Fuad Salie

M.Pharm Thesis

The antimicrobial screening of four South African Asteraceae species and the preliminary structural investigation of an antipseudomonal compound from *Arctotis auriculata*

Department of Pharmaceutical Chemistry
School of Pharmacy
University of the Western Cape

The antimicrobial screening of four South African Asteraceae species and the preliminary structural investigation of an antipseudomonal compound from *Arctotis auriculata*

by

FUAD SALIE B.PHARM (U.W.C)

Submitted in partial fulfilment of the M. Pharm degree in the Department of Pharmaceutical Chemistry, University of the Western Cape.

Supervisor: Professor P.F.K.Eagles
M.Pharm (U.W.C.)
Ph.D.(U.C.T.)

Co-supervisor: Doctor H.M.J.Leng
M.Pharm (U.W.C.)
M.Sc. (U.S.)
Ph.D.(U.C.T.)

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DEDICATION

To my Mother, Anesa, for all the interest, support, love and encouragement throughout my years of study.

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Summary

Infectious diseases represent one of the main causes of morbidity and mortality in developing countries, like South Africa. The indiscriminate use of antibiotics has also resulted in the emergence of a number of resistant bacterial strains.

Four plants belonging to the Asteraceae (Daisy) Family, which forms part of the Fynbos Biome, were screened for their phytochemical composition and antimicrobial activity. The plants investigated were: *Helichrysum crispum*, *Felicia erigeroides*, *Eriocephalus africanus* and *Arctotis auriculata*. The plants were selected on the basis of their ethnobotanical use in various infectious diseases.

The results from the phytochemical identification showed that all the plants tested positive for tannins. Flavonoids were detected in the leaves and stems of *A. auriculata* and *F. erigeroides* and the stems of *E. africanus*. Saponins were present in the leaves of *H. crispum* and the leaves and roots of *F. erigeroides*. Triterpene steroids were found in the stems of *E. africanus* and *F. erigeroides*. Alkaloids were only detected in the leaves of *A. auriculata* and cyanogenic glucosides were in the stems of *H. crispum* and the leaves of *A. auriculata*. None of the plants tested positive for quinones.

The disc diffusion method was used to determine the antimicrobial potential of the selected plant species. The results from this initial study showed that the organic extracts of *A. auriculata* and *H. crispum* inhibited the growth of *Mycobacterium smegmatis*. The same extracts, together with the organic extracts of *F. erigeroides*, were active against *Pseudomonas aeruginosa*. Antifungal activities against *Candida albicans* were exhibited by the organic extracts of *E. africanus*, *F. erigeroides* and *H. crispum*. Organic extracts of *A. auriculata* and *E. africanus*, as well as the aqueous extract of the latter plant, were active against *Staphylococcus aureus*. Hereafter, the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), minimum fungicidal concentration (MFC) and minimum mycobactericidal concentration (MMC) of the most active solvent extracts of selected organs of the four plants were done.

The chloroform extract of *Arctotis auriculata* which had significant antipseudomonal activity, was selected for further study. The active component of this extract was identified using the bioautographic method. It was situated at an R_f value of 0.83 on an analytical thin-layer chromatography plate. Larger quantities of this active compound were now collected using column chromatography and preparative thin-layer chromatography. The active compound was then subjected to various spectroscopic methods such as ultraviolet-visible spectroscopy, infrared spectroscopy, nuclear magnetic resonance spectroscopy and gas chromatography-mass spectroscopy to ascertain its chemical structure.
However, a conclusive structure for this compound could not be established. This may be due to the active compound having more than one chiral centre or that it may be a mixture of enantiomers. On the basis of the active compound's NMR and GC-MS data, it was hypothesised that the active compound closely resembled a sesquiterpene lactone isolated recently from *Arctotis auriculata* called 4-hydroxy-10(14),11(13)-guaiadien-12,6-olide.

Hereafter, the active compound was retested against *P. aeruginosa*. Its potency had significantly increased against the pathogen compared to the initial crude chloroform extract.

In conclusion, further studies are required to determine the active antipseudomonal compound's chemical structure and mechanism of action.

CHAPTER 1

INTRODUCTION

Traditional medicines are as old as human civilization.The utility of traditional medicines has been described in books such as the *Rig Veda* which dates back to 4800 B.C.[1] Higher plants have been used for centuries as remedies for human diseases. This has encouraged scientists to screen higher plants for various biological activities. [2]
Traditional medicines are used all around the world to "cure" a wide array of diseases. These diseases range from asthma, snake bite, fever, infection, menstrual disturbances, "warding off" of evil spirits, treating pain and to induce abortions.[3] It has been estimated that 75-80% of the world's population rely on traditional medicines for their health needs. The majority of traditional medicines are derived from plants. Western medical practice is also very reliant on plant-derived drugs as about 30% of prescription drugs have their origins in the plant kingdom. Approximately 75% of the latter arose following the investigation of traditional plant remedies.[4]

In order for traditional medicines to be used to maximum advantage in the formal health sector, there should be:

- Comprehensive documentation on traditional medicines.
- The safety, quality and clinical efficacy of these medicines should be scientifically studied.
- The therapeutically active compounds used should be identified.
- Adverse reactions related to the use of traditional medicines should be evaluated.
- The carcino-, nephro- and hepatotoxicity of traditional medicines should be investigated.
- Extensive collaboration with traditional healers should exist.[5]

Appropriate steps must be taken to ensure conservation of our medicinal plants. As increasing demands are being placed on the wild stocks of these plants due to the population growth, it has led to the eradication of certain popular species. Conservation measures at this stage are inadequate and

poorly managed so that the potential loss in biodiversity is grave. An inventory of the conservation status of medicinal plant species is needed. The Chiang Mai Declaration of 1988, called upon all people to commit themselves to "Save the Plant that Saves Lives." It was recognised that medicinal plants are essential to primary health care.The health care professionals and plant specialists were alarmed at the loss of plant diversity around the world and they were very concerned that plants providing traditional and modern drugs were threatened.[5]
The declaration urged the United Nations, its agencies and member states to recognise:

- The vital importance of medicinal plants in health care.
- The unacceptable loss of these medicinal plants due to habitat destruction and bad harvesting practices.
- The significant economic value of traditional medicinal plants used and the good potential of the plant kingdom to provide new drugs.
- The continuing disruption and disappearance of indigenous cultures which often hold the key to finding new medicinal plants.
- The urgent need to establish programmes for conservation of medicinal plants to ensure adequate quantities are available for future generations.

The study of traditional medicines, which has its origins from clinical observations, and which were gradually refined over many centuries, stimulates the mind and leads on to further discoveries and developments. New sources from which medicinal compounds can be extracted must be found. The discovery of new natural products as medicinal agents requires a thorough investigation of in-depth cultural knowledge, scientific expeditions to identify the nature of the drugs, chemical analysis and pharmacotoxicological tests, to result in final clinical confirmation.[6]

The indiscriminate use of antibiotics has resulted in the emergence of a number of pathogenic micro-organisms resistant to standard drug regimens.[7] This, coupled with the high cost of the newer and the more effective antimicrobial drugs, makes the search for less expensive, alternative substances imperative. The **fynbos**, with its richness and diversity, represents an important potential source of new drugs, including drugs for

the treatment of infectious diseases.[8] Many fynbos plants have long been used as herbal remedies by the descendants of the original inhabitants, the Khoi and the San people. [10] Other local communities have also displayed interest in these traditional medicines due to, amongst others, the high cost of conventional medicines.

1.2 But what is **fynbos**?

The flora of the Western Cape, commonly known as the cape fynbos, forms part of the Cape Floral Kingdom which consists of 8 550 species distributed over an area of 90 000km^2 .[9] Originally fynbos referred to a large group of evergreen plants with small, hard leaves eg. the Ericaceae. The fynbos biome is situated at the tip of Africa. It has an abundance of very attractive flowers and stretches in a crescent arch from Vanrhynsdorp to the Cape Peninsula and the Boland mountains and from there eastward to Port Elizabeth and Grahamstown. (see Figure 1.1). There is nowhere where the fynbos is more than 200km from the seashore and these areas normally have a rainfall of more than 250mm annually. The biome somewhat resembles the vegetation of the Mediterranean regions and other winter rainfall regions in that it had to adapt to wet winters and dry summers. It differs from these areas, however, in that it has a significantly higher number of plant species.

The fynbos is characterised by:

- A large variety of species, i.e. 8 550 species occur in the fynbos area and more than 6 000 species are endemic to certain regions.
- At the community level, fynbos is quite unique as a wide variety of species may occur within the same community in a very confined area.
- fynbos is a plant type that has adapted to fire due to the process of evolution.
- Some families or genera may predominate. There is always a restioid (reed) element, an erica (heather) element and a proteoid element in fynbos.
- The soils on which the fynbos are usually found are poor and infertile.

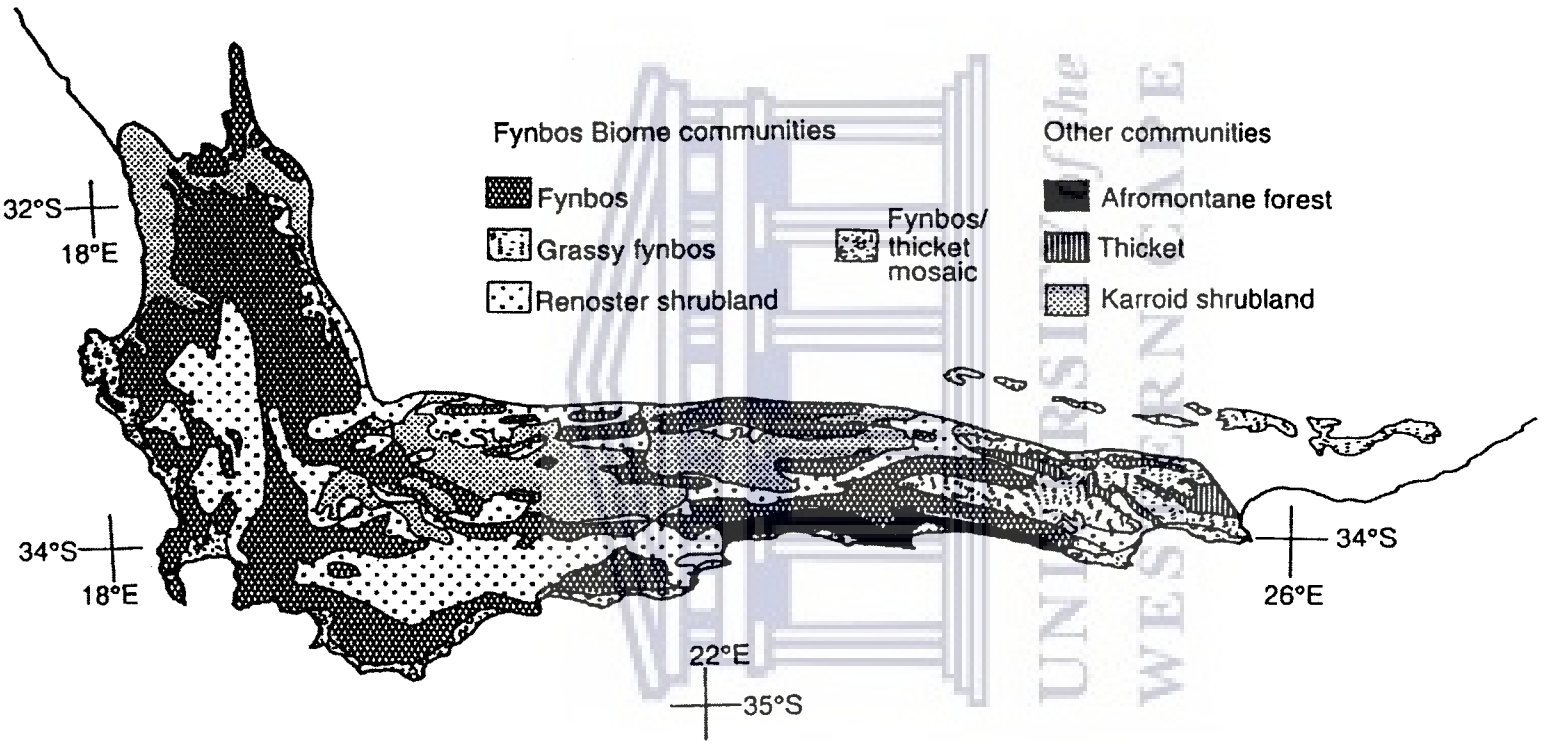


Figure 1.1: Major vegetation communities in the Cape Floristic Region.[26]

- fynbos communities are present on many types of landscapes ranging from coastal plains to subalpine peaks.
- The fynbos has characteristic distribution patterns.
- fynbos occurs in areas where the rainfall is between 250-3 000mm annually. (see Figure 1.2)
- Trees are rarely found in the biome. If they occur, they are normally isolated eg. *Virgilia oroboides* (Blossom tree), *Chironia baccifera* and *Aloe ferox*.[27]

1.3 Why study Ethnopharmacology?

Ethnopharmacology is the science that observes, identifies, describes and experimentally investigates the ingredients and the effects of indigenous drugs. Ethnopharmacological research is based on botany, pharmacology, toxicology, and chemistry, but other disciplines make vital contributions especially anthropology.
Ethnopharmacology can be defined as:

"the interdisciplinary scientific exploration of biologically active agents employed or observed by man"

The study of traditional medicines is not to advocate a return to the usage of these remedies in their crude form, nor to exploit traditional medicine. The objectives of ethnopharmacology are to rescue and document an important cultural heritage before it is lost, and to research and evaluate the agents employed.[11]

Plants provide people with food, shelter, fuel and a major use of plants in South Africa is in traditional medicines. Despite urbanization and developments that have taken place, traditional medical practice continues to play an important role for many people. In South Africa, it appears that that up to 80% of black people visit traditional doctors on a regular basis and make use of the treatments based on locally available plants. The estimated annual cost for plant-based traditional remedies is about R500-million.[5] In the South Western Cape, the demand for medicinal plants even from this species rich area is expected to increase. Already among the most threatened genera are the buchus, which are very popular.

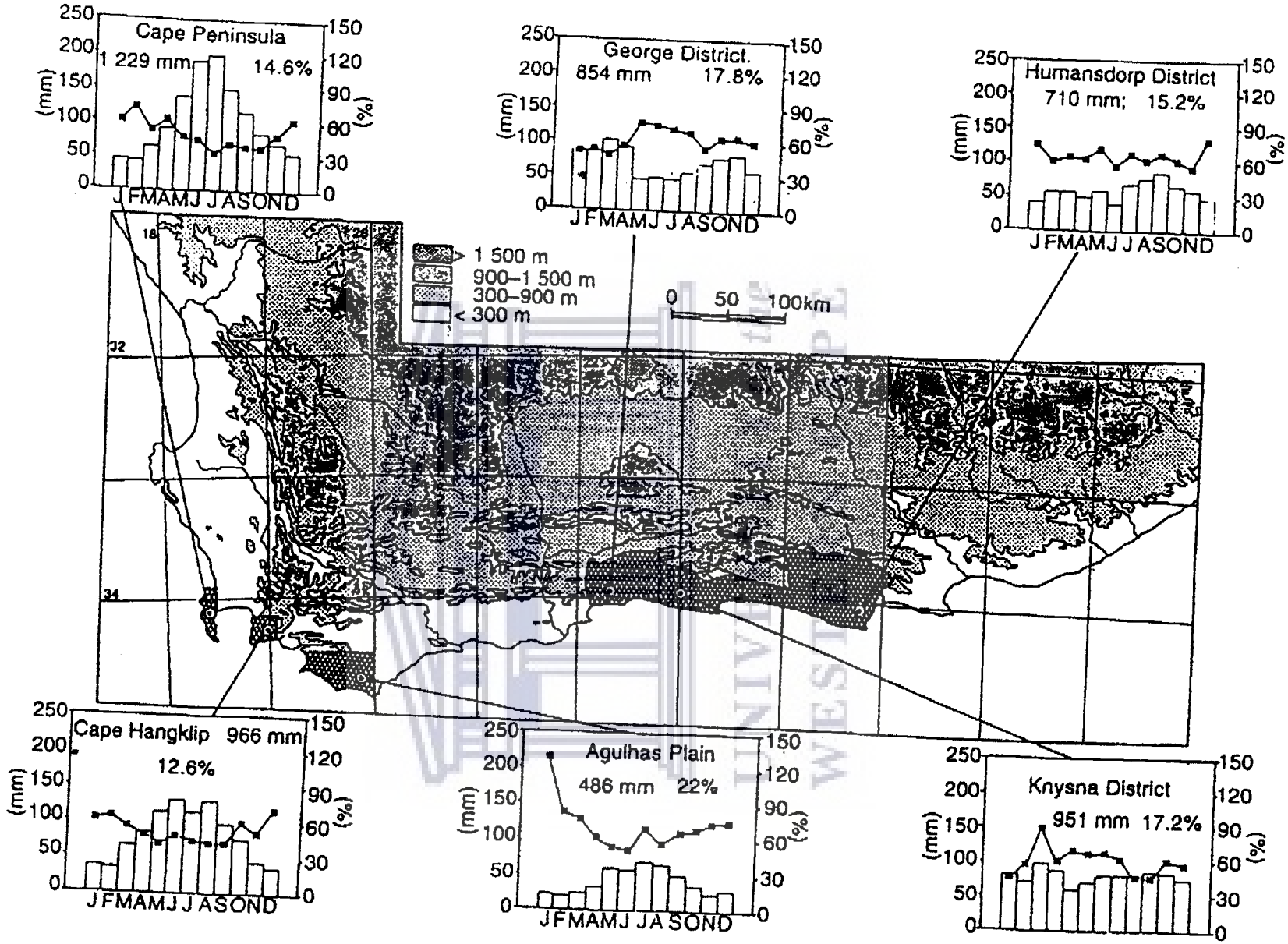


Figure 1.2: Location and representative rainfall data for the areas of the south-western and south-eastern floras. Bars refer to mean monthly rainfall and lines to coefficients of variation for monthly rainfall.[26]

The Department of Pharmaceutical Chemistry at the University of the Western Cape has decided to focus on the antimicrobial potential of fynbos species used in traditional medicine for various "infectious" conditions. The reason why this area of research was chosen is that infectious diseases represent one of the main causes of morbidity and mortality in developing countries, such as South Africa. The Western Cape region alone has one of the highest incidences of the tuberculosis in the world.[12] Hepatitis and meningococcal infections are also prevalent in this region. Thus, there is a real need to investigate the antimicrobial potential of plants used in traditional medicines for infectious conditions, especially due to increasing bacterial resistance to conventional drugs. The demand for inexpensive treatment methods to meet primary healthcare needs has increased interest in traditional medicines. Besides the above reasons, there is a possibility that novel antimicrobial compounds can also also be isolated from these plants as many of the fynbos species have not yet been fully researched. The research done in this project can also be used in the proposed pharmacopoeia for plants used in traditional medicines. But more importantly, consumers and traditional healers could then be informed of the findings. And, in collaboration with the Environmental Unit at the University of the Western Cape, the importance of conserving these plants can be emphasized. Also, there is a need to collect information on traditional medicines as the knowledge is being "lost" because the transmission between the older traditional healers and younger generations is not always assured.[13] It is the author's firm believe that our indigenous plants can contribute to the growth of our economy due to their economic potential. A very good example of this is *Aspalathus linearus,* commonly known as "rooibos tea".

1.4 Is there any danger in using traditional medicines?

To ask whether the use of traditional medicines can entail a health risk, is a rhetorical one. There has been numerous reports in the past that traditional medicines can cause serious illnesses such as liver and renal failure, and eventually death.[14]

Recently, a study was done in North Western Pakistan covering 16 000 households. Of the 5600 live births about 69 neonates died due to tetanus which was related to Ghee, a clarified butter, which is applied to the

umbilical wound stump. What had happened, was that the butter was made from unpasteurized milk. Although the butter was heated enough to kill non-sporulating organisms, the process was unlikely to eliminate *Clostridium tetani* spores. The researchers concluded that there would be an approximate 43% decline in neonatal tetanus deaths if this practice was discontinued.[15]

A major cause of hepatic failure in humans using traditional medicines is due to the consumption of pyrrolizidine alkaloids.[16] The World Health Organisation has listed over 280 species belonging to more than 40 different genera of plants containing hepatotoxic pyrrolizidine alkaloids.[17] It is interesting to observe that nine genera of the Asteraceae Family contain these alkaloids.
It becomes thus clear **that the benefit/risk principle** which is used in Western medicine **must also apply to traditional medicines**. [14]

1.5 Why study the Asteraceae (Daisy) Family?

Plants of the Asteraceae family, which is the largest family in the fynbos biome, was chosen to be researched for the following reasons:[18]

- Most traditional medicines used by the indigenous communities are, in fact, derived from plants belonging to this family.[19]
- The good availability of the plant material.
- The cost of plant material was within budgetary constraints.

Four species of the Asteraceae were investigated for antimicrobial activities: *Arctotis auriculata* Jacq., *Eriocephalus africanus* L., *Felicia erigeroides* DC. and *Helichrysum crispum* (L.) D. Don.

1.5.1 *Helichrysum crispum*

H.crispum is a common shrub growing throughout the Western Cape and is known as 'Hotnotkooigoed' or 'Imphepho'. It is an aromatic perennial herb with dense hairy leaves and persistent flower heads. The plant parts

usually used in traditional medicine are the leaves and twigs. A tea made from its leaves is taken for heart conditions, kidney ailments, backache and as a calmative.[20,21] Infusions of the plant are reported to be effective in the treatment of coughs, bronchitis, coronary thrombosis and hypertension. In the Transkei, an infusion of *H.crispum* is especially used for urinary tract infections. In the Western Cape it has also been utilized in the treatment of tuberculosis.[21,22]

Active ingredients isolated from *Helichrysum* species include flavonoids, sesquiterpenoids and acylated phloroglucinols. *H. odoratissimum* contains an antimicrobial flavonoid, helichrysetin. This species is also rich in essential oil, with alpha-pinene and alpha-humulene as the main compounds. *H. caespititium* has also yielded an antimicrobial compound, caespitin.[32]

1.5.2 *Eriocephalus africanus*

E.africanus is a fast growing evergreen, about 60-120 cm in height, with simple linear leaves. Its grey-green foliage has a characteristic fragrance.[23] The attractive white or slightly purplish flower heads are followed by conspicuous tufts of seed hairs, giving the shrub a distinctive appearance. It is these tufts that resemble snow (Afrikaans: kapok) hence the Afrikaans vernacular name.Amongst the locals the plant is commonly referred to as 'wild rosemary', 'kapokbos','asmabossie' or 'tontelbossie'.[23,24] The plant parts normally utilized in traditional medicine are the leaves and twigs. Infusions of the plant have been used in the treatment of asthma and chest complaints, and a tea made from the leaves has been used for treating coughs, colds, flatulence and colic. It has also been used as a diaphoretic and a diuretic.[24]

The aerial parts of *E. africanus* contains a mixture of sesquiterpenoid lactones with 4,11-eudesmanediol being the main constituent. Smaller quantities of ivangustine and dehydrofalcarinol have also been isolated.[32]

1.5.3 *Felicia erigeroides*

F.erigeroides is a perennial, Cape Peninsula plant approximately 30-60 cm in height and has lilac flowers.[25] Preparations of the plant has been used as an anthelmintiic and for the treatment of skeletal and genital infections.[20] Active ingredients isolated from *Felicia* species include oligopeptides,

glycosides and triterpenoid saponins.[36]

1.5.4 *Arctotis auriculata*

A.auriculata is a trailing perennial with a short, branched rhizome and leaves that are pinnately cleft.[26] It has yellow to orange flowers from August to September and is found on the hillsides of Clanwilliam and Namaqualand. Extracts of the plant are applied to sore breasts and have also been used as a douche for uterine cancer.[20]

Phytochemicals isolated from *Arctotis* species include 16-kauranol,[33,34] germacronolides, farnesol derivatives and sesquiterpene lactones.[35]

Figure 1.3 Photograph of *Arctotis auriculata*

CHAPTER 2

The antimicrobial screening and the phytochemical analysis of the four plants

The aims of the work in this chapter were to determine, firstly, whether the four selected plants contain certain classes of phytochemicals **and** if the various plant extracts have the ability to inhibit the growth of the four selected micro-organisms.

2. METHODOLOGY

2.1 **Plant material**

The plants were obtained from the National Botanical Institute at Kirstenbosch and the Cape Flats Nature Reserve in Bellville. The identity of the plants was verified by a taxonomist, Mr.Frans M. Weitz, and voucher specimens were deposited in the Herbarium of the Botany Department of the University of the Western Cape. After cleansing the plants with distilled water, they were separated into leaves, stems and roots and dried in an oven at 40^{0}C for 72 hours. The dried plant materials were then milled to a fine powder (mesh size: 1 mm). One third of the powdered materials from each plant was used for phytochemical assays and the remainder for solvent extraction.[8]

2.2. **Phytochemical assays**

The powdered organs were screened for the following biologically active secondary metabolites: alkaloids, cyanogenic glucosides, flavonoids, quinones, saponins, tannins, and triterpene steroids.
The phytochemical identification was carried out according to the following methods: [28]

2.2.1 **Alkaloids**

One part of the dried and powdered plant material was mixed with 4 parts of ammonium hydroxide. The wet, alkalinised powder was placed in a beaker

and extracted with chloroform in a waterbath for 15 minutes. The chloroform extract was then divided into 2 portions. One portion was subjected to thin layer chromatography, using Dragendorff's reagent for detection. The other portion was evaporated to dryness and the residue redissolved in 3% hydrochloric acid and tested by Mayers' test. The appearance of an orange-red spot on the thin layer chromatography plate and the formation of a precipitate with Mayers' test indicates the presence of alkaloids.

2.2.2 **Tannins**

The decoction was treated with 1% $FeCl_3$ solution. The appearance of a blue-black colour or a precipitate indicates the presence of tannins.

2.2.3 Flavonoids

The powdered material (10g in 100ml water) was boiled for 2-3 minutes over a waterbath. To 3 ml of the filtrate was added 3 ml of acid-alcohol (EtOH: H_2O: conc. HCl 1: 1: 1), solid magnesium, and about 1 ml of tertiary amyl alcohol. The appearance of a rose-orange or violet colour indicates the presence of flavonoids.

2.2.4 Saponins

The preceding decoction (1ml) was shaken vigorously for 10 seconds in a test tube, allowed to settle for 10 minutes, and the presence of a foam indicated the presence of saponin(s). The height of any foam expressed was measured in mm.

2.2.5 Quinones

The powder (10g) was moistened with a 10% HCl solution and was allowed to stand in an ether-chloroform mixture (3:1, 40ml). After filtration, 1 ml of the extract was treated with 1 ml of 10% sodium hydroxide. A red coloration indicates the presence of quinones.

2.2.6 Triterpene steroids

1 gram of the powder was extracted for 24 hours in 20 ml ether. 1 ml of the extract was evaporated to dryness and the residue redissolved in 4-5 drops of acetic anhydride. Several drops of 98% sulphuric acid were added and the presence of triterpene steroids is indicated by a green colour. A comparable test was done by using sulphuric acid on its own.

2.2.7 Cyanogenic glucosides

1 to 2 grams of the moistened powder was treated in a test tube with 2-3 drops of toluene. The tube was stoppered with a paper strip impregnated with Grignard's reagent. A positive test is demonstrated by the appearance of a red tint on the paper strip.

2.3 Solvent extraction

The dried powdered plant material was successively extracted in a Soxhlet extractor with petroleum ether, chloroform, ethanol and methanol (Merck). Extraction by each solvent was carried out for 6 to 8 hours. The organic solvent extracts were concentrated to approximately 10 ml *in vacuo* at 40^{0}C with a rotary evaporator (Eyela, Model NE-1, Tokyo) and then dried to constant mass in a Speedvac at 43^{0}C (Savant Instruments Inc., Model SC110, Farmingdale, NY). Aqueous extracts were prepared by adding the powdered plant material to warm water (50^{0}C) for 30 minutes, following the methanol extraction. After allowing the aqueous extracts to cool to room temperature, they were freeze-dried (LSL Secfroid SR, Model 3021, Switzerland). After determining the yields, the extracts were stored at 4^{0}C until further use.[8]

2.4. Antimicrobial screening

2.4.1. **Micro-organisms and growth media**.

The micro-organisms used were *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 25614), *Mycobacterium smegmatis*, and *Candida albicans*. *S.aureus* and *P.aeruginosa* were purchased from the American Type Culture Collection (Maryland, USA). *M.smegmatis* was a gift from Professor Paul van Helden of the Department of Biochemistry and Physiology at the University of Stellenbosch, and *C. albicans* was obtained from the Microbiology Department of the University of the Western Cape. *S.aureus* was maintained on Trypticase soy agar, *P. aeruginosa* on Bacto

nutrient agar, *M.smegmatis* on Bacto Mycobacteria 7H11 agar supplemented with Middlebrook OADC enrichment, and *C. albicans* on Sabouraud dextrose agar. All the media were purchased from Difco (Detroit, MI, USA).

2.4.2. **Preliminary screening for the antimicrobial activities**.

The dried plant extracts were dissolved in 10% aqueous dimethylsulfoxide (DMSO) to a final concentration of 10 mg/ml and sterilised by filtration through a 0.45 µm membrane filter (MSI, Westboro, MA). Sterile 9 mm discs (Schleicher and Schuell) were impregnated with 50 µl of extract and placed on the surface of agar plates inoculated with a microbial culture. Each extract was tested in quadruplicate. Control discs contained 50 µl sterile 10% aqueous DMSO. Ciprofloxacin (40 µg/disc) served as a positive control for *S. aureus*, *P. aeruginosa* and *M. smegmatis*, whereas amphotericin B (25 µg/disc) was the control for *C. albicans*.
Agar plates containing the bacteria and mycobacteria were incubated at 37^{0}C for 24 and 48 hours respectively, whereas plates inoculated with *C. albicans* were incubated at 30^{0}C for 48 hours. After incubation, inhibition zones were recorded in millimetres (mm) as the difference in diameter between the discs and the growth free zones around the discs.[8]

2.4.3 **Minimum inhibitory concentration (MIC)**

The liquid dilution method was used.[29] A stock solution (40 mg/ml) of each extract was prepared in 10% aqueous DMSO. An aliquot of this solution was serially diluted (two fold) with nutrient broth to obtain a concentration range of 10-0.0195 mg/ml. To each dilution was added 50 µl of a microbial culture with an optical density of 0.4 (log phase) at 620 nm using a Beckman DU-40 spectrophotometer. After careful mixing, the test preparations were incubated at 37^{0}C (bacteria and mycobacterium) or at 30^{0}C (fungus). Each extract was assayed in triplicate. Controls were prepared in the same manner, except that 50 µl of broth was added in place of the microbial culture. The minimum inhibitory concentration was determined by measuring the absorbance of the preparations at 620 nm with a multiplate reader (Multiskan, MCC 340, Labsystems, Finland) against the corresponding control. The lowest concentration that gave a zero absorbance reading, i.e. no turbidity, was taken as the MIC of the test extract.

2.4.4. **Minimum bactericidal, mycobactericidal and fungicidal concentration (MBC, MMC and MFC)**

This was determined by adding 50 µl aliquots of the preparations which did not show any growth after incubation during the MIC assays, to 200 µl of broth. These preparations were incubated at 37^{0}C for 24 hours (bacteria) and 48 hours for mycobacteria. The fungal test preparations were incubated at 30^{0}C for 48 hours. The microbicidal concentration for each micro-organism was regarded as the lowest concentration of extract which did not produce an absorbance reading at 620 nm.[8]

2.5 RESULTS

2.5.1. Phytochemistry

Table 2a. Phytochemical composition[a] of plants investigated

Plant	Plant organ	ALK	FLA	GLUC	QUIN	SAP	STER	TAN
Arctotis auriculata	LF	+	+	+	-	-	-	+
	ST	-	+	-	-	-	-	+
	RT	-	-	-	-	-	-	+
Eriocephalus africanus	LF	-	-	-	-	-	-	+
	ST	-	+	-	-	-	+	+
	RT	-	-	-	-	-	-	+
Felicia erigeroides	LF	-	+	-	-	+	-	+
	ST	-	+	-	-	-	+	-
	RT	-	-	-	-	+	-	-
Helichrysum crispum	LF	-	-	-	-	+	-	+
	ST	-	-	+	-	-	-	+
	RT	-	-	-	-	-	-	+

[a]ALK, alkaloids; FLA, flavonoids; GLUC, cyanogenic glucosides; QUIN, quinones; SAP, saponins; STER, triterpene steroids; TAN, tannins.
[b]LF, leaves; ST, stems; RT, roots.

All the plants tested positive for tannins (Table 2a). In *H. crispum*, *A. auriculata* and *E. africanus*, the tannins were distributed throughout the plant; however, for *F. erigeroides*, the tannins were only located in its leaves.Saponins were found only in the leaves of *H. crispum* and the leaves and roots of *F. erigeroides*. Flavonoids were detected in the leaves and stems of *A. auriculata* and *F. erigeroides* and and the stems of *E. africanus*. Triterpene steroids were found in the stems of *E. africanus* and *F. erigeroides*.

Alkaloids were detected only in the leaves of *A. auriculata*. Cyanogenic glucosides were found to be present in the leaves of *A. auriculata* and the stems of *H. crispum*.

None of the plants tested positive for quinones.

2.5.2 Antimicrobial activity

Table 2b

Zones of inhibition of the four Asteraceae species against different micro-organisms[a]

Organ	Extract[b]	Yield %w/w	*S.aur*[c]	*P. aer*	*M.smeg*	*C.alb*
Arctotis auriculata						
Leaves	PE	0.29	0	1+	1+	0
	Ch	0.12	1+	1+	0	0
	Et	0.004	0	0	1+	0
	Me	1.03	0	0	0	0
	Wt	0.30	0	0	0	0
Stem	PE	0.32	0	1+	1+	0
	Ch	0.08	0	1+	2+	0
	Et	0.13	0	0	0	0
	Me	0.10	0	0	0	0
	Wt	0.99	0	0	0	0
Roots	PE	0.02	0	0	0	0
	Ch	0.50	0	1+	1+	0
	Et	0.03	0	0	1+	0
	Me	0.44	0	0	1+	0
	Wt	0.24	0	0	0	0
Eriocephalus africanus						
Leaves	PE	0.18	0	0	0	1+
	Ch	1.27	0	0	0	1+
	Et	1.30	0	0	0	0
	Me	0.45	0	0	0	0
	Wt	0.77	0	0	0	0
Stems	PE	0.12	1+	0	0	0
	Ch	0.04	0	0	0	0
	Et	0.54	0	0	0	0
	Me	0.47	0	0	0	0
	Wt	3.30	0	0	0	0
Roots	PE	0.23	0	0	0	0
	Ch	0.39	0	0	0	0
	Et	1.36	0	0	0	0
	Me	0.73	1+	0	0	0
	Wt	2.34	0	0	0	0

Organ	Extract[b]	Yield %w/w	*S.aur*[c]	*P. aer*	*M.smeg*	*C.alb*
Felicia erigeroides						
Leaves	PE	0.31	0	0	0	0
	Ch	0.10	0	1+	0	0
	Et	0.01	0	0	0	0
	Me	1.03	0	1+	0	1+
	Wt	2.72	0	0	0	0
Stems	PE	0.16	0	1+	0	0
	Ch	0.06	0	0	0	0
	Et	0.17	0	0	0	1+
	Me	0.45	0	0	0	0
	Wt	1.22	0	0	0	0
Roots	PE	0.21	0	0	0	0
	Ch	0.13	0	1+	0	0
	Et	0.11	0	1+	0	2+
	Me	1.65	0	0	0	1+
	Wt	1.47	0	0	0	0
Helichrysum crispum						
Leaves	PE	1.33	0	3+	1+	0
	Ch	0.26	0	2+	0	0
	Et	0.08	0	1+	0	0
	Me	0.30	0	0	0	0
	Wt	2.33	0	0	0	0
Stems	PE	0.28	0	2+	0	0
	Ch	0.02	0	1+	0	1+
	Et	0.02	0	1+	0	0
	Me	0.17	0	0	0	0
	Wt	2.39	0	0	1+	0
Roots	PE	0.61	0	1+	0	0
	Ch	0.09	0	1+	0	0
	Et	0.05	0	1+	0	0
	Me	0.72	0	0	0	0
	Wt	2.87	0	0	0	0
Cipro[d]			5+	5+	5+	-
Ampho			-	-	-	3+
DMSO			0	0	0	0

[a]*S. aur*, Staphyllococcus aureus; *P. aer*, Pseudomonas aeruginosa; *M. smeg*, Mycobacterium smegmatis; *C. alb*, Candida albicans.
[b]PE, petroleum ether; Ch, chloroform; Et, ethanol; Me, methanol; Wt, water.
[c]0: no inhibition; 1+: 0-3 mm; 2+: 3-6 mm; 3+: 6-9 mm; 4+: 9-15 mm; 5+: >15 mm.
[d]Cipro, ciprofloxacin; Ampho, amphotericin B; DMSO, dimethylsulfoxide

Table 2c

Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), minimum fungicidal concentration (MFC) and minimum mycobactericidal (MMC) of the most active solvent extracts of selected organs of *Arctotis auriculata* Jacq. (*A. aur*), *Eriocephalus africanus* L. (*E. afr*), *Felicia erigeroides* DC. (*F. erig*), and *Helichrysum crispum* (L.) D. Don (*H. cris*)

Organ extracts[a]	Test organisms	MIC (mg/ml)	MBC/MFC/MMC (mg/ml)
PE leaves *A. aur*	*P. aeruginosa*	5	10
PE leaves *A. aur*	*M. smegmatis*	8.5	>10
Ch leaves *A. aur*	*S. aureus*	9.1	9.8
Me roots *E. afr*	*S. aureus*	10	10
Ch roots *F.erig*	*P. aeruginosa*	9.9	10
Et roots *F. erig*	*C. albicans*	2.5	5
PE leaves *H. cris*	*P. aeruginosa*	10	>10
Ch stems *H. cris*	*C. albicans*	10	>10

[a]PE, petroleum ether; Ch, chloroform; Et, ethanol; Me, methanol

All the plants exhibited some degree of antimicrobial activity (Table 2b). *E. africanus* proved the least potent. The petroleum ether stem and methanol root extracts of this plant only effected slight inhibitions in the growth of *S. aureus*. The MIC of the methanol root extract against *S. aureus* was 10mg/ml (Table 2c).

All plant organs of *F. erigeroides* exhibited activity against *P. aeruginosa* and *C. albicans*. The lipophilic extracts (chloroform and petroleum ether) inhibited the growth of *P. aeruginosa*, whereas the semipolar extracts (ethanol and methanol) were active against *C. albicans*. The ethanol extract of the root of this plant was very active against *C. albicans*, with an MIC of 2.5mg/ml and an MFC of 5mg/ml.

H.crispum also exhibited selective activity. It proved inactive against *S. aureus* but was very active against *P. aeruginosa*.

In fact, the whole plant inhibited the growth of *P. aeruginosa*, with the lipophilic extracts exhibiting the greatest activity. This was not unexpected since the cell wall of the micro-organism has a high lipid content. The petroleum ether leaf extract had an MIC of 10mg/ml against *P.aeruginosa*. This extract and the aqueous stem extract also caused slight inhibitions in the growth of *M. smegmatis*. Only the chloroform stem extract showed significant activity against *C. albicans,* with an MIC of 10 mg/ml.

A. auriculata was inactive against *C. albicans* and exhibited minimal activity against *S. aureus*. The whole plant, however, proved inhibiting to the growth of *P. aeruginosa* and *M. smegmatis*. *P. aeruginosa* was sensitive to the lipophilic extracts. The MIC of the petroleum ether extract of the leaf was 5mg/ml. *M. smegmatis* was susceptible to the nonpolar and semipolar extracts. The nonpolar extracts were, however, more potent with the MIC of the petroleum ether leaf extract being 8.5 mg/ml.

2.6 DISCUSSION

Four indigenous Asteraceae species were evaluated for *in vitro* antimicrobial activity. The plants which form part of the Fynbos Biome in the Western Cape are commonly used by the local inhabitants to treat a number of infectious diseases.[20,21,22] The results obtained in this investigation appear to justify their ethnopharmacological use. Two of the four plants, *A. auriculata* and *H. crispum*, exhibited antimycobacterial activity. The three plant organs of *A. auriculata* were equally active against *M. smegmatis*, with the active ingredients being mainly lipophilic in nature. In the case of *H. crispum*, the activity resided in a lipophilic extract of the leaves and the aqueous extract of the stems. As mentioned before, the Western Cape has one of the highest incidences of tuberculosis in the world and many patients are developing resistance to the standard drug regimen.[12] These two plants may, therefore, represent sources of new drugs for the treatment of tuberculosis, especially resistant forms of the disease.

Three of the plants demonstrated activity against the fungus, *C. albicans*. They include *E. africanus*, in which the activity resided in a lipophilic extract

of the leaves, *H. crispum*, in which a lipophilic extract of the stems caused the activity, and *F. erigeroides* in which semipolar extracts of all three plant organs produced the activity. Antifungal drugs are amongst the most expensive antibiotics. These three plants, particularly *F. erigeroides* which is a perennial, may, therefore, serve as readily accessible and inexpensive alternative remedies for the treatment of fungal infections.[8]

The growth of *P. aeruginosa* was inhibited by the lipophilic extracts of all three plants organs of *A. auriculata*, *F. erigeroides* and *H. crispum*. Infections caused by *P. aeruginosa* are amongst the most difficult to treat with conventional antibiotics.[30] These three plants may, thus, yield drugs that could improve the treatment of infections caused by this micro-organism.

S. aureus proved sensitive to lipophilic extracts of the leaves of *A. auriculata* and stems of *E. africanus* as well as to the aqueous extract of the roots of the latter plant. The observed inhibitions were, however, slight. It is thus doubtful that the plants would be effective for treating infections caused by *S. aureus*.

In conclusion, the four Asteraceae species exhibited selective antimicrobial activity to varying degrees.

After completing the antimicrobial screening of the four plants, a decision had to be taken as to which plant would be analysed further. Initially *H. crispum* was selected for its very potent activity against *P. aeruginosa*. However, it proved very difficult to separate the petroleum ether extract using chromatographic means. This is due to petroleum ether being the most nonpolar solvent.

Arctotis auriculata **was then chosen** as the plant was available in abundance and displayed antipseudomonal activity.

CHAPTER 3

Determination of the number of antipseudomonal compounds in the chloroform extract of *Arctotis auriculata.*

The aim of the work in this chapter was to determine which component(s) in the chloroform extract of *Arctotis auriculata,* using the bioautographic method,[31] were active against *Pseudomonas aeruginosa.*

3. METHODOLOGY

3.1 Plant material

Forty *Arctotis auriculata* plants were obtained from the National Botanical Institute at Kirstenbosch during the spring season. The plants were cleansed using distilled water and then dried in an oven at 40^{0}C for 72 hours. The dried plant material was then milled to a fine powder (mesh size: 1 mm). All of the plant material was then used for solvent extraction.

3.2 Solvent extraction

The dried, powdered plant material was successively extracted in a Soxhlet extractor with petroleum ether, chloroform and methanol. Extraction with each solvent was carried out for 6 to 8 hours. These extracts were then concentrated to approximately 10 ml *in vacuo* at 40^{0}C with a rotary evaporator and then dried to a constant mass using a Speedvac at 43^{0}C. After determining the yields, the extracts were stored in sealed glass tubes at 4^{0}C until further use.

3.3 Bioautographic method

This is a method whereby a plant extract is separated on a thin-layer chromatography (TLC) plate into its components and, thereafter, the thin-layer plate's surface is covered with an agar suspension of the micro-organism to be tested. The agar is allowed to set and the plate is then incubated for 18 hours. When the plate is removed from the incubator, zones of inhibition can be seen at R_f values representing components in the extract which are active against a certain micro-organism.

3.3.1 Thin layer chromatography

The dried chloroform extract was reconstituted in chloroform to yield a 100 mg/ml solution. An analytical thin-layer chromatography plate (glass plate covered with

0.1mm silica 20X5 cm) was spotted and allowed to develop in a TLC tank which was previously saturated with the mobile phase. The mobile phase used was 1 part ethyl acetate : 8 parts chloroform : 2 parts n-butanol : 1 part methanol. When the solvent front had ascended about 90% of the TLC plate, it was removed from the tank. The solvent front was marked and the TLC plate allowed to dry. This was done in duplicate so that one plate would serve as a reference and the R_f value of an active component could be determined

.

3.3.2 **Preparation of Pseudomonas aeruginosa culture**

A *P.aeruginosa* colony was picked from the stock nutrient agar plate aseptically and it was added to a McCarthy bottle containing 10 ml of nutrient broth. This culture was then placed in an incubator at 37^{0}C. When the optical density of the microbial culture was 0.4 (log phase) at 620 nm, 0.3 ml of this culture was then added to 30 ml of freshly prepared (45^{0}C) agar. This solution was then spread evenly over the entire surface of the TLC plate (containing the separated plant components) under aseptic conditions.

The TLC plate was then placed in a partially opened plastic container lined with sterile filter paper dampened with sterile water. This was done so as to prevent the agar from drying out completely during incubation.The TLC plate was then incubated at 37^{0}C for 20 hours and the results were then observed.

3.4 RESULTS

3.4.1 **Solvent extraction**

Mass of 40 plants = 2.701 kg
Dry mass of plants = 430 g

Mass of chloroform extract = 5.10g
% yield (dry) = 1.18%

3.4.2 **Bioautographic method**

The results clearly showed a zone of inhibition on the agar-covered TLC plates. The zone of inhibition corresponds to a compound on the reference plate having an R_f value of 0.83. (Figures 3.1; 3.2 and 3.3)

Figure 3.1: Thin-layer chromatography plate of chloroform extract of *Arctotis auriculata* under UV light at 254 nm.
Mobile phase - 1 part ethyl acetate : 8 parts chloroform : 2 parts n-butanol

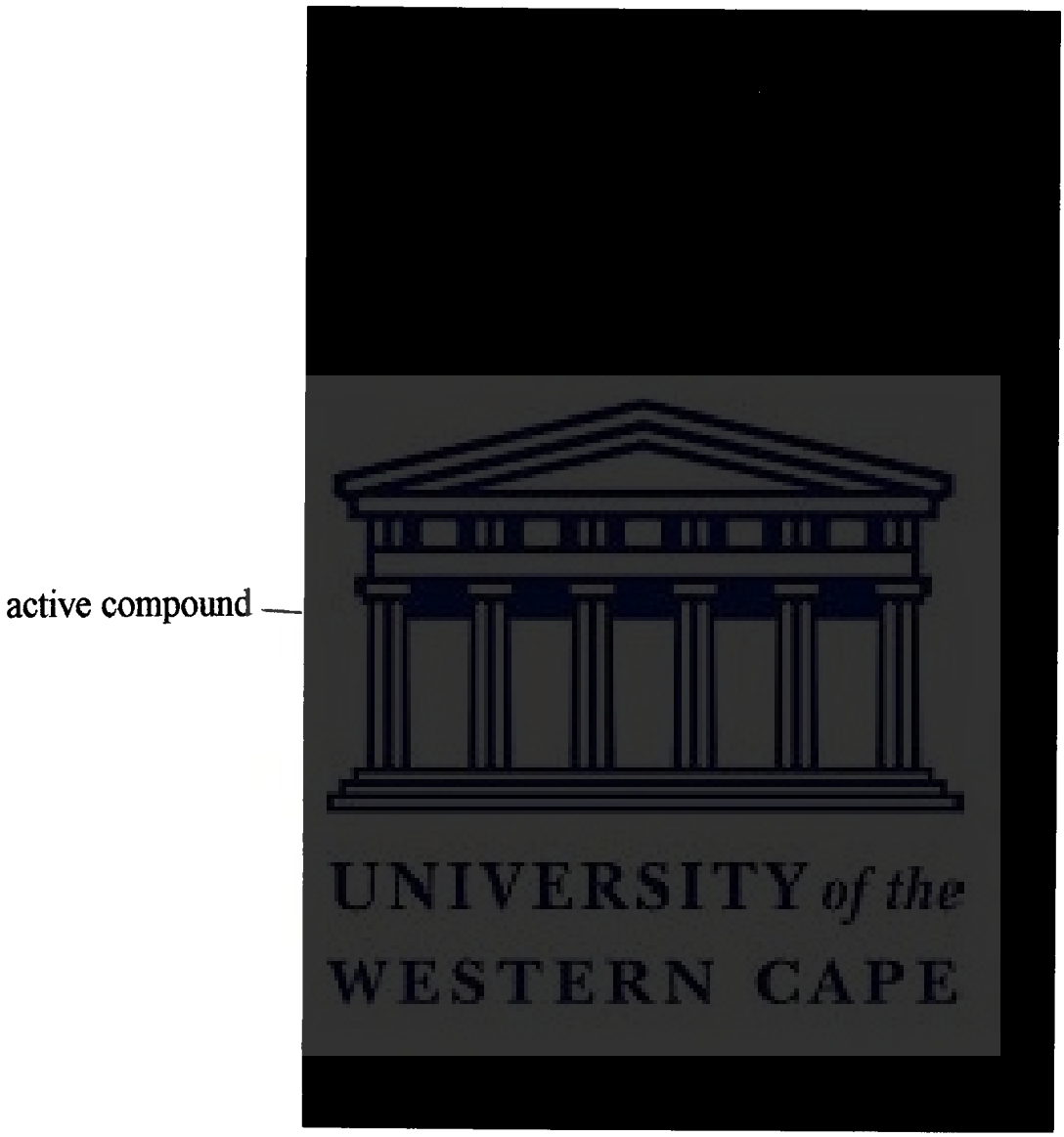


Figure 3.2: Thin-layer chromatography plate of chloroform extract of *Arctotis auriculata* under UV light at 365 nm.
Mobile phase- 1 part ethyl acetate: 8 parts chloroform: 2 parts n-butanol

Figure 3.3: Bioautographic plates of chloroform extract of *Arctotis auriculata* against *Pseudomonas aeruginosa*.

3.5 **DISCUSSION**

The percentage yield of the chloroform extract compared to the dry mass of the plant was only 1.18%. This percentage yield is however, quite normal for higher plants.

The bioautographic method was successful as one could easily see the zone of inhibition on the surface of the incubated thin-layer chromatography plate. Also, by comparing this plate to the reference TLC plate, the component of the extract responsible for the antipseudomonal activity could easily be identified. The active component resided at an R_f value of 0.83, indicating a relatively lipid-soluble compound. This component, when viewed under UV light, had a very characteristic colour. It had a very intense purple colour at 366 nm and a slight mauve colour at 254 nm. A major strength of the method is its reproducibility. It was a quick and convenient method for determining which phytochemical in an extract was active.

CHAPTER 4

Isolation and preliminary structural investigation of the active antipseudomonal compound of *Arctotis auriculata* using various analytical methods

The aim of the work in this chapter was to isolate and investigate the preliminary structural features of the active phytochemical using methods such ultraviolet-visible spectroscopy (UV-VIS), infrared spectroscopy (IR), nuclear magnetic resonance spectroscopy (NMR) and combined gas chromatography - mass spectroscopy (GC-MS).

4.1 THEORY OF THE METHODS USED

4.1.1 ULTRAVIOLET VISIBLE SPECTROSCOPY

The absorption spectra of plant constituents can be measured in a very dilute solution against a solvent blank using a spectrophotometer. Traces of material are required, since spectrophotometric cells normally hold about 3 ml of solution. The value of UV and visible spectra in identifying unknown plant constituents is related to the complexity of the spectrum and the general position of the wavelength maxima. If a compound shows a single absorption band between 250 and 260 nm , it would be difficult to try to identify such a substance using UV-VIS on its own. The reason for this is that the absorption band could belong to any one of the following compounds: a simple phenol; a purine; a pyrimidine; or an aromatic amino acid. However, if the compound shows three distinct peaks in the region between 400 and 500 nm, with little absorption elsewhere, it is almost certainly a carotenoid. Other classes of compounds which have characteristic absorption bands are polyacetylenes, aromatic compounds and ketones. Sometimes, the UV-VIS analysis of a compound may yield no spectrum. This usually indicates the presence of saturated lipids or alkanes or compounds which do not possess chromophores..

4.1.2 INFRARED SPECTROSCOPY

IR is indispensable in the analyses of phytochemicals. The absorption of IR radiation by molecules results in useful vibrational bands for all but a few compounds. Solids, liquids and gases can be studied using this method. The range of measurement is from 4000 to 667 cm^{-1} or 2.5 to 15 μm. The region in the IR spectrum above 1500 cm^{-1} normally shows spectral bands due to vibrations of

functional groups in the phytochemical under examination. For example carbonyl, frequencies normally occur between 1800 and 1650cm^{-1}. The region below 1500 cm^{-1}, referred to as the fingerprint region, is very difficult to interpret without a reference compound as the bands seen are due to vibrations of the entire molecule. The fact that many functional groups can be identified by their vibrational frequencies, makes the IR spectrum the simplest and usually a very reliable method in assigning a compound to its particular class.

Recently, Fourier Transform infrared (FTIR) spectroscopy has been introduced. It has several advantages over "normal" IR, including increased sensitivity of the spectrophotometer and an increased ability to obtain IR spectra from low molecular mass compounds.

4.1.3 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Proton NMR spectroscopy provides a way of determining the structure of an organic molecule by measuring the magnetic moments of hydrogen atoms. It provides the phytochemist with information such as the number of hydrogen atoms in an organic molecule and the environment surrounding these hydrogen atoms.
It does not give any information on the nature of the carbon atoms in the molecule. This information may be obtained by doing carbon 13 (^{13}C) NMR spectroscopy. This method is complementary to proton NMR and their combination provides a powerful means of obtaining structures for compounds like terpenoids, alkaloids and flavonoids. The sample in a solution of an inert solvent, such deuterochloroform or carbon tetrachloride, is placed between the poles of a strong magnet, and the hydrogen atoms experience different chemical shifts depending on their molecular surroundings. These are measured relative to a standard, tetramethylsilane (TMS) which is inert and is added to the sample solution. A major advantage of NMR over mass spectroscopy is that the sample is not destroyed during analysis.

4.1.4 GAS CHROMATOGRAPHY - MASS SPECTROMETRY

GC is a method used to separate components in a mixture that are volatile or that can be made volatile (e.g., by esterification of the mixture). It requires a mobile phase which is usually nitrogen or argon to carry the volatilised sample to a column where the separation of the mixture would occur. It provides both quantitative and qualitative data on plant substances. Small quantities of the sample are required, since a GC system can easily detect parts per million (ppm). The value of MS is that it requires microgram amounts of the compound that needs to be analysed. It

provides an accurate molecular mass and it may also provide a fragmentation pattern, by which a compound may be identified.

Thus, if GC is combined with MS, volatile mixtures may be separated into their components and the molecular mass and, sometimes, the identification of each of these components can be determined. A disadvantage of this method is that the sample is destroyed in the mass spectrometer due to it being bombarded with a high energy electron stream (70eV) in the ionization chamber.

In general the four analytical methods mentioned cannot individually give conclusive data on the possible structure of a phytochemical. These methods need to be used in conjunction with one another. However, before the above methods could be attempted, more of the active compound needed to be isolated from the chloroform extract. This was accomplished by doing preparative thin-layer chromatography.

4.2 METHODOLOGY

4.2.1 PREPARATIVE THIN-LAYER CHROMATOGRAPHY (PTLC)

100 mg of the chloroform extract of *Arctotis auriculata* was dissolved in 1 ml of chloroform (analytical grade, AnalaR assay 99-99.4%). Using a microsyringe (Hamilton), this solution was slowly streaked onto the line of origin on the surface of the PTLC plate (Silicagel 60 F_{254} , pre-coated 2 mm thick, 20x20 cm) .Care was taken to ensure that the width of the streak was not more than 3 mm wide.. Hereafter, the plate was introduced into a TLC tank saturated with mobile phase. The mobile phase used was 1 part ethyl acetate: 8 parts chloroform : 2 parts n-butanol : 1 part methanol. The plate was allowed to develop and only removed when the mobile phase had ascended about 19 cm of the PTLC plate. The plate was allowed to air dry and then it was viewed under UV light. The characteristic purple band of the active component was seen and pencil marked. Hereafter, the band (silica + active) was scraped from the PTLC plate using a scalpal and the active compound was redissolved in about 20 ml chloroform (analytical grade, Hipersolv min.assay 99.8%). This solution was filtered and the filtrate was then dried to a constant mass in a Speedvac at room temperature.
The resultant mass of the active compound was then purified using the same PTLC method as above.

4.2.2 ULTRAVIOLET-VISIBLE (UV-VIS) SPECTROSCOPY

A dilute chloroform (Hipersolv grade) solution (5 mg/ml) of the active was scanned between 200 and 900 nm using a Beckman DU-400 UV-VIS spectrophotometer, versus a blank (chloroform).

4.2.3 INFRARED SPECTROSCOPY

A methylene chloride solution of the active (10 mg/ml) was scanned between 500 and 3000 cm^{-1} in a Fourier Transform infrared spectrophotometer (Perkin-Elmer, Paragon 1000PC) versus a blank (methylene chloride), using NaCl windows.

4.2.4 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR)

a. Proton magnetic resonance spectroscopy (PMR)

A portion of the active (5 mg) was analysed at the Department of Chemistry at the University of Cape Town by Dr. W. Mabusela. The sample was dissolved in deuterochloroform and analysed using a 200MHz NMR machine. The procedure was done at 25^{0}C, and the number of repetitions was 512. After 31.9 minutes, the analysis was completed yielding the PMR spectrum of the compound.

b. CARBON MAGNETIC RESONANCE SPECTROSCOPY (CMR)

This analysis was also done at the Chemistry Department at the University of Cape Town. The sample (10 mg) was dissolved in deuterochloroform and introduced into the NMR machine. This time, however, the sample was exposed only to a frequency of 50MHz. The analysis was again done at 25^{0}C but the number of repetitions was now 1024. After 64 minutes, the analysis was completed yielding the CMR spectrum of the compound was obtained.

4.2.5. GAS CHROMATOGRAPHY - MASS SPECTROSCOPY (GC-MS)

The GC - MS analysis was done at the Chemistry Department at the University of Stellenbosch by Professor Burger. The sample (10 mg) was dissolved in chloroform (Merck residue analysis grade) and introduced into a gas chromatograhy - mass spectrometer (Carlo Erba QMD 1000) and analysed. The analysis was done using a PS.089, column which consists of 5% phenyl : 95% dimethyl stationary phase, and nitrogen as the mobile phase. The analysis was completed after 90 minutes.

4.3 RESULTS

4.3.1 PREPARATIVE TLC

Each 100 mg of crude (chloroform) extract separated on the PTLC plate only yielded 5 mg of active (5% yield). However, after the second purification step, 5.10 g of crude extract yielded only 130 mg of active (2,54% yield).

4.3.2 ULTRAVIOLET-VISIBLE SPECTROSCOPY (UV-VIS)

The compound absorbs between 200 - 460 nm, with a λ maximum at 239.2 nm (Figure 4.1).

4.3.3 INFRARED SPECTROSCOPY (IR)

High transmittance was observed at 3080 cm^{-1} and 2917 cm^{-1} with smaller transmissions between 1500 and 2000 cm^{-1} (Figure 4.2).

4.3.4 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

a. PROTON MAGNETIC RESONANCE SPECTROSCOPY

A complex PMR spectrum (Figure 4.3) was obtained. Numerous resonance signals were observed between 1 to 3 ppm and between 6 to 7.5 ppm, indicating that the active consisted of numerous functional groups.

b. CARBON MAGNETIC RESONANCE SPECTROSCOPY

A simple CMR spectrum (Figure 4.4) was obtained. A triplet was observed between 76.3 to 77.65 ppm and a singlet was observed at 29.67 ppm.

4.3.5 GAS CHROMATOGRAPHY - MASS SPECTROSCOPY

A major peak (Figures 4.6; 4.7 and 4.8) was observed after 70 minutes. This peak had a molecular mass of approximately 244.

Figure 4.1. UV-VISIBLE spectrum of the active compound

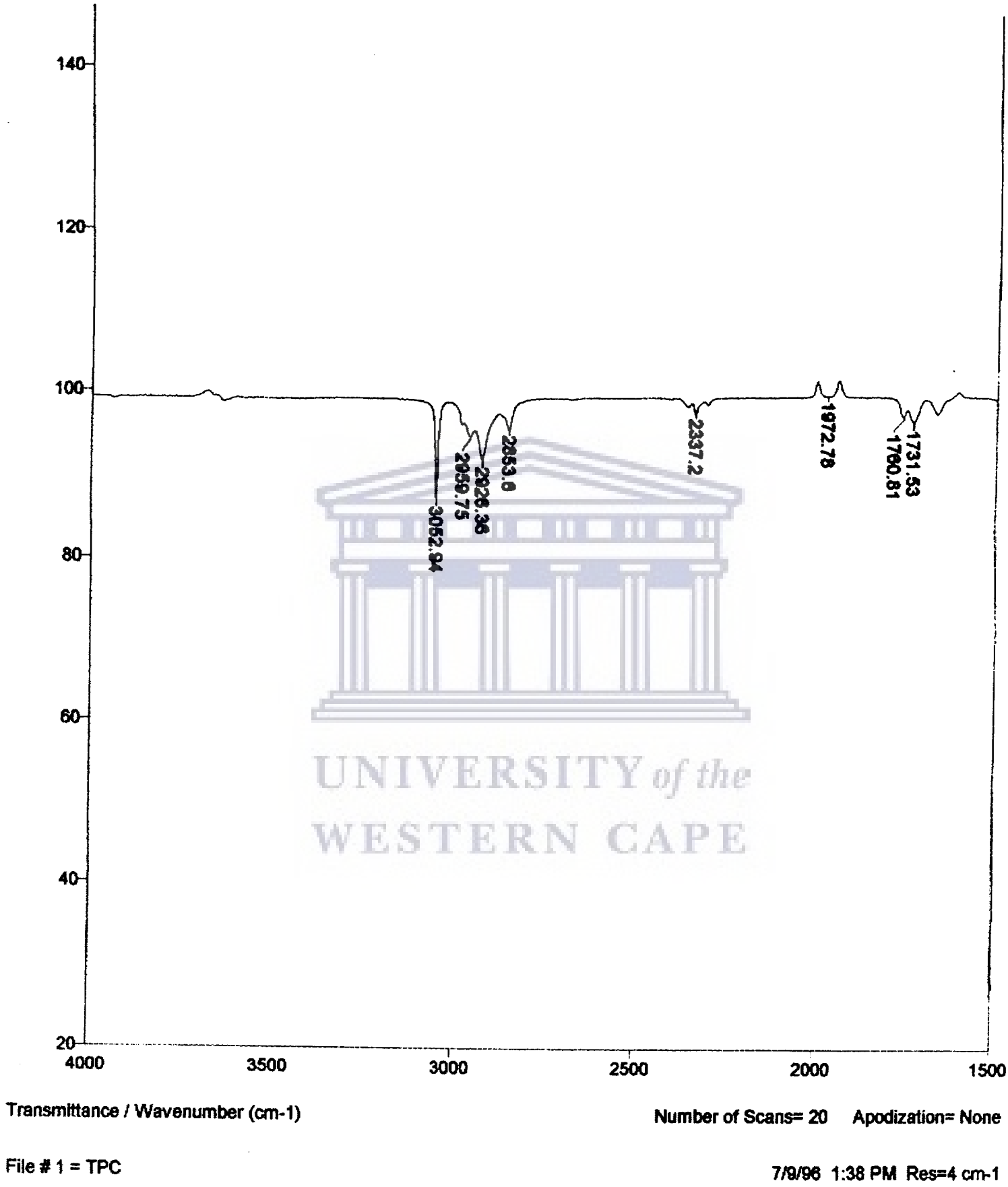


Figure 4.2. Infrared spectrum of the active compound in methylene chloride

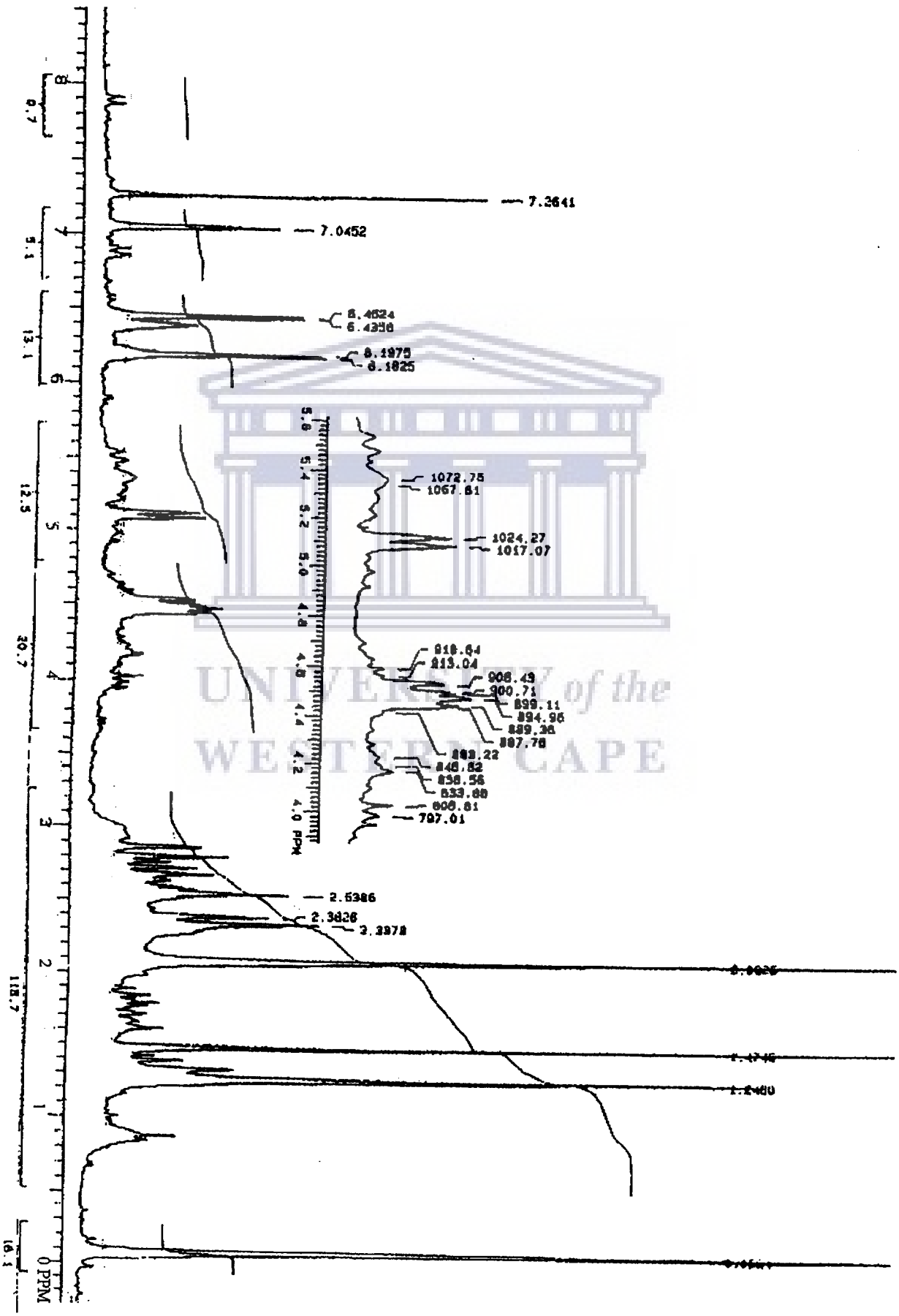


Figure 4.3. Proton magnetic resonance spectrum of the active compound

Figure 4.4. Carbon magnetic resonance spectrum of the active compound

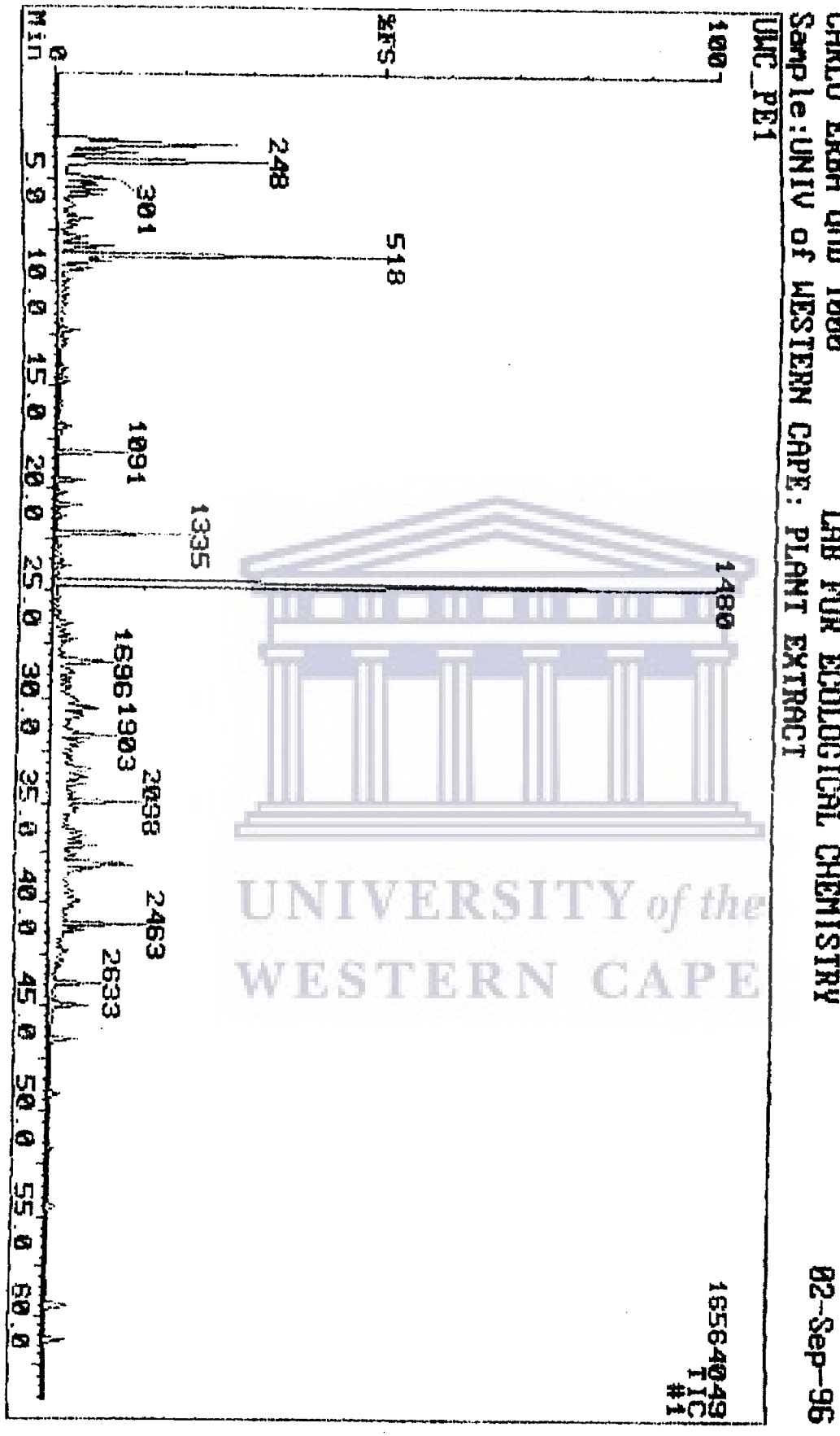


Figure 4.5. Gas chromatogram of active compound after 60 minutes

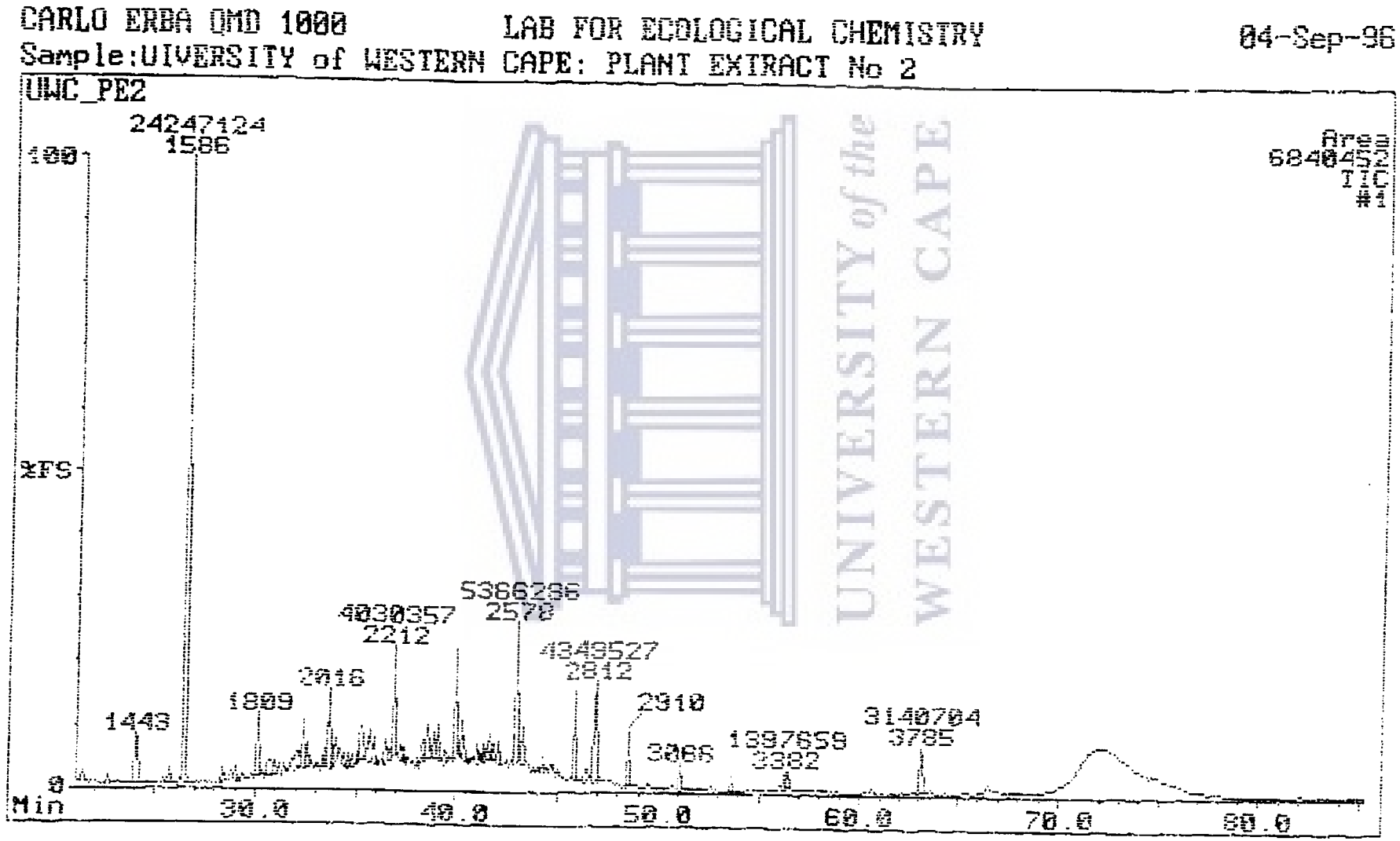


Figure 4.6. Gas chromatogram of the active compound after 90 minutes

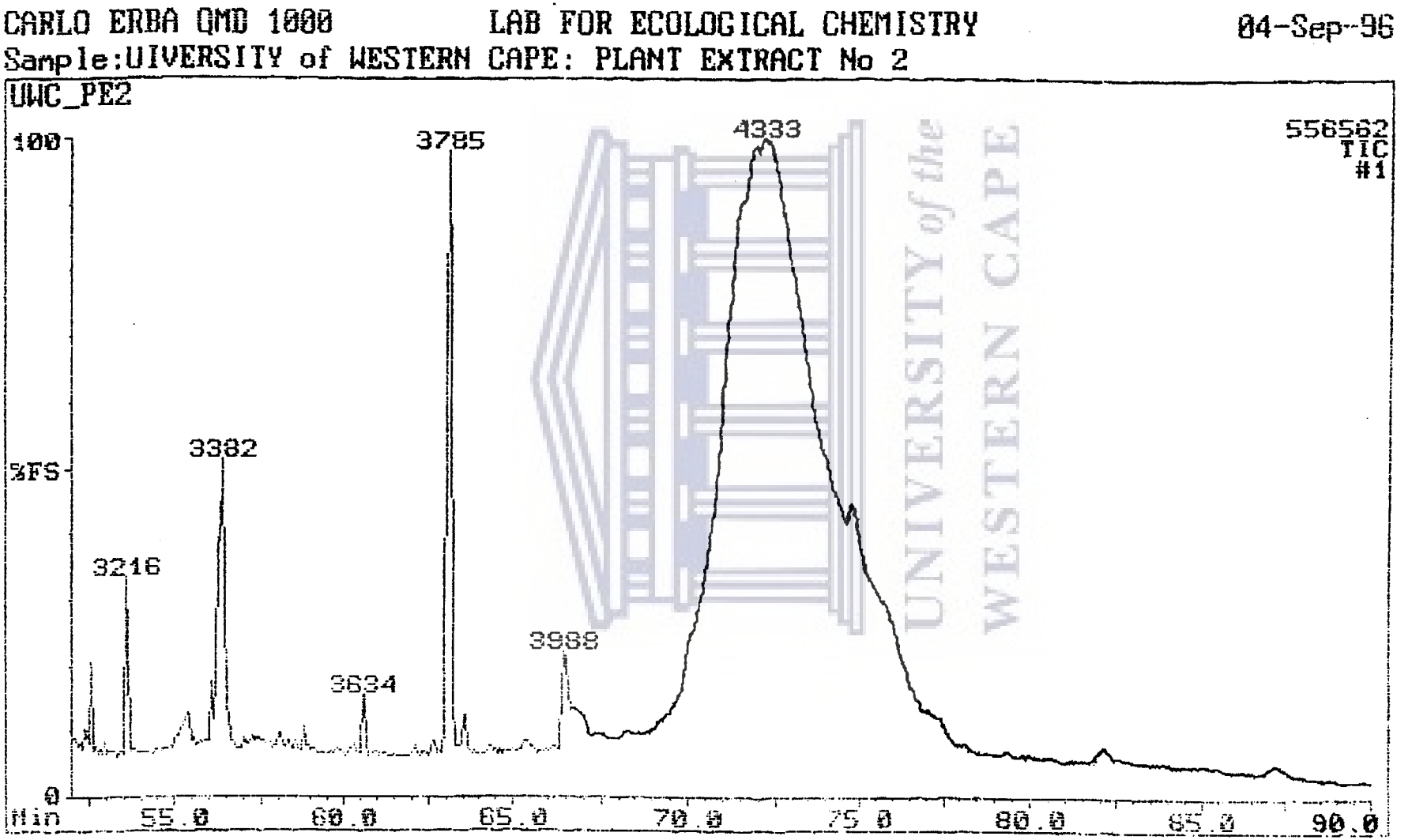


Figure 4.7: Gas chromatogram of the active compound after 90 minutes indicating the most abundant peak.

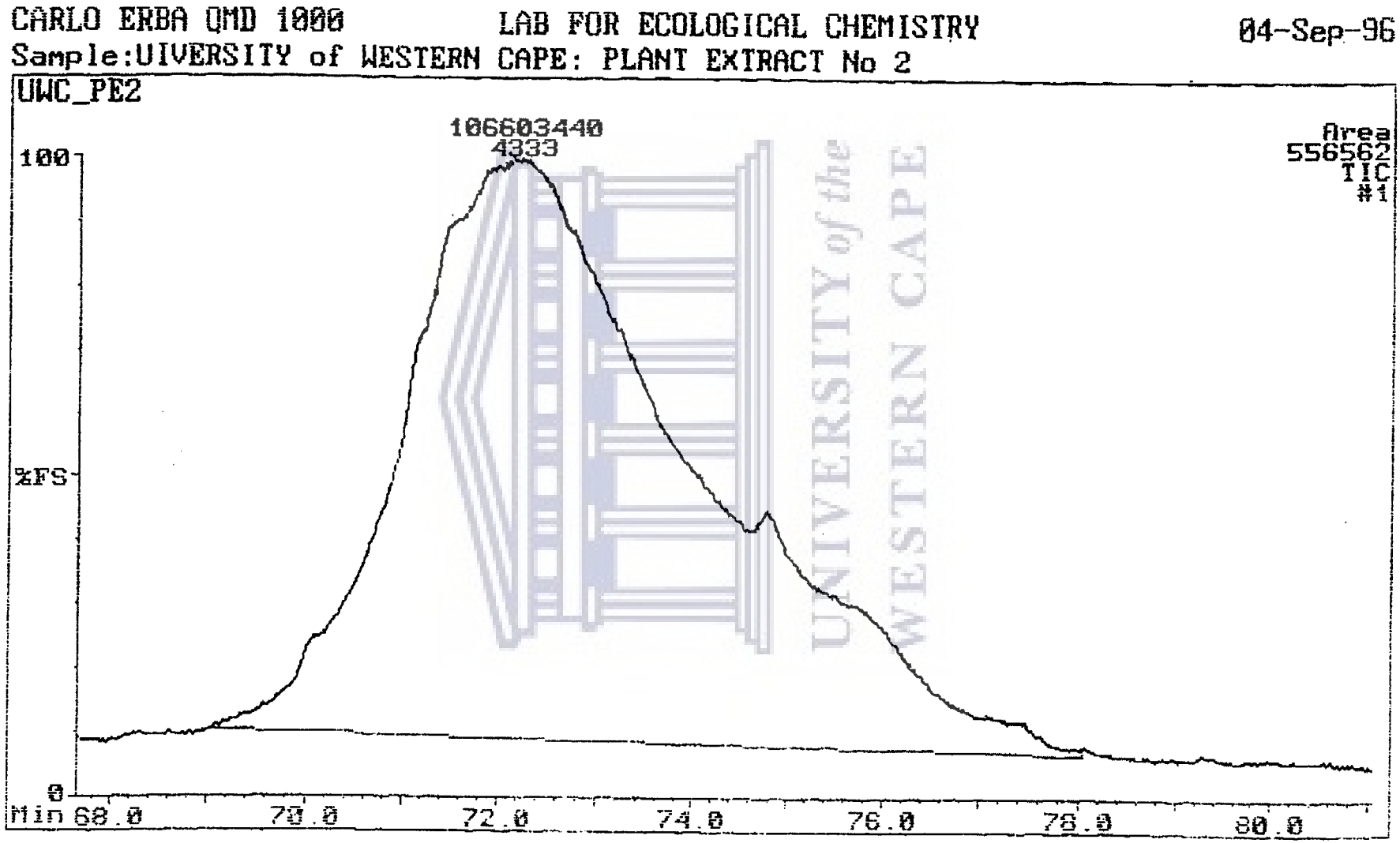


Figure 4.8. Gas chromatogram showing the retention time and area of the most abundant peak.

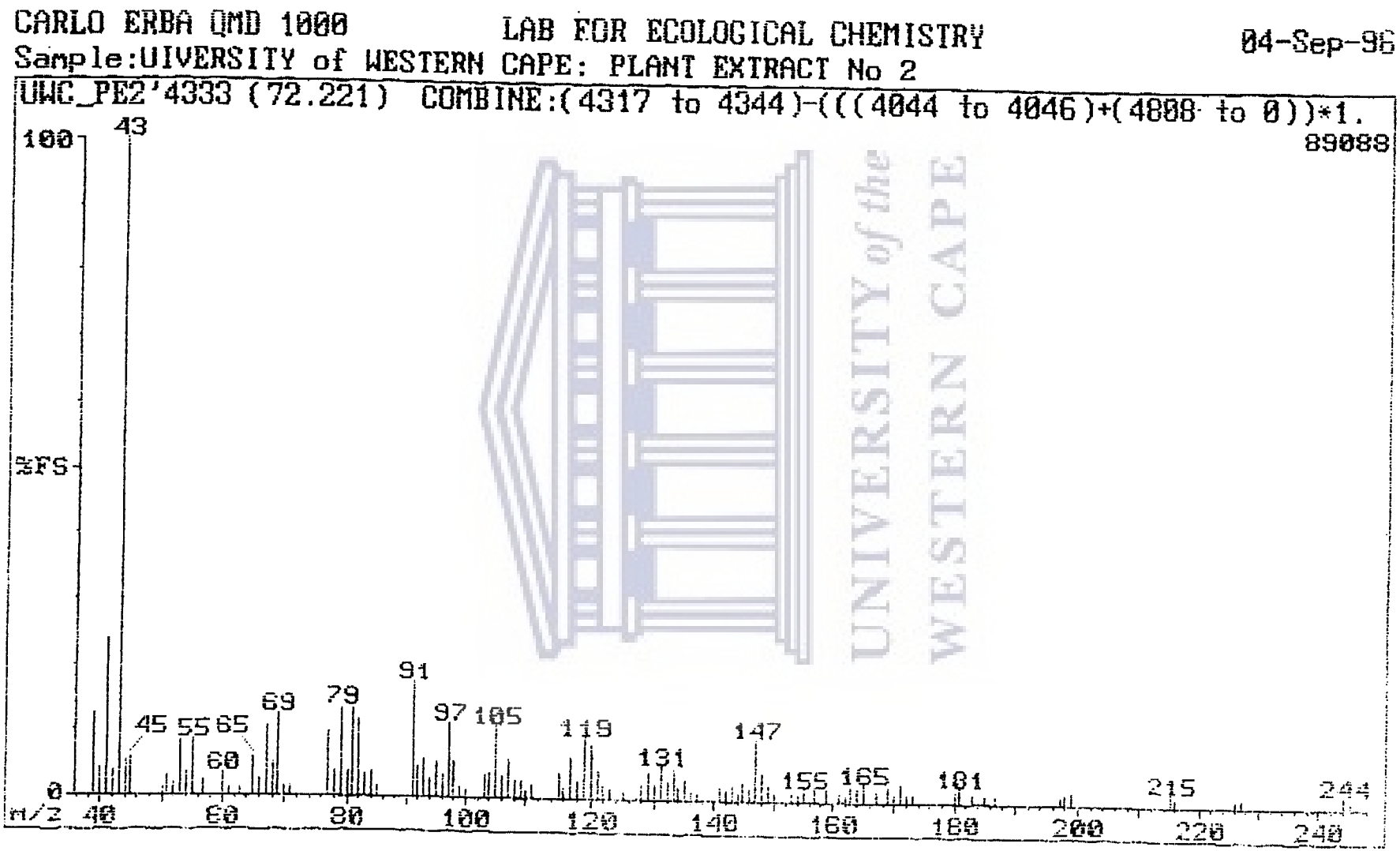


Figure 4.9. The mass spectrum of the major peak of the active compound

4.4 DISCUSSION

4.4.1 PREPARATIVE TLC

Initially, the yield of the active compound using this method was 5%.This was due to the crude chloroform extract of *Arctotis auriculata* containing many compounds when viewed under UV and normal light. However, when the active compound was isolated from the initial PTLC plate, and re-run on another PTLC plate so as to increase the purity of the compound, a yield of 2.54% was achieved.
The method was time consuming (6 hours) and the reproducibility was not particularly good.
Prior to using this method, column chromatography was employed using the same solvent system, but this method was very wasteful and more time consuming than the PTLC method.

4.4.2 ULTRAVIOLET - VISIBLE SPECTROSCOPY (UV-VIS)

The UV-VIS spectrum obtained clearly showed that the active absorbs light between 200 and 460 nm. A few high intensity absorbances occurred in the region between 200 and 230 nm, which may be due to trace impurities. A maximum wavelength of approximately 240 nm was observed for the active. The result clearly shows that the compound has a degree of unsaturation, i.e. it contains at least one double bond. The double bond(s) could either be associated with cyclic and aromatic structures or with aliphatic compounds.

4.4.3 INFRARED SPECTROSCOPY

Initially, the FTIR spectrum was done using chloroform as a solvent. However, due to the problematic light scattering and absorbances of chloroform, methylene chloride was used. The FTIR spectrum (using methylene chloride as solvent) obtained for the active antipseudomonal yielded some interesting results. It was possible to assign functional groups to particular wavenumbers (cm^{-1}) obtained from the FTIR spectrum. Firstly, the transmission at 3080 cm^{-1} is very characteristic of a hydroxyl group. The transmittance of the active at 2917 cm^{-1} is that of a lactone group.The transmittance seen between 1500 and 2000 cm^{-1} is normally assigned to methylene groups. These results support previous work that have shown that sesquiterpene lactones have been isolated from plants in the *Arctotis* genus.[35] Also, because a lactone group was identified, this showed that the molecule was definitely unsaturated. Thus, this result is in agreement with the UV-VIS spectrum of the active molecule.

4.4.4 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

a. **Proton magnetic resonance spectroscopy**

The PMR spectrum although not very clear, revealed valuable information about the active antipseudomonal. Firstly, the resonance signal at 1.248 ppm revealed a methyl (CH_3) group . The signals between 1.4 and 3.0 ppm were indicative of methylene groups.
The resonance signals between 3.5 and 5.0 ppm were difficult to interpret and it was suggested that it was maybe due to impurities. The analysis was then re-run, but the result remained the same.
The two sets of doublets between 6.0 and 6.5 ppm suggested protons that were present in unsaturated structures that contained electronegative atoms and this result was very positive as it could indicate a methylene group (CH_2) attached to a lactone structure.

b. **Carbon magnetic resonance spectroscopy**

The CMR spectrum revealed limited information. The signal at 29.67 ppm normally represents a carbon atom that is aliphatic in nature. The three signals seen between 76.33 and 77.60 ppm represented carbon atoms present in a cyclic non-aromatic structure.

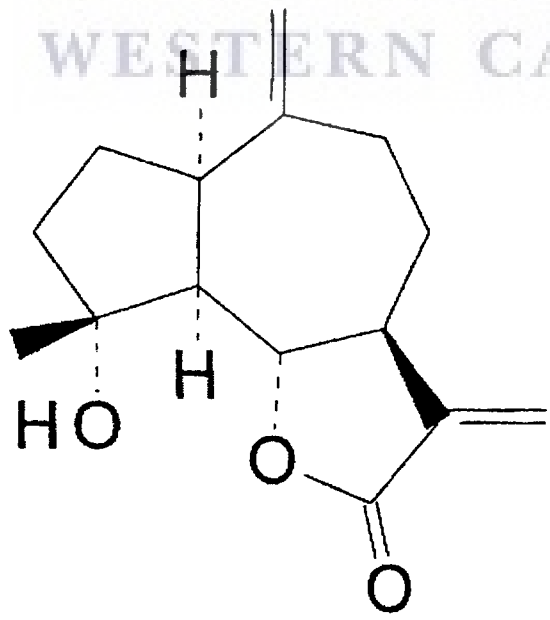
4.4.5 GAS CHROMATOGRAPHY - MASS SPECTROSCOPY

Initially, when the sample was put through the GC-MS, no significant peaks were seen on the gas chromatogram after 60 minutes.(Figure 4.5). The mass spectra of these peaks represented inorganic solvent residues. Prof. Burger, who did the analysis, then decided to extend the analysis time from 60 to 90 minutes. The gas chromatogram (Figure 4.6; 4.7 and 4.8) showed a significant peak 4333 eluting between 68 and 78 minutes. This peak represents an extremely nonpolar compound. The total ion count (TIC) was done on this peak and it confirmed that the peak represented only one compound. This compound represented 82% of the total mass of the sample. Although the mass spectrum (Figure 4.9) of this compound was not very clear, its molecular ion mass was approximately 244. The compounds isolated thus far from this plant genus have molecular masses ranging from 236 to 290 mass units.[35]

Table 4a Overview of spectroscopic results of the active compound.

Method	Result/chemical group present
Ultraviolet- visible spectroscopy	Active contains at least one double bond
Infrared spectroscopy	hydroxyl, lactone and methylene groups
Proton magnetic resonance spectroscopy	methyl, methylene and lactone
Carbon magnetic resonance spectroscopy	carbon atoms in non-aromatic cycle
Gas chromatography - mass spectroscopy	The major compound with an approximate molecular mass of 244

On the basis of the NMR and GC-MS data collected, the suggestion in terms of structure and what is found in the genus, the structural features of the active compound probably closely resembles a compound isolated recently from *Arctotis auriculata* called 4-hydroxy-10(14),11(13)-guaiadien-12,6-olide .
This compound has a molecular mass of 248.321 and has a molecular formula, $C_{15}H_{20}O_3$. It is a **sesquiterpene lactone (SL)** and it has the following structure:



SL

From the IR results, the active compound contains a hydroxyl group, a lactone group and methylene groups. All of these groups are seen in the above structure.(SL)
The degree of unsaturation of this compound(SL) is similar to that of the active compound (from UV data).The PMR data of the active compound also indicates a lactone structure, methylene groups and a methyl group.
The PMR data coupled with the CMR data proposes that the active compound also contains a ring structure of some kind.
The molecular masses of the two compounds are also similar. If one ignores the mass unit at 43 which normally indicates an acetyl group as being an impurity, then the base peak of the active is at 91 mass units which is exactly the base peak value of 4-hydroxy-10(14),11(13)-guaiadien-12,6-olide.[35] In addition, the active is very lipid soluble and slightly volatile which could indicate it to be a sesquiterpene lactone.
Further work , however, is needed before the structure of the active can be confirmed.

CHAPTER 5

Determination of the minimum inhibitory concentration and minimum bactericidal concentration of the active compound.

The aim of the work in this chapter is to determine the minimum inhibitory concentration and the minimum bactericidal concentration of the active compound and to establish whether the potency of the active compound was significantly increased upon further purification.

METHODOLOGY

Minimum inhibitory concentration (MIC)

A stock solution of 20 mg/ml of the active was prepared in 10% aqueous DMSO. An aliquot of this solution was serially diluted with nutrient broth to obtain a concentration range of 10- 0.0195 mg/ml. To each dilution was added 50 µl of the pseudomonal culture with an optical density of 0.4 units at 620 nm. After careful mixing, the test preparations were incubated at 37^{0}C for 18 hours. Each test preparation was done in quadruplicate. Controls were prepared in the same manner, except that 50 µl of broth was now added in the place of the microbial culture.
The minimum inhibitory concentration was then determined by measuring the absorbance of the preparations at 620 nm using a Multiskan multiplate reader against a corresponding control. As before, the lowest concentration that gave a zero absorbance reading (no turbidity) was considered to be the MIC of the test extract.

Minimum bactericidal concentration (MBC)

This was determined by adding 50 µl aliquots of the preparations which did not show any growth after incubation during the MIC assays, to 200 µl of broth. These preparations were incubated at 37^{0}C for 24 hours. The MBC was considered to be the lowest concentration of extract that did not produce an absorbance at 620 nm.

RESULTS

Table 5a

	Test organism	MIC mg/ml	MBC mg/ml
Active compound	*P. aeruginosa*	0.3175	5

The minimum inhibitory concentration and minimum bactericidal concentration of the active compound.

DISCUSSION

The results obtained conclusively showed that the potency of the active compound had significantly increased. The MIC was now 0.3125 mg/ml as opposed to 5 mg/ml when the active was still part of the crude chloroform extract of *Arctotis auriculata*, i.e. the potency had increased by a factor of 16 during the purification process. However, the MBC did not significantly change after the isolation procedure. The potency was only increased by a factor of 2.

What can be concluded is that at low concentrations there was inhibition by the active compound whereas at higher concentrations, the *Pseudomonal* cells were killed.

CHAPTER 6

Conclusion

The phytochemical analysis suggested that the selected plant species contained various classes of plant chemicals. All four plants tested positive for tannins. Alkaloids were only detected in the leaves of *A. auriculata* and cyanogenic glucosides in the stems of *H. crispum* and the leaves of *A. auriculata*. Flavonoids were detected in the leaves and stems of *A.auriculata* and *F. erigeroides* and the stems of *E. africanus*. Triterpene steroids were found in the stems of *E. africanus* and *F. erigeroides*.

All the plants investigated in this preliminary study exhibited some degree of activity against the chosen micro-organisms. The organic extracts of *A. auriculata* and *H. crispum* inhibited the growth of *M.smegmatis*. The same extracts, together with the organic extracts of *F. erigeroides*, were active against *P. aeruginosa*. Antifungal activities against *Candida albicans* were exhibited by the organic extracts of *E. africanus*, *F.erigeroides* and *H. crispum*. The organic extracts of *A. auriculata* and *E. africanus*, were active against *Staphylococcus aureus*.
These results thus support their uses as traditional medicines.

But more importantly, it indicated that traditional medicines can most definitely **contribute to the discovery of lead compounds** from which pharmaceuticals can eventually be derived. It also proves that the claims made for certain traditional medicines are in fact correct. The author is of the opinion that one should not dismiss any medicinal claims of traditional medicines until it can be disproved scientifically.

The present study has shown that a compound isolated from *Arctotis auriculata* possessed significant antipseudomonal activity. This result is of great significance as *Pseudomonas aeruginosa* causes serious infections like sepsis, pneumonia, severe external otitis and urinary tract infections. Sepsis caused by *Pseudomonas* has a mortality rate of over 50%. Also, *P. aeruginosa* is resistant to many antibiotics so that its pharmacotherapy is very difficult. The drugs used to treat these infections include azlocillin, ceftazidime and usually an aminoglycoside such as gentamycin.[30,38]

Thus, the isolated antipseudomonal compound could serve as a lead compound in the fight against *Pseudomonas aeruginosa* infections.

The actual chemical structure of this antipseudomonal compound was difficult to determine as it is possible that the compound has more than one chiral centre. It is also probable that the active could be a mixture of enantiomers, thus adding to the difficulty. It is proposed by the author that the compound most probably closely resembled a sesquiterpene lactone. Further studies are required to obtain the actual structure of the active compound. This would include work on its synthesis and mechanism of action.

Although traditional medicines are cheap and affordable to many of South Africa's people, they need to be investigated more thoroughly. There are still too many people being treated in hospital for suspected adverse reactions due traditional medicines. Ethnopharmacology should strive not only to discover Western rationale in traditional healing and find therapeutic novelties in exotic medicines, but that it should also identify which traditional drug therapies do more harm than good. It is the duty of ethnopharmacologists to disseminate their findings beyond the privacy of their scientific domain. The providers and consumers of traditional health care need to be informed about ethnotoxicological findings. This process will not be easy, but it is the responsibility of researchers to contribute to the welfare of the nation and to discourage harmful traditional practices. Ethnotoxicology can only have an optimal impact on public health when we combine our toxicological findings with anthropological skills.[14]

As the traditional medicines industry generates more than R1-billion per annum, there must be control measures to limit unskilled persons from pretending to practice as traditional healers.[37] The healers must fall under a Council that "regulates and controls" all aspects of traditional medicinal practice especially the preparation of these medicines. If traditional medicines are to be accepted in primary health care, then information on the plant source(s), preparation, medicinal use, dosage, extraction procedure and storage of these medicines must be obtained.

For a real ecological crisis to be averted, traditional medicinal healers and other users need to take cognisance of the fact that the supplies of wild plant

species are finite and that, ultimately certain popular species may become extinct if harvesting is uncontrolled. Scientists, communities and traditional medicinal healers need to agree on ways for the crops and land to "recover" and a solution might be to cultivate certain plant species in nurseries or under controlled conditions.

The World Health Organisation guidelines for the conservation of medicinal plants focus on promoting : [39]

- Increasing ethnobotany surveys to document the extensive traditional knowledge that exists.
- The cultivation of indigenous medicinal plants to develop the resource base.
- The sustainable harvesting of medicinal plant populations.
- The more efficient harvesting, storage and production of indigenous plant products.
- The conservation of plants in developed and wild environments and communication of conservation issues.

It is encouraging to see that natural products such as feverfew (antimigraine remedies), yohimbine (male impotence remedies) and karela (Type 2 Diabetes remedies) have shown good clinical results recently, and they may still show us the way to therapeutic discoveries.[40] Although the number of discoveries of outstanding natural compounds are low when compared to synthetic drug programs, this may actually be related to the extent of funding that synthetic drug programs receive.

It should also be recognised that traditional medicines may have non-pharmacological benefits. It is well established that therapeutic dosage forms without an active may provide substantial relief in various diseases. This placebo effect is likely to be more powerful when administration takes place in a non-secular traditional setting which uses holistic approaches to treatments.

In order to protect our natural products from commercial exploitation both locally and abroad, the following code of practice is suggested: [38]

- Legislation should be enacted at national level to control the collection and

export of biological material in co-operation with relevant professional organizations.

- A strict code of ethics should be formulated to ensure that research recipients (traditional healers) and members of herbaria are fully informed of the objectives and the commercial aspects of the research.

- Equitable compensation should be given to individuals that are assisting.

- The relevant national organization should receive fair royalty payments.

- National requirements for plant collection must be observed.

- Maximum use of local expertise must be made within developing countries to extract and screen important compounds.

- It must be that any plant material collected for export and use outside the country has the full approval of the authorities, and that collection has been carried out with the co-operation of the host country and the local communities involved.

- It must be that collections comply with conservation and quarantine regulations of the host country and the destined country.

Better use of the research capabilities of the tertiary institutions of South Africa is required so that progress in the field of ethnopharmacology can be increased substantially. For any institution to develop a good ethnopharmacological unit, there is a need for vigorous methodology and a multidisciplinary approach

We, as South Africans, need to appreciate that traditional healers are assets to our great country as they possess vital knowledge needed for the study of ethnopharmacology. We need to urgently collect data on the uses of traditional medicines so that we can rescue our cultural heritage. We must investigate our plants thoroughly as to avoid foreign exploitation of our natural resources. There is always a possibility that novel plant compounds may be isolated. We need to become more protective and proud of our indigenous plants.

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Appendix A

Abstract: Oral presentation at the First International Conference on Pharmaceutical and Pharmacological Sciences (South Africa) September 22-25, 1996.

ACTIVITY-DIRECTED ISOLATION AND STRUCTURAL ELUCIDATION OF AN ANTIPSEUDOMONAL COMPOUND FROM *ARCTOTIS AURICULATA*

F Salie[a], W Mabusela[b], HMJ Leng[a], PFK Eagles[a]

[a] Department of Pharmaceutical Chemistry, University of the Western Cape

[b] Department of Chemistry, University of the Western Cape, Private Bag X17, Bellville

Infectious diseases represent one of the main causes of morbidity and mortality in developing countries, like South Africa. The indiscriminate use of antibiotics has also resulted in the emergence of a number of resistant bacterial strains.

Four plants within the Asteraceae family were screened for antimicrobial activity (Salie et al., 1996). The results showed that certain plants possessed significant antipseudomonal (AP) and antistaphylococcal activity.

An extract of *Arctotis auriculata* which had a significant AP activity was selected for further study. The active component of the above extract was determined using the bioautographic method. The active component was isolated using preparative thin layer and high pressure liquid chromatography. Its structure was elucidated using nuclear magnetic resonance spectroscopy, mass spectroscopy, infrared and ultra violet spectroscopy. The pure compound was then tested against *Pseudomonas aeruginosa* to determine its minimum inhibitory and minimum bactericidal activity.

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Salie, F., Eagles, P.F.K. and Leng,H.M.J.(1996) Preliminary antimicrobial screening of four South African Asteraceae species. *Journal of Ethnopharmacology* 52,27-33.

Appendix B

Paper: "Preliminary antimicrobial screening of four South African Asteraceae species."
Journal of Ethnopharmacology 52 (1996)

ELSEVIER

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Preliminary antimicrobial screening of four South African Asteraceae species

F. Salie, P.F.K. Eagles, H.M.J. Leng*

Department of Pharmaceutical Chemistry, University of the Western Cape, Private Bag X17, Bellville, 7535, South Africa

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Abstract

Organic and aqueous solvent extracts of *Arctotis auriculata* Jacq., *Eriocephalus africanus* L., *Felicia erigeroides* DC., and *Helichrysum crispum* (L.) D. Don, were investigated for selective antimicrobial activities. Organic extracts of *A. auriculata* and *H. crispum* inhibited the growth of *Mycobacterium smegmatis*. The same extracts, together with organic extracts of *F. erigeroides*, were active against *Pseudomonas aeruginosa*. Antifungal activities against *Candida albicans* were exhibited by organic extracts of *E. africanus*, *F. erigeroides*, and *H. crispum*. Organic extracts of *A. auriculata* and *E. africanus*, as well as the aqueous extract of the latter plant, were active against *Staphyllococcus aureus*.

Keywords: Antimicrobial; Fynbos; Asteraceae; Tuberculosis

1. Introduction

The flora of the Western Cape, known as Cape fynbos, forms part of the Cape Floral Kingdom which consists of 8550 species distributed over an area of 90 000 km^2 (Cowling et al., 1992). Many fynbos plants have long been used as herbal remedies by descendants of the original inhabitants, the Khoi and San people (De Selincourt, 1992). Other local communities have also displayed interest in traditional medicines due to, amongst others, the high cost of conventional medicines.

The indiscriminate use of antibiotics has resulted in the emergence of a number of resistant bacterial strains. In addition, the Western Cape is reported to have one of the highest incidences of tuberculosis in the world (Heap and Ramphele, 1991). This, coupled with the high cost of the newer and more effective antimicrobial drugs, makes the search for less expensive, alternative substances imperative. The fynbos, with its richness and diversity, represents an important potential source of new drugs, including drugs for the treatment of infectious diseases.

The Asteraceae is the largest family in the fynbos biome (Cowling et al., 1992). Most traditional medicines used by the indigenous communities are in fact derived from plants belonging to this family

* Corresponding author.

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S. aureus was maintained on Trypticase soy agar, *P. aeruginosa* on Bacto nutrient agar, *M. smegmatis* on Bacto Mycobacteria 7H11 agar supplemented with Middlebrook OADC enrichment, and *C. albicans* on Sabouraud dextrose agar. All media were purchased from Difco (Detroit, MI, USA).

2.4.2. Preliminary screening for antimicrobial activities. The dried plant extracts were dissolved in 10% aqueous dimethylsulfoxide (DMSO) to a final concentration of 10 mg/ml and sterilised by filtration through a 0.45 μm membrane filter (MSI, Westboro, MA). Sterile 9 mm discs (Schleicher and Schuell) were impregnated with 50 μl of extract and placed on the surface of agar plates inoculated with a microbial culture. Each extract was tested in quadruplicate. Control discs contained 50 μl of sterile 10% aqueous DMSO. Ciprofloxacin (40 μg/disc) served as positive control for *S. aureus*, *P. aeruginosa* and *M. smegmatis*, whereas amphotericin B (25 μg/disc) was the control for *C. albicans*.

Agar plates containing bacteria and mycobacteria were incubated at 37°C for 24 and 48 h, respectively, whereas plates inoculated with *C. albicans* were incubated at 30°C for 48 h. After incubation, inhibition zones were recorded as the difference in diameter between the discs and the growth-free zones around the discs.

2.4.3. Minimum inhibitory concentration. The liquid dilution method of Rois et al. (1988) was used. A stock solution (40 mg/ml) of each extract was prepared in 10% DMSO. An aliquot of this solution was serially diluted (two-fold) with nutrient broth to obtain a concentration range of 10–0.0195 mg/ml. To each dilution was added 50 μl of a microbial culture with an optical density of 0.4 (log-phase) at 620 nm (Beckman DU-40 spectrophotometer). After careful mixing, the test preparations were incubated at 37°C (bacteria and mycobacterium) or at 30°C (fungus). Each extract was assayed in triplicate. Controls were prepared in the same manner, except that 50 μl of broth was added in place of the microbial culture.

The minimum inhibitory concentration (MIC) of each extract was determined by measuring the absorbance of the preparations at 620 nm with a multiplate reader (Multiskan, MCC 340, Labsystems, Finland) against the corresponding control. The lowest concentration which gave a zero absorbance reading (i.e., no turbidity) was taken as the MIC of the test extract.

The minimum bactericidal, mycobactericidal and fungicidal concentrations (MBC, MMC and MFC) were determined by adding 50 μl aliquots of the preparations which did not show any growth after incubation during the MIC assays, to 200 μl of broth. These preparations were incubated at 37°C for 24 h (bacteria) and 48 h (mycobacterium). The fungal test preparations were incubated at

Table 1
Phytochemical composition[a] of plants investigated

Plant (voucher specimens)	Plant organ[b]	ALK	FLA	GLUC	QUIN	SAP	STER	TAN
Arctotis auriculata Jacq.	LF	+	+	+	–	–	–	+
(Salie 1001)	ST	–	+	–	–	–	–	+
	RT	–	–	–	–	–	–	+
Eriocephalus africanus L.	LF	–	–	–	–	–	–	+
(Salie 1002)	ST	–	+	–	–	–	+	+
	RT	–	–	–	–	–	–	+
Felicia erigeroides DC.	LF	–	+	–	–	+	–	+
(Salie 1003)	ST	–	+	–	–	–	+	–
	RT	–	–	–	–	+	–	–
Helichrysum crispum	LF	–	–	–	–	+	–	+
(L.)D. Don (Salie 1004)	ST	–	–	+	–	–	–	+
	RT	–	–	–	–	–	–	+

[a]ALK, alkaloids; FLA, flavonoids; GLUC, cyanogenic glucosides; QUIN, quinones; SAP, saponins; STER, triterpene steroids; TAN, tannins.
[b]LF, leaves; ST, stems; RT, roots.

Table 2 (*Continued*)

Organ	Extract[b]	Yield (%w/w)	*S. aur*[c]	*P. aer*	*M. smeg*	*C. alb*
Stems	PE	0.12	1+	0	0	0
	Ch	0.04	0	0	0	0
	Et	0.54	0	0	0	0
	Me	0.47	0	0	0	0
	Wt	3.30	0	0	0	0
Roots	PE	0.23	0	0	0	0
	Ch	0.39	0	0	0	0
	Et	1.36	0	0	0	0
	Me	0.73	1+	0	0	0
	Wt	2.34	0	0	0	0
Felicia erigeroides						
Leaves	PE	0.31	0	0	0	0
	Ch	0.10	0	1+	0	0
	Et	0.01	0	0	0	0
	Me	1.03	0	1+	0	1+
	Wt	2.72	0	0	0	0
Stems	PE	0.16	0	1+	0	0
	Ch	0.06	0	0	0	0
	Et	0.17	0	0	0	1+
	Me	0.45	0	0	0	0
	Wt	1.22	0	0	0	0
Roots	PE	0.21	0	0	0	0
	Ch	0.13	0	1+	0	0
	Et	0.11	0	1+	0	2+
	Me	1.65	0	0	0	1+
	Wt	1.47	0	0	0	0
Helichrysum crispum						
Leaves	PE	1.33	0	3+	1+	0
	Ch	0.26	0	2+	0	0
	Et	0.08	0	1+	0	0
	Me	0.30	0	0	0	0
	Wt	2.33	0	0	0	0
Stems	PE	0.28	0	2+	0	0
	Ch	0.02	0	1+	0	1+
	Et	0.02	0	1+	0	0
	Me	0.17	0	0	0	0
	Wt	2.39	0	0	1+	0
Roots	PE	0.61	0	1+	0	0
	Ch	0.09	0	1+	0	0
	Et	0.05	0	1+	0	0
	Me	0.72	0	0	0	0
	Wt	2.87	0	0	0	0
Cipro[d]			5+	5+	5+	–
Ampho			–	–	–	3+
DMSO			0	0	0	0

[a]*S. aur, Staphyllococcus aureus*; *P. aer, Pseudomonas aeruginosa*; *M. smeg, Mycobacterium smegmatis*; *C. alb, Candida albicans.*
[b]PE, petroleum ether; Ch, chloroform; Et, ethanol; Me, methanol; Wt, water.
[c]0: no inhibition; 1+: 0–3 mm; 2+: 3–6 mm; 3+: 6–9 mm; 4+: 9–15 mm; 5+: >15 mm.
[d]Cipro, ciprofloxacin; Ampho, amphotericin B; DMSO, dimethylsulfoxide.