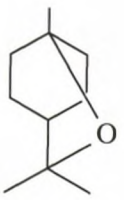
β-pinene



linalool



phellandrene



1,8 cineole

Diosma prama I. Williams

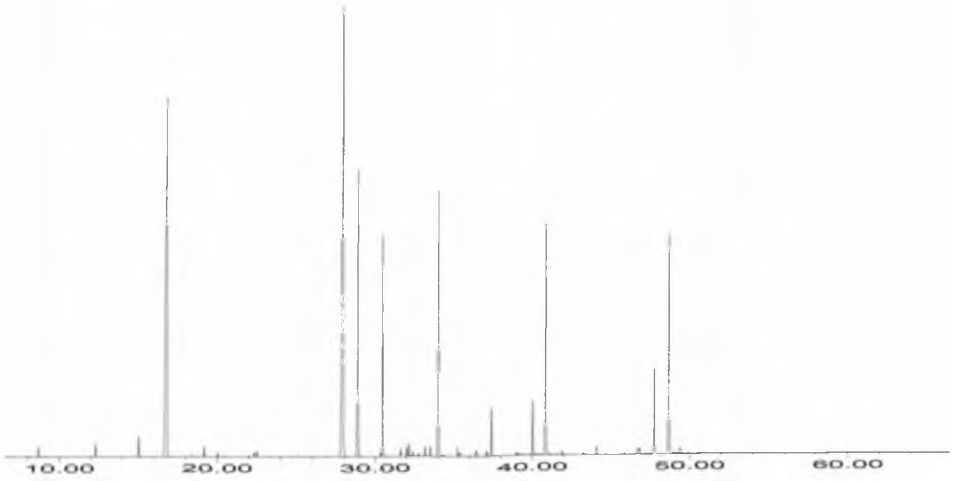


Figure 30: GC chromatogram displaying the partition of the essential oil components of *Diosma prama* (sample B).

Essential oil composition of Sample B:

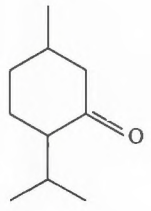
Essential oil obtained by hydrodistillation (3 hrs) from plants collected from Rooiberg (Ladismith). The oil yield is 0.52 % (wet wt).

Table 15: GC/MS data and integration percentages for *Diosma prama* (sample B) essential oil.

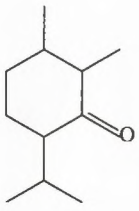
RRI	Component	Area %
1032	α-pinene	0.42
1118	β-pinene	0.53
1174	myrcene	0.72
1203	**limonene**	**26.62**
1246	(Z)-β-ocimene	0.08
1266	(E)-β-ocimene	0.40
1280	p-cymene	0.18
1337	geijerene	0.19
1386	3-methyl-cyclohexane	0.12
1475	**menthone**	**28.10**
1503	**isomenthone**	**10.77**
1553	**linalool**	**6.68**
1598	trans-isopugelone	0.14
1583	cis-isopugelone	0.24
1604	neo-menthol	0.32
1611	terpinen-4-ol	0.13
1624	trans-dihydrocarvone	0.13

RRI	Component	Area %
1632	neo-isomenthol	0.27
1638	menthol	0.25
1662	**pulegone**	**10.84**
1706	α-terpineol	0.28
1748	piperitone	0.18
1772	citronellol	0.20
1845	trans-carveol	0.10
1875	trans-p-menthane-8-thiol-3-one	1.17
1904	cis-p-menthane-8-thiol-3-one	4.37
1748	piperitone	0.12
2100	heneicosane	0.08
2131	hexahydrofarnesylacetone	0.13
2144	spathulenol	0.21
2148	(Z)-3-hexen-1-ol benzoate	0.14
2187	trans-3-oxo-p-menthane-8-thiol acetate	1.25
2223	cis-3-oxo-p-menthane-8-thiol acetate	3.09
2255	α-cadinol	0.20
2655	benzyl benzoate	0.22
	TOTAL	**98.87**

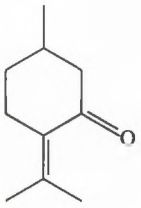
This sample of *Diosma prama* (B) was totally different in its major compound expression compared to the *Diosma prama* (A). Menthone, a ketone was present to the largest extent with 28.10 %. The cyclic monoterpene, limonene, so often found in citrus was also present in large quantities (26.62 %).



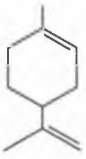
menthone



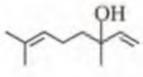
isomenthone



pulegone



limonene



linalool

The two samples of *Diosma prama* differ significantly in their chemical composition. *Disoma prama* (A) resembled *Coleonema album* more than it did the second specimen. *Diosma prama* (B) has many menthol derivatives instead. The oil yield of A was much higher than that of B.

Microbial Results

The antimicrobial results are summerised in the following series of tables. Gram-positive bacteria, gram-negative bacteria, yeasts and fungi were used as the test organisms. *Acmadenia sheilae, Coleonema aspalathoides, Coleonema pulchellum* (A) and *Diosma prama* (A) are the most antimicrobially active. Essential oil samples ran out for the following: *Adenandra obtusata, Coleonema aspalathoides* and *Coleonema pulchellum* (B). Differences in activity became apparent within the species.

Table 16: Results of disc diffusion assay as expressed in mm from the edge of the disc.

		A	B	C	D	E	F	G	H	I	J	K	L
1.	*Acmadenia alternifolia*	1	-	-	-	-	-	2	3	<1	-	<1	-
2.	*Acmadenia obtusata*	1,5	<1	-	-	-	-	-	2	-	-	1	-
3.	*Acmadenia sheilae*	2,5	<1	4	-	-	2	2	3	<1	3	2	2
4.	*Adenandra obtusata*	-	-	-	-	-	-	-	2	ND	ND	ND	ND
5.	*Adenandra villosa*	<1	-	2	-	-	-	2	1	-	2	2.5	<1
6.	*Coleonema album*	-	<1	-	-	-	-	-	-	-	<1	-	-
7.	*Coleonema aspalathoides*	3	2	3	-	-	1	3	2	ND	ND	ND	ND
8.	*Coleonema calycinum*	-	<1	-	-	-	-	-	-	-	1	-	-
9.	*Coleonema pulchellum* (A)	2	1	5	-	-	2	3	3	-	-	1	-
10.	*Coleonema pulchellum* (B)	-	<1	-	-	-	-	1	-	ND	ND	ND	ND
11.	*Coleonema pulchellum* (C)	-	<1	-	-	-	-	-	-	1	1	-	-
12.	*Coleonema virgatum*	-	<1	-	-	-	-	-	-	-	1	-	-
13.	*Diosma prama* (A)	2	1	2	-	-	1	1	4	-	1	1	-
14.	*Diosma prama* (B)	-	-	-	-	-	-	1	-	-	1	1	-
15.	Positive control	10	5	3	2	7	7	5	4	6	10	10	12
16.	Negative control	-	-	-	-	-	-	-	-	-	-	-	-

A – *Staphylococcus aureus*
B – *Escherichia coli*
C – *Enterococcus faecalis*
D – *Pseudomonas aeruginosa*
E – *Aspergillus niger*
F – *Candida albicans*
G – *Cryptococcus neoformans*
H – *Candida tropicalis*
I – *Serratia odorifera*
J – *Bacillus subtilis*
K – *Bacillus cereus*
L – *Staphylococcus epidermidis*

(-) - No diameter was noted – organisms showed no antimicrobial activity

ND – Not Determined

Table 17: Minimum inhibitory concentration in mg/ml for *Escherichia coli.*

1	**2**	**3**	**4**	**5**	**6**	**7**
32	32	32	32	32	32	32
16	16	16	16	16	16	16
8	8	8	8	8	8	8
4	4	4	4	4	4	4
2	2	2	2	2	2	2
1	1	1	1	1	1	1
0.5	0.5	0.5	0.5	0.5	0.5	0.5
0.25	0.25	0.25	0.25	0.25	0.25	0.25

KEY:

1 - *Acmadenia sheilae*

2 - *Adenandra villosa*

3 - *Coleonema aspalathoides*

4 – *Coleonema calycinum*

5 - *Coleonema pulchellum* (A)

6 - *Coleonema pulchellum* (B)

7 - *Diosma prama* (A)

Non-bolded Values – growth

Bolded Values - no growth

In Table 17 none of the essential oils showed a clear MIC. All seven samples tested had zones less than 2 mm (Table 16).

Table 18: Minimum inhibitory concentration in mg/ml for *Staphylococcus aureus.*

1	**2**	**3**	**4**	**5**	**6**	**7**
32	**32**	**32**	32	**32**	**32**	**32**
16	**16**	**16**	16	**16**	**16**	**16**
8	8	8	8	**8**	8	8
4	4	4	4	4	4	4
2	2	2	2	2	2	2
1	1	1	1	1	1	1
0.5	0.5	0.5	0.5	0.5	0.5	0.5
0.25	0.25	0.25	0.25	0.25	0.25	0.25

1 - *Acmadenia sheilae*
2 - *Adenandra villosa*
3 - *Coleonema aspalathoides*
4 - *Coleonema calicynum*
5 - *Coleonema pulchellum* (A)
6 - *Coleonema pulchellum* (B)
7 - *Diosma prama* (A)

Non-bolded Values – growth

Bolded Values – no growth

MIC values for *Staphylococcus aereus* ranged from 8 mg/ml for *Coleonema pulchellum* (A) to 32 mg/ml for *Acmadenia sheilae*.

Table 19: Minimum inhibitory concentration in mg/ml for *Enterococcus faecalis.*

1	**2**	**3**	**4**	**5**	**6**	**7**
32	**32**	**32**	**32**	**32**	**32**	**32**
16	**16**	**16**	**16**	**16**	**16**	**16**
8	8	**8**	8	**8**	**8**	**8**
4	4	4	4	4	4	4
2	2	2	2	2	2	2
1	1	1	1	1	1	1
0.5	0.5	0.5	0.5	0.5	0.5	0.5
0.25	0.25	0.25	0.25	0.25	0.25	0.25

1 - *Acmadenia sheilae*
2 - *Adenandra villosa*
3 - *Coleonema aspalathoides*
4 - *Coleonema calicynum*
5 - *Coleonema pulchellum* (A)
6 - *Coleonema pulchellum* (B)
7 - *Diosma prama* (A)

Non-bolded Values – growth

Bolded Values – no growth

The results for *Enterococcus faecalis* were more promising across the seven samples tested. All except *Adenandra villosa* and *Coleonema calicynum* had MICs of 8 mg/ml.

Table 20: Summary of MIC values obtained for the various species tested.

Plant Specimen	**Organism**	**MIC**
Acmadenia sheilae	*Escherichia coli*	Growth
Adenandra villosa	*Escherichia coli*	Growth
Coleonema aspalathoides	*Escherichia coli*	Growth
Coleonema calycinum	*Escherichia coli*	Growth
Coleonema pulchellum (A)	*Escherichia coli*	Growth
Coleonema pulchellum (B)	*Escherichia coli*	Growth
Diosma prama (A)	*Escherichia coli*	Growth
Acmadenia sheilae	*Staphylococcus aureus*	>32 mg/ml
Adenandra villosa	*Staphylococcus aureus*	>16 mg/ml
Coleonema aspalathoides	*Staphylococcus aureus*	>16 mg/ml
Coleonema calycinum	*Staphylococcus aureus*	Growth
Coleonema pulchellum (A)	*Staphylococcus aureus*	>8 mg/ml
Coleonema pulchellum (B)	*Staphylococcus aureus*	>16 mg/ml
Diosma prama (A)	*Staphylococcus aureus*	>16 mg/ml

Plant Specimen	**Organism**	**MIC**
Acmadenia sheilae	*Enterococcus faecalis*	>8 mg/ml
Adenandra villosa	*Enterococcus faecalis*	>16 mg/ml
Coleonema aspalathoides	*Enterococcus faecalis*	>8 mg/ml
Coleonema calycinum	*Enterococcus faecalis*	>16 mg/ml
Coleonema pulchellum (A)	*Enterococcus faecalis*	>8 mg/ml
Coleonema pulchellum (B)	*Enterococcus faecalis*	>8 mg/ml
Diosma prama (A)	*Enterococcus faecalis*	>8 mg/ml

Table 21: Minimum inhibitory concentration in mg/ml using DMSO.

1	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
32	**32**	**32**	**32**	32	32	32	32	32	32	**32**	**32**
16	**16**	**16**	**16**	16	16	16	16	16	16	**16**	**16**
8	**8**	8	**8**	8	8	8	8	8	8	8	8
4	4	4	4	4	4	4	4	4	4	4	4
2	2	2	2	2	2	2	2	2	2	2	2
1	1	1	1	1	1	1	1	1	1	1	1
0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Staphylococcus aureus				***Escherichia coli***				***Enterococcus faecalis***			

Using DMSO as a solvent, it was decided to observe only four due to the activity displayed in the previous assays

1 - *Acmadenia sheilae*

2 – *Coleonema aspathaloides*

3 - *Coleonema pulchellum* (A)

4 – *Diosma prama* (A)

Non-bolded Values – growth

Bolded Values – no growth

Table 22: Summary of MICs using DMSO

Plant Specimen	**Organism**	**MIC**
Acmadenia sheilae	*Escherichia coli*	Growth
Coleonema aspalathoides	*Escherichia coli*	Growth
Coleonema pulchellum (A)	*Escherichia coli*	Growth
Diosma prama (A)	*Escherichia coli*	Growth
Acmadenia sheilae	*Staphylococcus aureus*	>8 mg/ml
Coleonema aspalathoides	*Staphylococcus aureus*	>8 mg/ml
Coleonema pulchellum (A)	*Staphylococcus aureus*	>16 mg/ml
Diosma prama (A)	*Staphylococcus aureus*	>8 mg/ml
Acmadenia sheilae	*Enterococcus faecalis*	Growth
Coleonema aspalathoides	*Enterococcus faecalis*	Growth
Coleonema pulchellum (A)	*Enterococcus faecalis*	>16 mg/ml
Diosma prama (A)	*Enterococcus faecalis*	>16 mg/ml

Discussion

Recent studies has shown that the "super bugs" that has modern medicine so concerned (such as "MRSA": methicillin-resistant *Staphylococcus aureus*), cannot survive in the presence of essential oils. Nor has there been any pathogen known to resist essential oils by mutating. This factor may very well be due to the fact that bacteria are able to develop genetic mutation and consequent resistance only versus a strictly specific drug – such as methicillin – but their genetic system seems not to be able to develop defensive mutations versus many natural antibacterial terpenic substances, such as those contained in essential oils. In fact, there can be several hundreds of different terpenic substances present in just one essential oil and their concentration may vary in relationship with the climatic conditions, harvesting time, humidity, sun exposition etc. All such variances make impossible the triggering of those genetic mechanisms which on the contrary are successful when they are targeted by one and only one specific chemical. http://www.bth.co.uk .

Essential oils are used in many industries for the scenting and flavouring of all kinds of finished consumer products. The germicidal properties of essential oils have been known for a long time. It is also known that the essential oils are more active against fungi than bacteria (Delespaul *et al.* 2000).

The bacteriostatic and fungistatic activities of essential oils were commonly evaluated by the filter paper disc and agar diffusion methods, using the undiluted oils. Quantitative results can be better achieved by estimating the minimum inhibitory concentration (MIC), using the serial dilution method. Although some essential oils have been screened for their bacteriostatic activity using this method, their bactericidal effect i.e. minimum bactericidal concentration (MBC) has received little attention. On the other hand, it has been well established that the composition of essential oils will depend on the plant species, the chemotypes and their climatic conditions; therefore, their antimicrobial activities could vary (Kivanç and Akgül, 1986).

It was observed that leaves from *Citrus limonum*, a member of the Rutaceae was also highly active against various microbes. *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli* in particular had large zones of inhibition (Kivanç and Akgül, 1986). Kivanç and Akgül (1986) reported that the biostatic data obtained by agar diffusion and serial dilution methods were not always comparable, and therefore in testing the bacteriostatic effects of essential oils it is advisable to carry out both techniques.

The samples collected represented members of the Rutaceae genus not yet studied. Very little has been known about these plants. They are however closely related to Buchu (*Agathosma*). Buchu leaves is a widely used household medicine in South Africa, usually as a brandy tincture or as a vinegar, known respectively as Buchu

brandy and buchu vinegar. These preparations have a great reputation in diseases of the kidney and urinary tract and as a local application to bruises and for the relief of rheumatic pains, although buchu has also been used for almost every disease which afflicts mankind (Watt and Breyer-Brandwijk, 1962).

Part of the objective of this study was to record any antimicrobial activity from taxonically closely related taxa. Essential oil extracts were tested against 12 microbes i.e. *Cryptococcus neoformans, Candida tropicalis, Aspergillus niger, Candida albicans, Staphylococcus epidermidis, Staphylococcus aureus, Escherichia coli, Enterococcus faecalis, Serratia odorifera, Pseudomonas aeruginosa, Bacillus subtilis* and *Bacillus cereus*. Of these, the first four were fungal and the latter eight were bacterial. The results of the screening are given in Table 16. It was noteworthy that while testing *Enterococcus faecalis* inhibition, *Acmadenia sheilae*, and *Coleonema pulchellum* (A) had zones of inhibition of 4 mm and 5 mm respectively from the edge of the disc. These results were larger than the control, Neomycin's zone of 3 mm. For *Staphylococcus aureus* seven essential oils were active and *Escherichia coli* was sensitive to ten extracts. However, the zones examined were far more distinct for *Staphylococcus aureus* than those for *Escherichia coli* where most zones were less than 1 mm. No inhibition was recorded for *Pseudomonas aeruginosa*, a virulent bacteria and the fungus *Aspergillus niger*. Monoterpenes in general perform poorly against *Pseudomonas aeruginosa* (Naigre *et al.* 1995). This holds true for most Gram negatives in general. Many oils were active against *Cryptococcus neoformans* and *Candida tropicalis.*

Disoma prama (A) in particular showed high activity against *Candida tropicalis* (Table 16). It revealed a zone of 4 mm from the edge of the disc which was the same as the control, Neostatin. The most active species in this study is A*cmadenia sheilae* which inhibited ten of the twelve microbes.

It was decided to take seven active specimens and subject them to MIC assay for *Escherichia coli, Staphylococcus aureus* and *Enterococcus faecalis*. The preceding microbes were chosen to perform MICs because they cause some of the most important infectious diseases known to man ranging from upper respiratory infections to urinary tract infections and gastro intestinal disorders. Of the initial fourteen species, *Acmadenia sheilae*, *Adenandra villosa*, *Coleonema calycinum*, *Coleonema aspalathoides*, *Coleonema pulchellum* (A and B) and *Diosma prama* (A) were chosen. Table 17 represents MICs for *Escherichia coli.* Inhibition seemed apparent on the first day for the MICs but these were short-lived. Thus, it has been determined as having growth instead. This development correlates with what was observed in the disc diffusion assay. None of the sample specimens had any significant zones compared to the positive (Table 16). In the *Staphylococcus aureus* MIC assay (Table

18) the best results were noted for *Coleonema pulchellum* (A) at 8 mg/ml. The only oil without any activity was *Coleonema calycinum.* With reference to *Enterococcus faecalis* (Table 19), all seven specimens had favourable MICs. Five of the seven samples had MICs of 8 mg/ml.

Pattnaik *et al.* (1997) reported variation between different laboratory methods for antimicrobial testing. Inconsistencies were evident due to the volatile nature of some of the essential oils. It was decided to explore whether there were any differences in the MIC values with different solvents as a base to explore solubility. In this case acetone and dimethylsulfoxide acid (DMSO) were used separately. Only four essential oils from the initial seven used for the acetone solvent method were retained as comparators. These four oils were *Acmadenia sheilae, Coleonema aspalathoides, Coleonema pulchellum* (A) and *Diosma prama* (A). Four factors are especially important when testing essential oils: the assay technique; the growth medium; the micro-organism; the essential oil (Janssen *et al.* 1986). The MICs for the acetone solvent method compared to the DMSO solvent method, differ substantially with the exception of *Escherichia coli.* Tabulation (table 20-22) reveals a clearer picture of the differences. This variation in results accurately correlates with what Pattnaik had established in his analysis. Delespaul *et al.* (2000) also found varying antifungal activity when direct screening methods were employed.

In a study conducted by Inouye *et al.* (2001) it was proven that among 14 essential oils, cinnamon bark, lemon-grass and thyme oils showed the lowest minimum inhibitory dose (MID), followed by essential oils containing terpene alcohols as major constituents. The essential oils containing ketones, ethers and, in particular, hydrocarbons had high MIDs. In addition to this the results indicated that the antibacterial action of essential oils was most effective at high vapour concentration for a short time (Inouye *et al.* 2001). If one imposed these findings on these results it holds true as well. For example, linalool and terpinen-4-ol are alcohols. Wherever they are present in large quantities as major compounds the zones are clearly defined (Table 16). *Acmadenia sheilae* also presented with numerous ketone derivatives. With reference to Table 16, ten of the twelve microbes were inhibited by *Acmadenia sheilae.*

An attempt will be made to correlate the GC and GC/MS results of the essential oils (Species Monographs) to the activity observed. To facilitate the process we will be discussing each genus individually.

The role of structure and molecular properties of terpenoids were evaluated in determining their antimicrobial activity. It was proven that terpene acetates and hydrocarbons tended to be relatively inactive regardless of their structural type, and

that activity appears closely related to their limited hydrogen bond capacity and water solubility. Ketones, aldehydes and alcohols showed activity but with differing specificity and levels of activity. Specificity and level of activity were not always defined by the functional group present but were associated with hydrogen-bonding parameters in all cases, and for Gram-negative organisms a combination of hydrogen-bonding parameters and molecular size parameters. The importance of subtle structural differences of the *p*-menthane terpenoids on molecular properties and activity, especially *Pseudomonas aeruginosa*, has also been demonstrated (Griffin *et al.* 1999).

In discussing *Coleonema pulchellum* a similar study was conducted by Brophy and Lassak (1986). This particular study focused primarily on GC and GC/MS data. The steam volatile leaf oil of *Coleoneam pulchellum* contained mainly monoterpenes with α- and β-pinene, myrcene and β-phellandrene accounting for approximately 50 % of the oil. Linalool and terpinen-4-ol accounted for 6 % of the oil, whilst the sesquiterpenes, caryophyllene, germacrene-D and bicyclogermacrene contributed 10%. Fifty-five compounds were detected. Similarities can be identified from what was observed to what was discovered in our plant specimens. In each case the presence of α- and β-pinene were consistent. The interesting fact however was that linalool is one of the major components for each of the three specimens investigated in this study. The antimicrobial activity of linalool is well known. Skaltsa *et al.* (1999) reported that linalool was active against the Gram-positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis* as well as Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa.* Of the three species, *Coleonema pulchellum* (A) was the most active. In addition to the α- and β-pinene, linalool and β-phellandrene, sabinene was one of the major compounds with 8.62 %. This does not imply that the compound in those ratios would cause sufficient inhibition. On the contrary, any one of the thirty-two compounds could be responsible for the recorded activity. Minute quantities of a particular compound may be highly effective. To illustrate this point, reference is made to the research paper by Couladis *et al.* (2000). It was proven that α-pinene was an active molecule and limonene was not. In fact nine organisms were used for the assays. Activity against *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, E. cloacae, Klebsiella peumoniae, E. coli Candida albicans, Candida tropicalis* and *T. glabrata* were determined, using the dilution technique, the minimum inhibitory concentration was measured for the oils. α-Pinene was active against all nine pathogens while limonene displayed no activity (Couladis *et al.* 2000). Thus, we can effectively rule out activity due to limonene even though it may be a major compound.

It has been proven that Tea Tree oil is an effective antifungal and antibacterial agent (Christoph *et al.* 2001). Many cosmetic houses have adopted it into their formulas for creams, toners etc. The dawning of the age of active cosmetics has begun. In a recent study it was demonstrated that Tea Tree oil was very effective not only against bacteria and moulds but even against dust mites where 0.05 % of Tea Tree oil was found as effective as 0.5 % benzyl benzoate over a 30 minute period. It was further revealed that at shorter exposure times Tea Tree oil was more effective, killing 79 % of the mites in 10 minutes. Through studies utilising GC and GC/MS we have established that the permutation of linalool and terpenen-4-ol are the key to the activity. Indeed, in our study we found that *Coleonema aspalathoides*, the most active *Coleonema* species has this very same combination (linalool and terpinen-4-ol). Upon comparison, this specimen's profile closely resembled that of *Croton stellulifer*, an endemic species from S. Tomé e Prīcipe (Martins *et al.* 2000). Similarities were found for the antimicrobial results (Table 16). The antimicrobial studies showed that the essential oil of *C. stellulifer* was moderately active against Gram-positive and Gram-negative bacteria, yeasts and most of the filamentous fungi. Conversely, no activity against *Aspergillus niger* was detected. The activity of the oil was suspected to be associated with the significant contribution of the monoterpenes linalool, *p*-cymene and α-pinene, which have been studied previously and are known to have antimicrobial activity (Martins *et al.* 2000).

Coleonema calycinum has minimal activity (Table 16). It displayed a zone of inhibition of 1 mm for *Bacillus subtilis* and there was a poorly defined zone of <1 mm for *Escherichia coli.* The main constituents were 1,8-cineole and β-phellandrene which represents 42.02 % of the total composition. Linalool was present in 9 % of the sample. Terpinen-4-ol and linalool are both present. The concentration of terpinen-4-ol is only 0.07 %. From this observation it may seem that the ratio of linalool to terpinen-4-ol must be consistent and high. This could account for its only slight antibacterial activity against *Bacillus subtilis* and *Escherichia coli.* There was growth in all other plates.

Coleonema album also had insignificant activity (Table 16). Both *Escherichia coli* and *Bacillus subtlis* were weakly inhibited with zones of < 1 mm. 1,8-Cineole and β-phellandrene were the major compounds. 1,8-Cineole is an extremely weak inhibitor. It does, however, produce a MIC of 9100 ppm against *Escherichia coli* and could therefore be the slightly inhibiting factor (Griffin *et al.* 1999).

Coleonema virgatum, a relatively inactive specimen produced myrcene as its major compound (Table 16). In fact about 63 % of the total composition was myrcene. In the study of *Pistacia lentiscus,* myrcene too was the major compound but very small zones of inhibition were observed (Prokopios, 1999).

The sample of *Coleonema aspalathoides* yielded good activity (Table 16). Of the eight microbes tested six were susceptible. Again, as with the others, *Aspergillus niger* and *Pseudomonas aeruginosa* revealed no activity. *Enterococcus faecalis* was inhibited by 3 mm from the edge of the disc. This was identical to the positive control. Linalool and terpinen-4-ol were both present as major compounds. This combination probably contributed to most of the activity. There were also many alcohol and ketone derivatives which contributed to its activity (Inouye *et al.* 2001).

Acmadenia alternifolia displays activity against *Cryptococcus neoformans, Candida tropicalis, Serratia odorifera, Bacillus cereus* and *Staphylococcus aureus*. Some active molecules were found in the trial (Table 16). These include α-pinene, linalool, terpinen-4-ol and α-terpineol. In the study conducted by Griffin *et al.* (1999), it was discovered that both terpinen-4-ol and α-terpineol were active. Furthermore it was found that the overall number of cells killed and initial death rate caused by α-terpineol is less than that of terpinen-4-ol suggesting that terpinen-4-ol has greater killing capacity and possibly greater outer membrane permeability.

Acmadenia obtusata showed activity against *Stapylococcus aureus, Escherichia coli, Candida tropicalis* and *Bacillus cereus*. A large concentration of α-pinene (27.44 %) was present which has already been established as an antimicrobially active molecule. The α-pinene (+) is twice as active (MIC 1680 ppm) as the α-pinene (–) (MIC 3400 ppm) molecule against *Stapylococcus aureus* (Griffin *et al.* 1999). In addition to this, lower concentrations of linalool, α-terpineol and terpinen-4-ol were also present. The combination of these molecules provided an antimicrobial effect. It was beyond the scope of the project to do chiral separations to establish the enantiomers present for each of the molecules.

Acmadenia sheilae showed good activity with ten of the twelve microbes being inhibited (Table 16) relative to the positive controls. Linalool and terpinen-4-ol were present in high concentrations in a ratio of 2:1 (species monographs). Linalool is active against *Escherichia coli* and *Staphylococcus aureus* with MICs of 1700 ppm for both and terpinen-4-ol has an MIC of 1900 ppm respectively (Griffin *et al.* 1999). Another active compound present is citronellol. Citronellol showed activity against mycobacteria at a concentration of 64 μg/ml (Cantrell *et al.* 2001). Perhaps it too added to the efficacy observed with *Acmadenia sheilae*.

Adenandra obtusata revealed poor activity against all organisms tested with the exception of *Candida tropicalis* where the MIC was 2 mg/ml (Table 16). A third of the sample is composed of α-pinene. Griffin *et al.* (1999) mentions that the (+)-α-pinene molecule is more active against *Candida albicans* but we find no activity. The inhibition of *Candida tropicalis* could be due to any of the other molecules.

Adenandra villosa showed moderate inhibition throughout. It was the most active against *Bacillus cereus*. Fifty percent of the sample was composed of myrcene which has been already proven to have minimal inhibition. There were however some very active molecules in minute quantities. These include citronellol, nerol, geraniol and α-terpineol as identified by Griffin. Many other molecules could also possibly contribute to its efficacy.

Diosma prama, (sample B) showed little activity compared to *Diosma prama* sample A. Its major compound profile closely resembled that of *Adenandra villosa.* In another related study of *Murraya exotica* L (El-Sahawy *et al.* 1998) it was ascertained that 62.5 % of the total leaf volatile oil was α-pinene. It has been proven before that α-pinene has antimicrobial activity. There was moderate inhibition against most Gram-positive and Gram-negative bacteria but poor coverage for the various *Candida* species. Thus, one can make the assumption that α-pinene is more inclined to kill bacteria than fungi (El-Sakhawy *et al.* 1998). Looking at the two *Diosma* specimens similarities and differences become evident. More of the α-pinene compound is present in the first sample as compared to the second sample. Also interesting was the fact they displayed such different profiles. *Diosam prama* (B) had much more menthol derivatives. Pulegone was one of its major compounds. Flamini *et al.* (1999) reported that the only one endowed with antibacterial effectiveness was pulegone, showing inhibition halos varying between 16mm and 20mm. Because pulegone, at a dose of 5 μL, corresponding to its percentage in the essential oil, maintained an inhibitory activity, it is possible to state that the effectiveness of the essence is almost exclusively due to this constituent. The *Diosma* sample (B) showed activity against only three pathogens. These include both *Bacillus* sp. and the yeast *Cryptococcus neoformans.* The assumption therefore is that its activity could be due to this compound. Sample (A) had a combination of active factors. All pathogens studied were susceptible with the exception of *Aspergillus niger, Candida albicans, Serratia odorifera* and *Staphylococcus epidermidis.* The active metabolites include linalool, terpinen-4-ol, α-terpineol, *p*-cymene, geraniol and α-pinene as discovered by Choa *et al.* (2000).

Several other authors have performed antimicrobial assays on other plant species. From these analyses, they have been able to draw some conclusions concerning activity of monoterpenic compounds. According to Griffin *et al.* (2001) it was established that pre-treatment with polymixin B nonapeptide (PMBN), an outer membrane permeabilising agent, significantly increased the initial rates and overall numbers of cells killed for all compounds. Therefore if PMBN was added to any of

the test samples the MICs and zones of inhibition would have been better especially on the Gram-negative organisms. Also, essential oil compounds pose some challenges due to their physico-chemical properties. All available assays are aqueous based and terpenoids are mostly apolar. Furthermore, the bioassays are all "open-air" systems and due to their volatile nature (especially after incubation for 24 hrs) some of the more volatile compounds could 'escape'. The MIC values reported in Tables 17 - 19 are low but given the problems encountered with solubility and volatility it remains an important technique for a rapid screen of many samples. Recently Inouye *et al.* (2001) illustrated that the MIC values of essential oils were lowered 2-8 fold when evaporation was prevented.

Structural differences between several compounds lead to subtle differences in molecular properties. For example, terpinen-4-ol has a lower surface area and polarity than α-terpineol yet a larger H-bond donor capacity. Differences in properties such as hydrogen bond donor capacity, molecular volume, molecular surface area and hydrophilic/lipophilic balance have also been associated with activity with *Pseudomonas aeruginosa.* These subtle differences in molecular properties must therefore be associated with how effectively the outer membrane of *Pseudomonas aeruginosa* can protect the organism from the killing effect of α-terpineol and terpinen-4-ol. This finding accounts for why many of the samples were ineffective in the disc diffusion assays. Naigre *et al.* (1995) investigations lead to the following postulations: The carbonylation of tested monoterpenes was shown to increase the bacteriostatic and fungistatic activities specifically by the contact method. In addition, it seems that the presence of an oxygenated function in the framework increases the antimicrobial properties. Monoterpenes were also more active using a micro-atmosphere method. Thus from the above we were able to make some predictions about activity of the different monoterpenes. Those monoterpenes which can be manipulated by carbonylation will have better kill rates. Ketones, like acetone, carvone etc. which have an oxygenated atom will be more active as well.

Conclusion

Paolo Rovesti who was considered internationally one of the top leading scientific experts in essential oils, wrote in 1980:

"It is indispensable to explain thoroughly the chemical, physical and biochemical properties of essential oils, which may briefly be summed up as follows:

The considerable bactericidal power of the whole or the oxygenated part of the essences, hence their use in disinfectant and antiseptic preparations of every kind. Each chemical function of the different constituent parts of the essential oils has a conspicuous bactericidal predilection. Thus, for example, phenol hydroxyl for *Staphylococcus* sp. and for choleric parasites, the aldehyde group for typhus and cholera, etc. The double bonds of the aliphatic chains of terpenic molecules amplify these microbial functions and if they are specially treated with certain natural gases, the microbicidal action is further increased...."

The aim of this pharmacognosy related study was: firstly to determine the biological activity of Rutaceous species. Then the task was undertaken to record the essential oils profiles and identify the major compounds in the oil.

With reference to our aims we were able to prove the biological activity of the various Rutaceous species. The activity was relatively poor with moderate activity observed for *Acmadenia sheilae*, *Coleonema pulchellum* (A) and *Disoma prama* (A). Then too there was no consistency between the different specimens in the genus or even within the subfamily. This would provide many difficulties in quality control due to the inter-species variation.

The fifteen oils were subjected to GC and GC/MS and the chemical profiles were recorded. More than 90% of the compounds were effectively identified. Based on prior studies conducted by Griffin *et al.* (1999) and Inouye *et al.* (2001), it was established that certain compounds contributed to the efficacy observed. Using *Acmadenia sheilae* as an example, many ketone and alcohol derivatives were found. This specimen had some of the best MIC and disc diffusion results. Also, it was proven that wherever terpine-4-ol was present in large enough concentrations and in comparable ratios with that of linalool there was better MIC readings. It was confirmed that using different solvents in Minimum Inhibitory Sensitivity (MIS) assay would lead to vastly differing results. This would suggest that standardization of laboratory techniques should be introduced.

From the preceding discussion it appears appropriate to place more emphasis on the moderately active compounds in terms of antibacterial activity. There are of course many alternative uses of essential oils and for this reason the other specimens which proved to be not too effective should not be discarded.

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