An assessment of three *Carpobrotus* species extracts as potential antimicrobial agents.

By

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A thesis in partial fulfillment of the requirements for the degree of Magister Scientiae (Pharmaceutical Sciences) in the in School of Pharmacy, Faculty of Sciences, at the University of the Western Cape.

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ABSTRACT

An assessment of three *Carpobrotus* species extracts as potential antimicrobial agents.

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For centuries, indigenous people in South Africa have used a variety of medicinal herbs to treat chronic infections. This investigation focused on three *Carpobrotus* species, in an attempt to assess their potential antimicrobial activity.

Extracts of varying polarities of the plants were prepared and tested against *Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans* and *Mycobacterium smegmatis*. For the disc diffusion method Ciprofloxacin (40μg/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*. A sample concentration of 10mg/ml was used. Minimum inhibitory concentrations (MIC) were determined by two-fold serial dilution. Phytochemical analysis was completed to test for the presence of flavanoids, hydrolysable tannins, phytosterols and aromatic acids. The ethyl acetate extracts {21μl of 95mg/ml} were used for bio-autography, together with TLC analyses and HPLC fingerprinting.

Carpobrotus mellei, Carpobrotus muirrii and *Carpobrotus quidrifidus* showed antimicrobial activity against *S. aureus* and *M. smegmatis* in the disc diffusion method and inhibition against *S. aureus* and *M.smegmatis* was observed by clear zones on the TLC plate. HPLC fingerprinting of the three species showed similarities with common peaks detected at 366 nm, and providing a phytochemical map of potentially important natural bioactives.

This investigation confirms that extracts of the three *Carpobrotus* species that are used as indigenous medicines, exhibits anti-bacterial activity. This scientific information can serve as an important platform, for the development of inexpensive, safe and effective natural anti-infective therapeutics.

STATEMENT

I declare that "An assessment of three *Carpobrotus* species extracts as potential antimicrobial agents" is my own work, that it has not been submitted for any degree or examination in any other University, and that all the sources I have used or quoted have been indicated and acknowledged by means of complete references.

Evan Springfield May, 2001

Signed:

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CHAPTER 1

Introduction

Indigenous knowledge exists in the community of older folks who know about the identification and uses of many medicinal plants. In South Africa, as in other parts of the world, the search for new drugs is underway and many aspects of traditional plants are being explored. While the uses of traditional medicine versus western medicine seem to rival each other generally, poverty is the deciding factor pertaining to the choice of medicine amongst those in the rural communities. However, the lack of knowledge in the rural communities creates the possibility that the wrong species may inadvertently be collected (if the collector is inexperienced) or may intentionally be substituted for the correct species, if the latter is scarce, expensive or difficult to find. Collection of the wrong plant species, including substitutions, leads to incorrect prescriptions of medicine; the result may be an ineffective treatment or harm to the patient if the substitute is toxic.

Correct identification of plant species is therefore an essential component of the quality assurance of herbal medicines, which is inextricably bound up, with issues of safety and efficacy. Except with licensed products, there is no guarantee that the herbal material used in preparation of the product was of good quality or correctly identified.

Different species of the genus *Carpobrotus* are used as traditional medicine. Correct identification of species within the genus *Carpobrotus* must be done by a

taxonomist because of their similar appearance. However, it is believed that these plants are being used interchangeably and substituted by one another in local communities. These plant species occur in abundance among communities and are widely distributed from the coastal towns to the inlands, of which the most popular species is *C. edulis*. Solvent extracts of *C. edulis* have been tested against various micro-organisms and found to exhibit antimicrobial activity.

The overall objective of this study was to evaluate the solvent extracts of *C. mellei, C. muirii* and *C. quadrifidus* as potential antimicrobial agents. The specific aim was to compile scientific information about these species in order to determine whether they can be substituted for or used interchangeably among one another. The scientific information includes antimicrobial investigation of the species as possible therapeutic agents and a detailed chemical profile to illustrate the major chemical class of components, which are present in all three species. To realize these objectives, various solvent extraction procedures were used to suit the appropriate antimicrobial technique.

CHAPTER 2

LITERATURE BACKGROUND

2.1. Antibiotics and Microorganisms

An antibiotic is any substance produced by a microorganism, which may harm or kill another microorganism (Rang et al., 1999). The first antibiotic, penicillin, was discovered in 1929 by Sir Alexander Fleming who observed the inhibition of staphylococci on a plate contaminated by a penicillin mould. By the mid 1940's, antibiotics were available for treatment against many bacterial infections including throat, pneumonia, skin, wound, scarlet fever, toxic shock syndrome and others. By the early 1950's, the discovery and introduction of streptomycin, tetracycline and other antibiotics led to the effective treatment of a vast array of formerly life-threatening infections, illnesses and diseases. Antibiotics are only effective in the treatment of bacterial infections. They have absolutely zero impact on viral infections (Medicapharma, 2000).

Despite the wide availability of clinically useful antibiotics and semi synthetic analogues, there remains a need for continual research for new anti-infective agents. Some of the major antibiotics, however, have indeed considerable drawbacks in terms of limited antimicrobial spectrum or serious side effects. Moreover, the combination of the genetic versatility of microbes and widespread overuse of antibiotics has led to increasing clinical resistance of previously sensitive microorganisms and the emergence of previously uncommon infections.

The discovery of new molecules exhibiting prominent activities against infectious pathogens such as toxogenic staphylococci, anaerobes, *Pseudomonas*, *Legionellae*, various fungi and others, showing no cross-resistance with the existing antibiotics, would therefore be essential (Vanden Berghe, 1991).

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. One source of potential medicines is the vast array of plants with medicinal properties, most of which have yet to be discovered (Schnitzler et al., 1996). This is also a valuable resource for diseases, which do not already have an effective treatment, as proofed in the study by Schitzler et al (1996).

When it comes to selecting microorganisms for antibiotic screening, one is faced with a vast amount of options. In order to simplify the selection, factors such as, laboratory conditions and the availability of the microorganisms should be considered. The gram-positive bacterium, *Staphylococcus aureus*, the gram-negative bacteria *Pseudomonas aeruginosa*, *Mycobacterium smegmatis* and the fungus, *Candida albicans*, are easily accessible and were used by Salie et al. (1996) in their study. *M.smegmatis* is closely related to the pathogen *Mycobacterium tuberculosis*, that is the causative organism of tuberculosis, but less virulent (Sneader., 1986).

S. aureus is pyogenic, an opportunistic pathogen and responsible for a range of infections including severe sepsis, pneumonia, endocarditis and soft tissue

infections. *P. aeruginosa,* normally a soil bacterium, is an opportunistic pathogen of humans who are immunocompromised. It can infect the wounds of victims with severe burns, causing the formation of blue pus. *C. albicans* is a dimorphic fungus that is an opportunistic pathogen of humans and a common aetiological agent for candidiasis and thrush. This species is found as part of the normal gastrointestinal flora (On-line Medical dictionary, 2002).

2.2. Medicinal plants

Scientists generally agree that the use of medicinal plants dates back to pre-historical times. However, the precise origins and patterns of use are disputed. In man's quest for food during the early nomadic period of his existence he would most certainly encounter some plants that were poisonous, others that would serve as adequate foods, and still others that produced bizarre and unusual effects by altering his consciousness. Among this latter group were those that would simultaneously relieve pain and counteract disease (Emboden, 1997).

The necessity to help sick people goes back to the origin of mankind. In many places of the world special ways of medical treatment were found. The experiences were passed on from generation to generation resulting in a scientific approach to a philosophical system and indeed 'Traditional Medicine' or 'Ethnopharmacology' has become an important area of enquiry. These considerations may contribute to the ongoing discussions between traditional and modern medicine (Vogel, 1991).

As forest ecosystems are damaged, traditional food- and medicinal supplies are disrupted, leading to a reduction in the nutritional status of forest people.

Historically, public health and medical projects have not worked closely with ethnobotany and forest conservation projects, even though an integration of these different disciplines can be mutually beneficial (King et al., 1996).

Despite the use of herbal medicines over many centuries, only a relatively small number of plant species has been scientifically studied for possible medical applications. Safety and efficacy data are available for an even smaller number of plants, their extracts and active ingredients and preparations containing them. The World Health Assembly (WHA) has adopted a number of resolutions drawing attention to the fact that a large section of the population in many developing countries still relies on traditional plant medicine, and that the work force represented by traditional medicine practioners is a potentially important resource for primary health care. The policy of the World Health Organization (WHO), regarding traditional plant medicine, conforms to the relevant WHA resolutions; to promote the rational use of traditional medicine through the development of technical guidelines and international standards in the field of herbal medicine (WHO, 1998).

As most herbal medicines still need to be studied scientifically, it is expected that the WHO identify safe and effective herbal medicines for use in a national health care system. A large number of South Africans consult traditional healers, mostly

in addition to medical practitioners. There is a large medicinal plant market in the country and indigenous herbal medicines are in the main materia medica.

The trade in crude indigenous herbal products is completely unregulated and if a health-related claim is made for a finished product, it has to go through the full drug evaluation procedure set by the Medicines Control Counsil (MCC) before marketing (WHO, 1998).

The general requirement for new drugs worldwide is quality, safety and efficacy. As far as efficacy is concerned, herbal drugs have to be compared with a synthetic drug as standard. However, there is no efficient therapy with synthetic drugs against many diseases, whereas experience gathered over centuries suggests that herbal drugs may be effective (Vogel, 1991). This is especially true for chronic diseases where there are no appropriate pharmacological models. Unfortunately, a few synthetic drugs have caused unexpected side effects, like foetal malformations by thalidomide. Therefore the requirements for development of a synthetic drug are rather stringent. Compared with modern medicine, traditional medicine has specific shortcomings, that includes not having double-blind studies or even well described case records. Therefore, at present, it is common that a traditional medicine may only be accepted in one cultural area (Vogel, 1991), and therefore comprehensive scientific assessment is critical for overall acceptance.

2.3. The *Carpobrotus* genus

The family Aizoaceae is well known in South Africa from the names Mesembryanthemum in English, "vygies" in Afrikaans and "iqina" in Xhosa

(Watt and Breyer-Brandwijk, 1962). Hermann made the first mention of a plant identifiable as a species of Carpobrotus in 1687 (Wisura et al., 1993). Knowledge of the genus spread more slowly than the plants themselves. A revision of the Southern African species of *Carpobrotus* was done by Wisura et al. to help with the identification of field-gathered specimens (Wisura et al., 1993).

2.3.1. Taxonomic character

The genus *Carpobrotus* is immediately recognizable both in the field and in the Herbarium by its trailing habitat and long, robust internodes. Plants in cultivation or in disturbed habitats form dense mats, but in natural conditions they tend to trail between other vegetation. All species of the genus are similar in habitat and this character is therefore of no significance at the specific level. The flowers of *Carpobrotus* are among the largest in the Mesembryanthema and are 50-120mm in diameter. This may be used as an accessory character both in distinguishing between species and in separating this genus from others in the family. In all species in the genus, the flowers appear to be solitary; this is not uncommon in other genera of the family with large flowers (Wisura et al., 1993).

2.3.2. Uses and preparation

The fleshy fruits of *Carpobrotus* are edible. Fruits of the species are allowed to dry on the plants, and then picked, of which the inside is eaten. Dried fruits are also soaked in the preparation of a traditional Cape recipe for making a jam or to be preserved (Wisura et al., 1993; Watt and Breyer-Brandwijk., 1962).

C. edulis is grown widely as a sand-binder. This ability to stabilize, combined with its edible fruits and its tolerance of a wide range of soils and climates, has led to naturalization in the inland parts of Southern Africa, and in Australia, Britain and California (Wisura et al., 1993). In indigenous medicine, *C.edulis* and *C.acinaciformis* are the most commonly used herbs from the genus *Carpobrotus*, of which a preparation are gargled to treat infections of the mouth and throat. They are also said to be effective against toothache, earache, and oral and vaginal thrush. The leaf juice which is an astringent and contains malic acid, citric acid and their calcium salts, was successfully used in the treatment of severe infantile eczema (van Wyk et al., 1997; Watt and Breyer-Brandwijk., 1962). The boiled fruit of *C. acinaciforme* L. Bol. is used in the treatment of Pulmonary Tuberculosis and other internal chest conditions. The fruits are also used for heart conditions (van Wyk et al., 1997). The widespread use of *Carpobrotus* species as anti-infective medicine compels us to further explore the potential of other understudied candidates. The current investigation evaluated the solvent extracts of *C. mellei, C. muirrii and C. quadrifidus* as potential antimicrobial candidates and to validate whether they can be used interchangeably.

2.3.3. Active principles

The genus contains malic and citric acids and their calcium salts (Watt and Breyer-Brandwijk, 1962). In a recent publication by Van der Watt et al. (2001), six active compounds were purified and identified as flavonoids. The flavonoids are rutin, neohesperidin, hyperoside, catechin and ferulic acid. The sixth compound, also a flavanoid, is still under investigation. The flavonoids showed

activity against *Bacillus subtilis*(+), *Staphylococcus epidermis*(+), *Staphylococcus aureus*(+), *Streptococcus pneumoniae*(+), *Moraxella cattharalis*(-) and *Pseudomonas auruginosa*(-) (Van der Watt et al., 2001).

2.4. New bioassay methods

It is widely accepted that the use of folk or traditional medicinal uses of plants indicate the presence of biologically active constituent(s) in a plant. The prolonged and continued use of a particular plant in an indigenous culture to treat certain disease conditions should provide a basis of efficacy. Collecting plants with such uses for drug testing is believed to increase the chances of discovering new medicines. In other words, the use of traditional medical plants represent 'leads' that could shortcut the discovery process in modern medicine (Soejarto, 1996).

The mode of action assays, together with *in vitro* anti-bacterial, anti-fungal, anti-viral and anti-parasitic assays have made it economically feasible to screen large numbers of substances in a wide range of assays, and in a relatively short time. New assays that can selectively detect biologically active molecules at very low levels, and chemical techniques that allow for the isolation and identification of minute amounts of active compounds, have together facilitated the current increase in the interest in natural products, while they have also resulted in the rise of other kinds of screening. These kinds of screening have the inherent advantage of explicitly knowing in advance which compounds are being evaluated in the assays, allowing the chemists to proceed rapidly with any lead that may be discovered (Borris, 1996).

The research (screening) done on medicinal plants serves only as a stepping-stone, from natural products as food supplements to natural products as templates for new drug discovery. Natural product source materials can and do provide valuable template molecules to aid in the discovery of new drugs, together with other emerging technologies, such as combinatorial libraries, whose objective is to create reproducible and defined chemical diversity (Turner, 1996).

2.5. Antimicrobial screening methods from higher plants.

Antimicrobial activity of plants can be detected by observing the growth response of various microorganisms to those plant tissues or extracts which are placed in contact with them. Many methods for detecting such activity are available, but since they are not equally sensitive or even based upon the same principle, the results obtained will also be profoundly influenced not only by the method selected, but also by the microorganisms used to conduct the test (Vanden Berghe, 1991).

2.5.1. Principle diffusion methods

A technique, which does require homogeneous dispersion in water is the agar – overlay method using a disk, hole or cylinder as reservoir. The reservoir containing the sample to be tested is brought into contact with an inoculated medium and after incubation, the diameter of the clear zone around the reservoir (inhibition diameter) is measured. This method was originally designed to monitor the amount of antimicrobial substances in crude extracts (Rios et al., 1988).

2.5.2. Dilution methods

Dilution techniques are those which require a homogeneous dispersion of the sample in water. They are used to determine, principally, the minimum inhibitory concentration (MIC) values of an extract, essential oil or pure substance. They can be used in the preliminary screening of antimicrobial activity (Rios et al., 1988).

2.5.3. Bioautographic methods

Bioautography permits the facile localization of an activity even in complex mixtures. The main feature of the direct assay is the elimination of the diffusion step. Well-defined zones of inhibition observed directly on the TLC plate improve the localization of the activity and enhance the sensitivity of the assay (Hamburger et al., 1987). Comparison with a chromatogram developed under identical conditions and visualized with an appropriate chromogenic reagent may provide extremely useful information about the chemical nature of the active principles (Rios et al., 1988).

The principle of the assay is the following: a suspension of a microorganism in a suitable broth is applied to a developed TLC plate. Incubation in a humid atmosphere permits growth of the bacteria. Zones of inhibition are then visualized by a dehydrogenase- activity- detecting reagent, e.g., a tetrazolium salt. Metabolically active bacteria convert the tetrazolium salt into the corresponding intensely coloured background. Thus, anti-bacterial compounds appear as clear spots against a colored background (Hamburger et al., 1987).

2.5.4. Microplate assay in the presence of redox indicator

This is a micro-dilution technique using 96-well microplates and tetrazolium salts to indicate bacterial growth. Tetrazolium salts are frequently used to indicate biological activity because the colourless compound acts as an electron acceptor and is reduced to a colored product by biologically active organisms. This method is robust, is not expensive, gives reproducible results, is about 30 times more sensitive than other methods used in the literature, requires a small quantity of sample, used for large numbers of samples, and requires little time (Eloff, 1998a).

Figure 1. *Carpobrotus mellei* (van Wyk et al., 1997)

Figure 2. *Carpobrotus quadrifidus* (van Wyk et al., 1997)

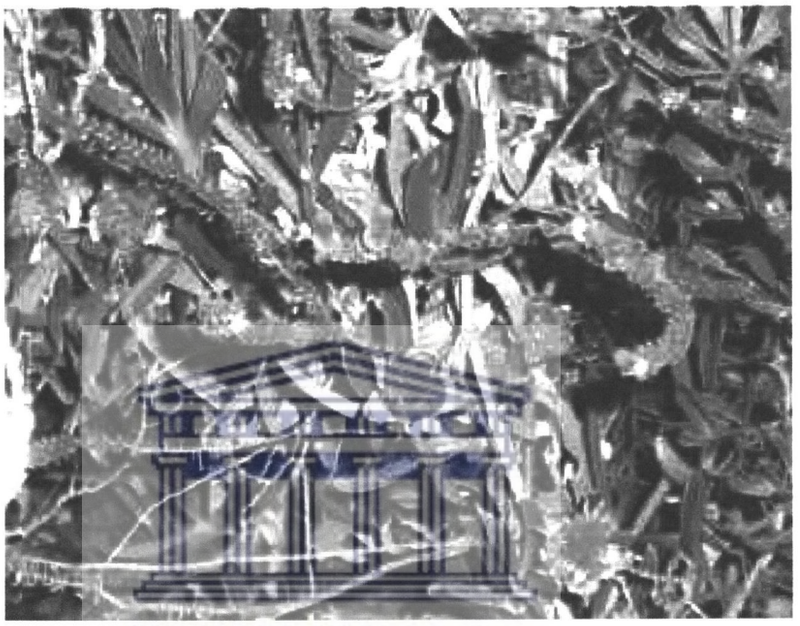

Figure 3. *Carpobrotus muirii* (author)

CHAPTER 3

MATERIALS AND METHODS

3.1.1.*C. mellei*

C. mellei has relatively small flowers and differs from all the other species by the extremely long stigmas overtopping the stamens. The leaves of *C.mellei* spread to erect, green or bluish green, sometimes with red margins and subacinaciform or almost straight, rather abruptly curving towards the apex and ending in a short reddish mucro. This species is restricted to the mountains of the south-Western Cape (Wisura et al., 1993).

For this study *C. mellei* was collected, on the 3rd of April 2001, in the Karoo area towards the Montagu baths next to the road in a soil substrate with a clay soil type. The specific location of the plant was captured with a GPS with grid reference: 33° 40' 15 S and 20° 07' 52 E at an altitude of I'500'. The habitat condition of *C. mellei* is best described as having a shale lithology, full sun exposer with the plant facing east and being flat on the ground. No flowers were present at the time of collection and the leaves had a bluish green colour.

3.1.2. *C. quadrifidus*

C. quadrifidus is the most robust species with the largest flowers (120-150mm diam.), largest leaves (15-20mm thick), and largest number of loculi. Its flowers are purplish, pale pink or white and the leaves being straight and often mucronate, rarely scimitar-shaped. This species occurs in the Western Cape coast at low altitude (Wisura et al., 1993).

C. quadrifidus was collected, on the 24th March 2001, along the Westcoast at SAS Saldanha with a grid reference, read with a GPS at 33°01'27 S and 17°56'57E. The plant vegetation type is "strandveld plantegroei". The plant was found on a semi disturbed beach dune in a well-drained sandy soil type. The collected plant had full sun exposure on a moderate slope, facing northwest.

3.1.3. *C. muirii*

C. muirii differs from other purple-flowered species in having small flowers with narrow leaves (5-6mm thick), and having the center of the ovary top depressed. The stigmas are shorter than stamens. This species is restricted to small parts of the southern Cape coast (Wisura et al, 1993).

C. muirii was collected, on the 11th May 2001, in the Bredasdorp area (behind the lime factory) with Grid ref: 34°30'39 S and 20°04'48E. The vegetation type is lime stone fynbos with a stony substrate. The sand is well drained. The collection was done against a gentle slope, in the shade among bushes, facing northwest.

Authentication of all the plant species was done by the curator of the University of the Western Cape Herbarium, Mr. Frans Weitz. Voucher specimens (Springfield001 for *C. quadrifidus*, Springfield002 for *C. muirii* and

Springfield003 for *C. mellei*) were prepared and deposited in the UWC Herbarium. The leaves were cut into smaller pieces and dried at 40°C in an oven for five days. The dried material was milled, to shorten the extraction period and passed through a 850 microns sieve (Eloff,1998b).

3.2. Solvent extraction

Several extraction methods were considered, which included not only well-known techniques associated with major phytochemical groups such as flavonoids, but also other approaches, based on solubilities in water and various solvent combinations (to determine hydrophilicity or lipophilicity), and on molecular weights, polarity, and degree of ionizability.

The milled plant material (100g) was shaken in 200ml of acetone: water (4:1) and further extracted according to Latté (1999) to obtain four fractions of different polarities. The flask was capped with foil and shook at room temperature for 6 hours at 120 RPM and allowed to stand overnight for a further 18 hours.

After the 24hours, the solution was centrifuged (5minutes at 1000RPM) and the supernatant was transferred into a separate covered flask and the residue was returned to the original flask. The remained residue in the original flask was extracted with a further two 200ml portions of acetone:water (4:1) mixture, repeating the shaking/standing procedure with each addition of solvent. The three +-200ml acetone:water extracts was combined and reduced to a 100ml volume on a Buchi rotary evaporator <40 °C. At this stage an aqueous extract is left. This method is designed to extract both hydrophilic and lipophilic compounds.

The aqueous extract is transferred to a separating funnel and extracted with 3 x 50ml aliquots of Petroleum Ether (BP 40-60 °C). The three aliquots are combined and reduced to-dryness on a Buchi rotary evaporator <40 °C.

The above step, treatment of the sample solution with Petroleum Ether, was also repeated with chloroform and then ethyl acetate. The dried organic residues were stored in a dessicator. The remaining aqueous solution was freeze-dried and the dried residue stored in the dessicator.

The extracts, from lowest to highest polarity, were Petrol ether (PE), Chloroform (CF), Ethyl acetate (EA) and water (WT). Each extract was evaporated to dryness on a Buchi Rotavapor (Labortechnik, Switzerland). A part of the ethyl acetate and water fractions respectively, were re-dissolved in 80% ethanol and the tannins were removed according to Van der Watt et al (2001) and Porter (1989).

3.3. Phytochemical analysis

Phytochemical analysis was carried out according to the methods of Latté (1999).

3.3.1. Detection of coumarins

A 50ul aliquot of the aqueous extract and ethyl acetate extract was applied at the base of two 5x10 cm TLC plates pre-coated with Kieselgel 60 F254 (Merck). The solvent system was ethyl acetate:water(19:1 V/V). Detection was done under fluorescence in UV light at 365nm wavelength. Coumarins, if present, are visible in UV light as blue to violet fluorescence bands at fairly high R-f values.

3.3.2. Detection of flavanoids

Procedure as for coumarins. Flavonoids, if present (ethyl acetate and n-butanol fractions), are visible in daylight on the finished, dried chromatograms as yellow bands, the colour of which is enhanced by spraying with a 1% methanolic solution of 2-aminoethyl diphenylborinate (Naturstoffreagenz A), followed by a 5% solution of polyethylene glycol 400 in methanol. Depending on the state of oxidation of the flavonoid B-ring, yellow, orange or peach colours are produced. Quercetin was used as marker.

3.3.3. Detection of hydrolysable tannins

Procedure as for coumarins and flavonoids. Hydrolysable tannins comprise esters of gallic and ellagic acid, and give characteristic blue-black or green-brown colours on finished chromatograms sprayed with a 10% solution of iron-III-chloride (FeCl3) in methanol (general reagent for phenols). Gallotannins can be distinguished on unsprayed chromatograms by their violet fluorescence in UV light or by the rose-pink colour obtained after spraying the finished chromatogram with saturated aqueous solution of potassium iodate. Ellagitannins can be detected by spraying chromatograms with an ice-cold 10% NaNO2 aqueous solution to which ice-cold 6% aqueous solution of acetic acid has been added immediately before use; a carmine red colour is obtained which changes after about 10 minutes to indigo blue. Markers: gallic acid, tannic acid.

3.3.4. Detection of condensed tannins

These colourless compounds usually remain in the water extract and can be visualized by spraying the finished chromatogram, developed in the same solvent system as used for coumarins, with 5% ethanolic hydrochloric acid. Oxidation of the leucoanthocyanindin to the corresponding anthocyanin gives a purple, red or orange colour. Marker: cyanidin.

3.3.5. Detection of alkaloids

The chloroform, ethyl acetate and water fractions were all examined for the presence of alkaloids by spraying the finished chromatograms, developed in acetone: water: ammonia (90:7:3) with Dragendorff's reagent. Alkaloids give an orange colour with this reagent. Marker: quinine.

3.3.6. Detection of phytosterols

The petrol ether and chloroform extracts were tested for phytosterols, separable on silica gel 60 F254 layers using toluene: acetone (4:1 V/V) as developing solvent. The finished chromatograms were sprayed with anisaldehyde/sulphuric acid reagent, followed by heating in an oven at 105 degrees for 5-10minutes. Marker: B-sitosterol.

3.3.7. Detection of aromatic acids

The ethyl acetate and water fractions were examined. Inspection of the finished chromatograms in UV light of both long and short wavelength should reveal aromatic acids as blue or green fluorescent bands. Spraying with 10%FeCl3 in

methanol gives a blue colour with compounds having three vicinal hydroxy groups, green or grey with compounds having two vicinal hydroxy groups and yellow with compounds having a single phenolic hydroxyl group. Markers: caffeic acid, p-hydroxy benzoic acid.

3.4. Antimicrobial screening

3.4.1. ***Microorganisms and growth media.*** The microorganisms used were *S. aureus* (ATCC 29213), *P. aeruginosa* (ATCC 27853), *C. albicans* (ATCC 10231) and *M. smegmatis*. *M. smegmatis* was a gift from Professor Paul van Helden of the Department of Biochemistry and Physiology at the University of Stellenbosch Medical School. The *S.aureus*, *P.aeruginosa* and *C.albicans* were obtained from the Medical Biosciences Department at the University of the Western Cape.

3.4.2. ***Preliminary screening for antimicrobial activities.*** The PE, CF, EA and WT extracts were dissolved in 100% methanol (MeOH) to a final concentration of 10mg/ml. All the solutions were sterilized by filtration through a 0.45 μm membrane filter (MSI, Westboro, MA). Sterile 9 mm discs were impregnated with 50μl of extract and placed on the surface of agar plates inoculated with a microbial culture. Each extract was tested in triplicate. Ciprofloxacin (40μg/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 the fungi, bacteria and mycobacteria were incubated at 37°C for 24 and 48 h, respectively (Salie et al., 1996). After incubation, inhibition zones were recorded as the diameter of the growth free zones around the disc.

For direct bioassay on thin layer chromatography (TLC) plates, 21µl EA extract (95mg/ml) was applied to 5x 20cm silica F254 (Merck) glass plate. Best separations were achieved with ethyl acetate: methanol: water (100:13,5:10) according to Van der Watt et al. (2001). The developed TLC plates were dried overnight and agar, which was inoculated with *S. aureus*, and *M. smegmatis* respectively, was poured over the plates. Agar plates containing the bacteria and mycobacteria were incubated at 24 and 48 h, respectively. After incubation, the plates were sprayed with a 2mg/ml solution of *p*-iodonitrotetrazolium violet (Sigma chemicals co.). Clear zones on the chromatogram indicated inhibition of growth (Beugue et al., 1972).

3.4.3. Minimum inhibitory concentration.

A stock solution of 100 mg/ml of each of the tannin-free ethyl acetate and aqueous residues was prepared in distilled water. Minimum inhibitory concentration (MIC) was determined by two-fold serial dilution of extracts beyond the concentration where no inhibition of growth of *Staphylococcus aureus* and *Mycobacterium smegmatis* was observed. The serial dilution technique using 96-well microplates method of Eloff (1998a) was used. The test solution (50-100µl) was serially diluted to 50% with water and 50 - 100µl of a 3-hour-old culture of one of *Staphylococcus aureus* or *Mycobacterium smegmatis* grown at 37°C in Muller-Hinton broth (Merck chemicals) were added to each well. The covered microplates were incubated overnight for *S. aureus* and 48 hours for *M. smegmatis*.

To test for bacterial growth, 40µl of *p*-iodonitrotetrazolium violet (INT) or –2,5-diphenyltetrazolium bromide (MTT) (Sigma chemical co.) dissolved in water are added to the microplate wells and incubated for a further 10-30 minutes. The bacterial suspension changes to red (INT) or blue (MTT) where bacterial growth occurs several times (Eloff, 1998a).

3.5. High performance liquid chromatography (HPLC)

The chromatographic system used was a Beckman HPLC system consisting of a double pump Programmable Solvent Module 126, Diode Array detector Module model 168, with 32 Karat Gold software supplied by Beckman; Column C18 Bondapak 5 µm and dimensions (250 x 4.6 mm). The chromatographic conditions were as follows: Mobile phase, solvent A: methanol (MeOH); solvent B: 5% acetic acid (CH_3COOH); Mode: gradient, increasing the organic phase (MeOH) from 20% to 90% over 18minutes; flow rate: 1ml/min; reference standard: Rutin (2.5 g dissolved in 10ml MeOH); Sample size: EA extract used in the direct bioassay (95mg/ml); Injected volume: 5 µl. The run time was 25 min.

CHAPTER 4

RESULTS

4.1. Phytochemical analysis.

Table1. Phytochemical composition[a] of plants investigated

Plant	Coum.	Flav.	H.Tan.	C.Tan.	Alk.	P.sterols	A.Acids
C.mellei	-	+	+	-	-	+	+
C.muirii	-	+	+	-	-	+	+
C.quid.-	+	+	-	-	+	+	

[a]Coum, coumarins; Flav, flavonoids; H.Tan, hydrolysable tannins; C.Tan, condensed tannins; Alk, alkaloids; P.sterols, phytosterols; A.Acids, aromatic acids.

All of the plants tested positive for flavanoids, hydrolysable tannins, phytosterols and aromatic acids.

4.2. Antimicrobial activity

4.2.1. Disc diffusion method

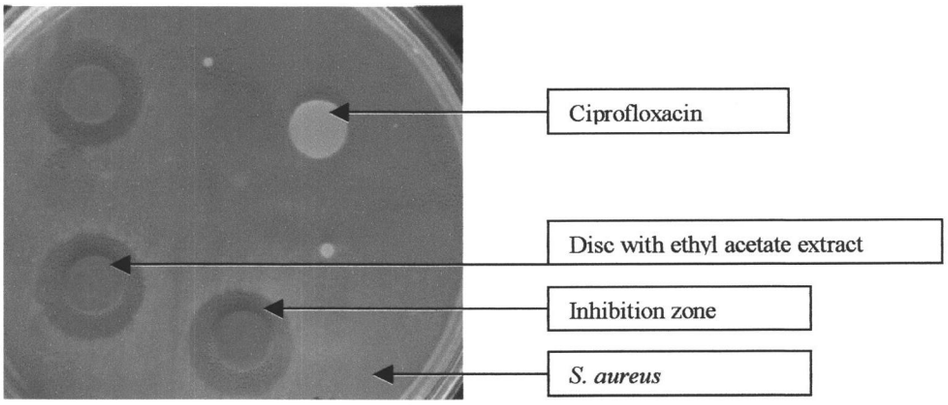

Figure 4. The growth inhibition activity of an Ethyl acetate extract against *Staphyloccus aureus.*

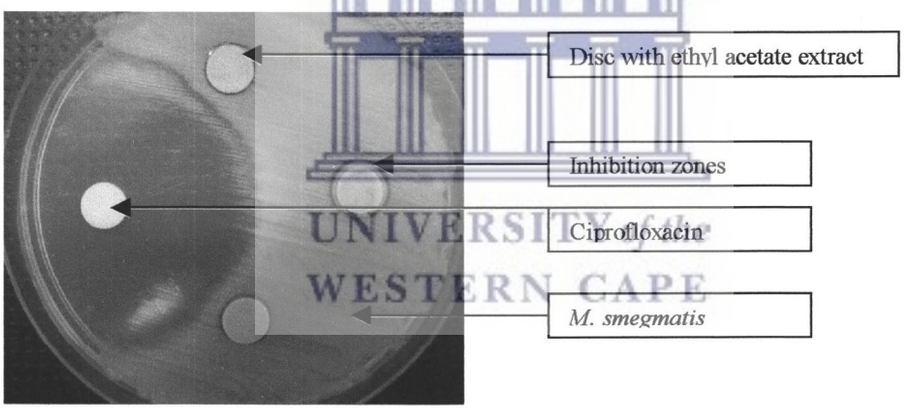

Figure 5. The growth inhibition activity of an Ethyl acetate extract against *M. smegmatis.*

Table 2. Growth inhibition activities of three *Carpobrotus* species against different micro-organisms[a].

Species	Plant extracts[b]	*S.aureus*	*P.aeruginosa*	*C. albicans*	*M. smegmatis*
C.mellei					
	P.ether	0	0	0	0
	$CHCl_3$	0	0	0	0
	E. Acetate	16.5	0	0	10
	H_2O	12.5	0	0	10
C. muirii					
	P.ether	0	0	0	0
	$CHCl_3$	0	0	0	0
	E. Acetate	16.5	0	0	11
	H_2O	12	0	0	10
C.quadrifidus					
	P.ether	0	0	0	0
	$CHCl_3$	0	0	0	0
	E. Acetate	12.5	0	0	10
	H_2O	11	0	0	10
	Ciprofloxacin	50	50	0	40
	Amphotericin	0	0	14	0

S.aureus, Staphyllococus aureus; *P.aeruginosa* , Pseudomonas aeruginosa; *C. albicans*,Candida albicans; *M. smegmatis*, Mycobacterium smegmatis.[b]P.ether, petroleum ether; $CHCl_3$, chloroform; E.acetate, ethyl acetate; H_2O, water.

Figures 4 and 5 both show the growth inhibition of ethyl acetate extracts of *C. mellei*, against *S. aureus* and *M. smegmatis* respectively. All the plants exhibited some degree of antimicrobial activity (Table 2), which was observed in the more polar extracts whereas the non-polar extracts didn't show any activity. Aqueous extracts appear to have less antimicrobial activity than the ethyl acetate extracts.

4.2.2. Bioautography

Table 3. Growth inhibition activity of tannin-free ethyl acetate fraction of *C. mellei* against *S. aureus* and *M.smegmatis*.

Zone of inhibition of micro-organisms (L x B/mm)			
Rf	*S. aureus* Zone	*M.smegmatis* Zone	Colour (UV366
0.05	4 x 20	-	Yellow
0.25	7 x 20	-	Light yellow
0.30	7 x 20	-	Orange
0.38	4 x 20	-	Orange
0.41	-	4 x 20	-
0.48	9 x 20	-	Brown
0.51	5 x 20	7 x 20	Light brown
0.59	10 x 20	4 x 20	Brown

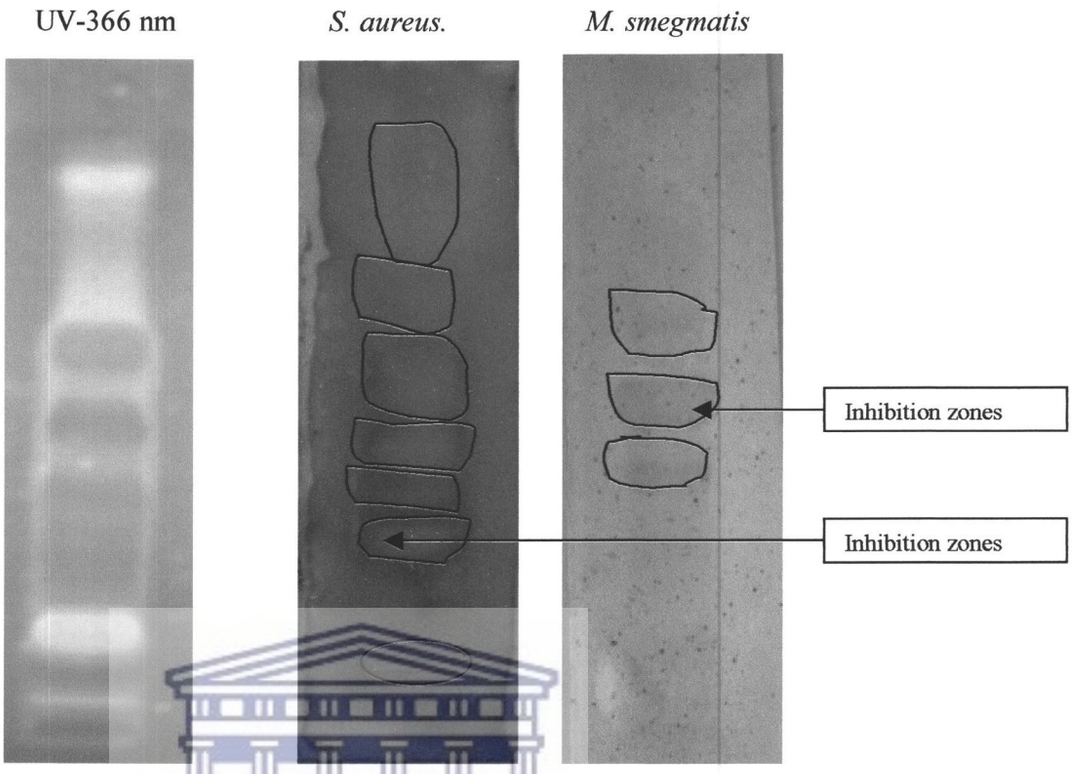

Figure 6. Bioautogram showing growth inhibition of *S. aureus* and *M. smegmatis* by *C. mellei.*

All the plates were developed in ethyl acetate: methanol: water (100:13,5:10). The left bioautogram show the developed TLC plate under UV-366 after being sprayed with *Naturstoff Reagenz* (NA) reagents. Both the middle and the right plates were overlayed with agar inoculated with *S. aureus* (middle) and *M.smegmatis* (right). Growth inhibition indicated by colourless areas on TLC.

Table 4. Growth inhibition activity of tannin-free ethyl acetate fraction of *C. muirrii* against *S. aureus* and *M. smegmatis*.

Zone of inhibition of microorganisms (L x B/mm)			
Rf	*S. aureus* Zone	*M.smegmatis* Zone	*Colour (UV366 nm)*
0.05	-	-	Orange
0.08	-	-	yellow
0.12	-	-	yellow
0.18	-	-	Blue
0.30	-	-	orange
0.54	-	-	Blue
0.64	-	-	yellow
0.49	1 x 2.5	-	-
0.54 – 0.89	-	7 x 3	-
0.59	1 x 2.5	-	-
0.69	1.25 x 2.5	-	-
0.80	1 x 2.5	-	-

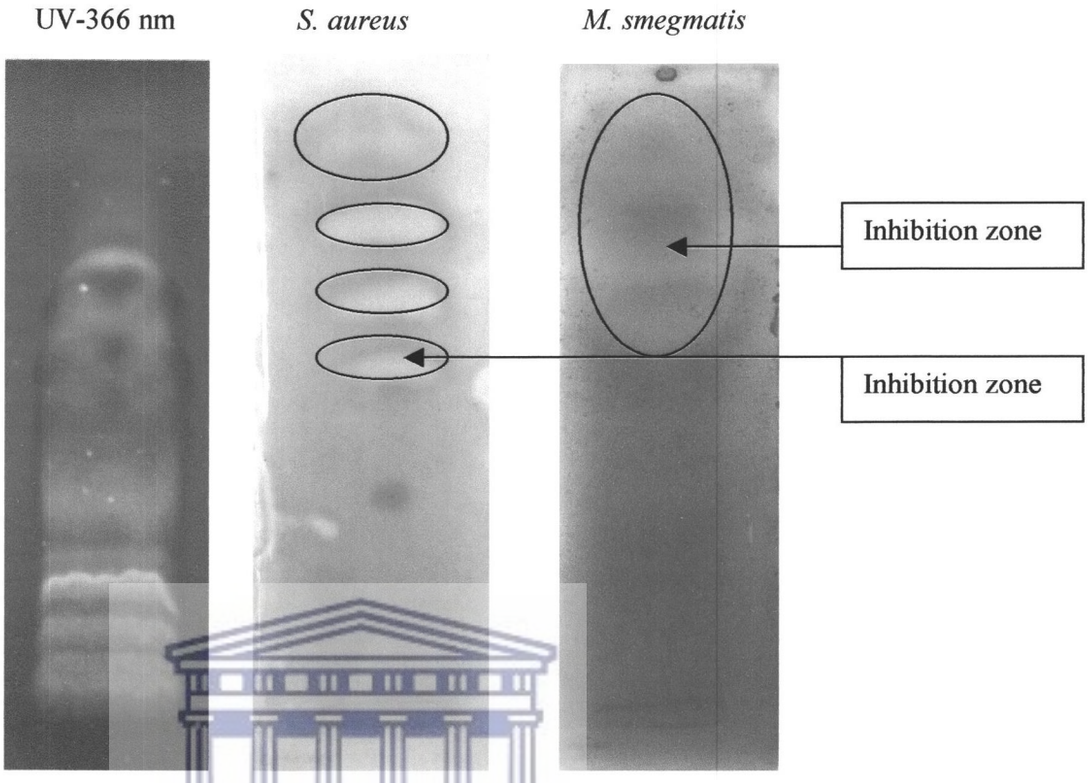

Figure.7. Bioautogram showing growth inhibition of *S. aureus* and *M. smegmatis* by *C. muirii*.

All the plates were developed in ethyl acetate: methanol: water (100:13,5:10). Left bioautogram show the developed TLC plate under UV-366 after being sprayed with *Naturstoff Reagenz* (NA) reagent. Both the middle and the right plates were overlayed with agar inoculated with *S. aureus* (middle) and *M.smegmatis* (right). Growth inhibition indicated by colourless areas on the TLC plates.

Table 5. Growth inhibition activity of tannin-free ethyl acetate fraction of *Carpobrotus quidrifidus* against *S. aureus* and *M. smegmatis*.

Zone of inhibition of micro-organisms (L x B/mm)			
Rf	*S. aureus* Zone	*M.smegmatis* Zone	Colour (UV366)
0.08	-	0.4 x 2.5	orange
0.36	-	0.5 x 2.5	blue
0.41	1.2 x 0.8	-	orange
0.55	1.2 x 0.8	-	Yellow
0.64	1.3 x 0.8	-	yellow
0.73	1.2 x 0.8	1 x 2.5	Yellow-brown
0.85	1.7 x 0.8	-	Peach

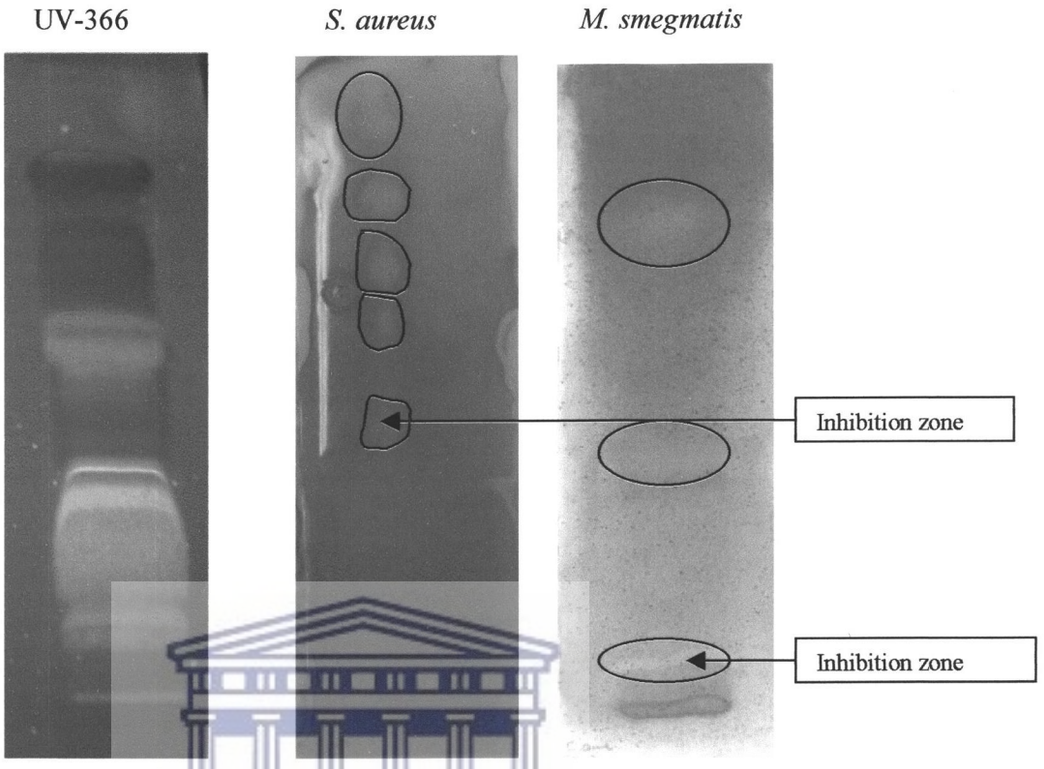

Figure 8. Bioautogram showing growth inhibition of *S. aureus* and *M. smegmatis* by *C. quadrifidus.*

All the plates were developed in ethyl acetate: methanol: water (100:13,5:10). Left bioautogram show the developed TLC plate under UV-366 after being sprayed with NA reagents. Both the middle and the right plates were overlayed with agar inoculated with *S. aureus* (middle) and *M.smegmatis* (right). Growth inhibition indicated by colourless areas on TLC.

2.4.3. Minimum inhibitory concentration.

Table 6. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of tannin free ethyl acetate- and aqueous extracts against *S. aureus* and *M. smegmatis*.

Plant extracts[a]	Test organism[b]	MIC (mg/ml)	MBC (mg/ml)
Mel-EA	*S. aureus*	7.5	7.5
	M.smegmatis	25	>25
Mel-H_2O	*S. aureus*	12.5	12.5
	M.smegmatis	50	>25
Mur-EA	*S. aureus*	7.5	15
	M.smegmatis	30	30
Mur- H_2O	*S. aureus*	25	25
	M.smegmatis	50	>50
Quad-EA	*S. aureus*	7.5	15
	M.smegmatis	30	30
Quad- H_2O	*Staph.*	25	25
	M.smegmatis	50	>50

[a] *Mel, mellei; Mur, muirii; Quad, quadrifidus*; EA, ethyl acetate; H_2O, aqueous;
[b] *S.aureus., Staphylococcus aureus; M. smegmatis, Mycobacterium smegmatis*

Tetrazolium salts are frequently used to indicate biological activity because the colourless compound acts as an electron acceptor and is reduced to a coloured product by biologically active organisms. The green colour of plant extracts makes it difficult to read data with a microplate reader. It was, however, usually easy to differentiate between the red product of INT reduction and the colour of the extract. MTT was used when the colour of the extract was reddish (Eloff, 1998a).

The minimal inhibitory concentration is defined as the lowest concentration, which visibly inhibits growth of the microorganism after overnight incubation. The ethyl acetate extracts were proven, to be more potent than the aqueous extracts.

2.5. HPLC analysis

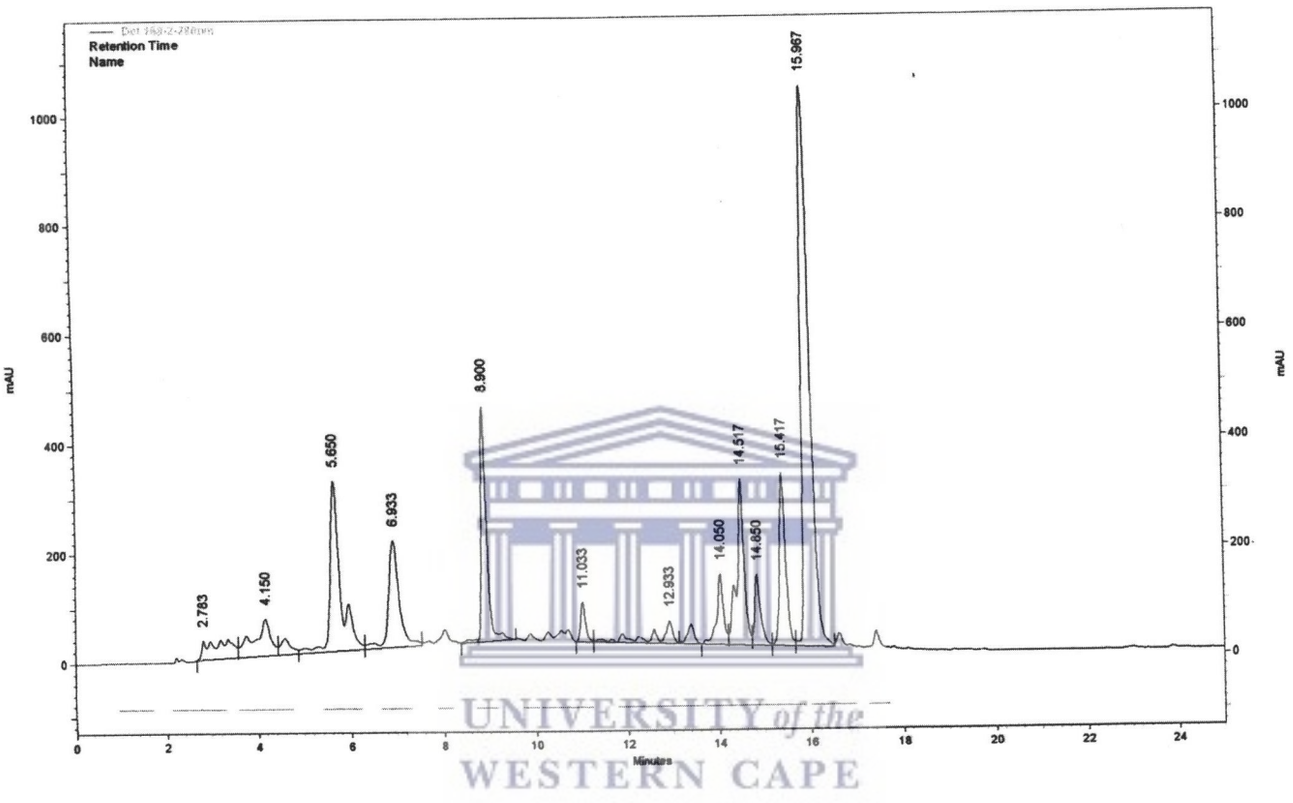

Fig.9. HPLC fingerprint of the active ethyl acetate extract of *C. mellei*.

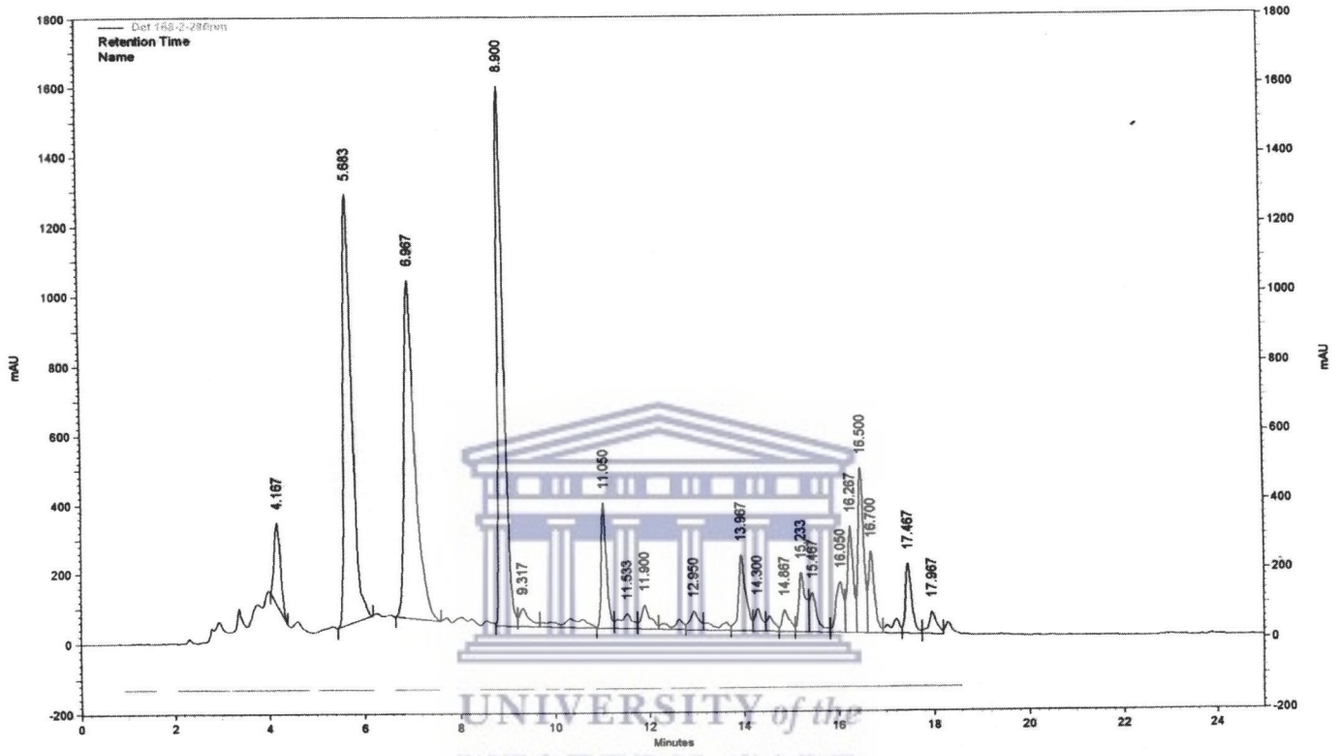

Fig.10 HPLC fingerprint of the active ethyl acetate extract of *C. muirii*.

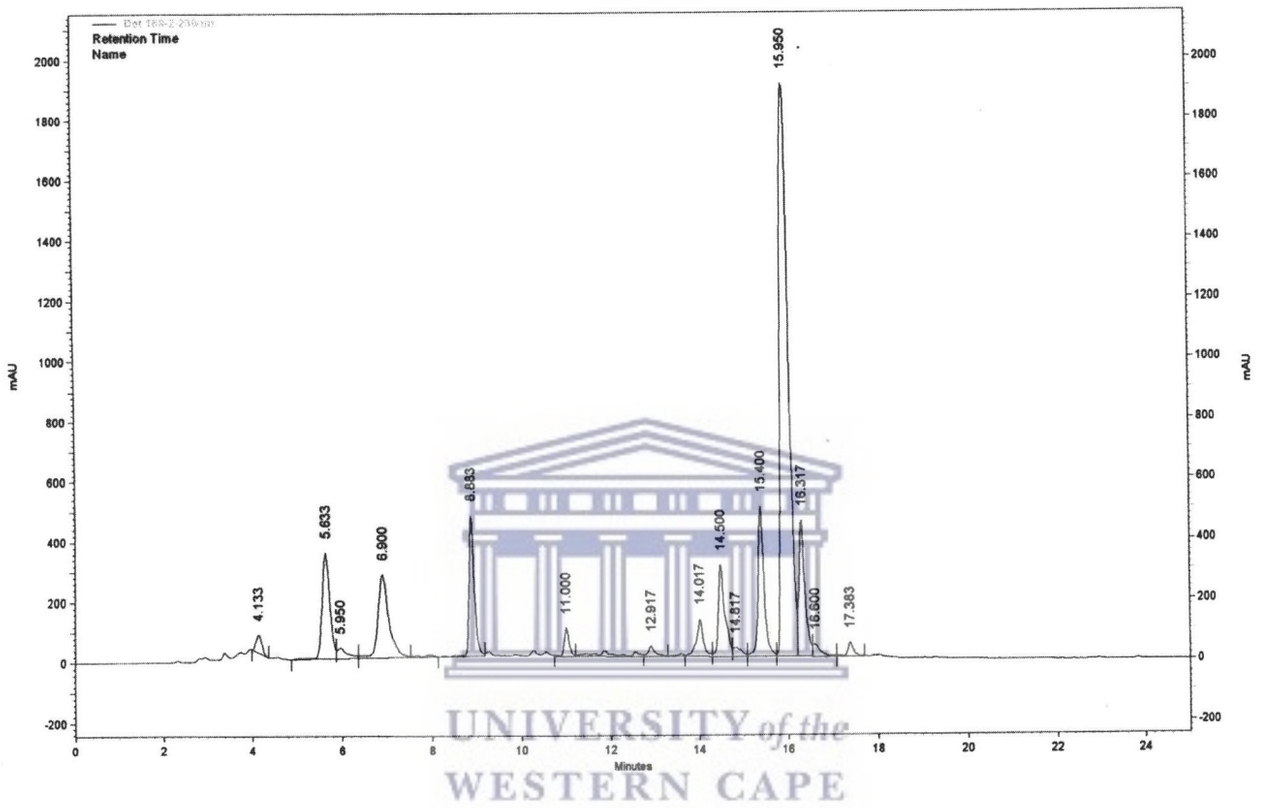

Fig.11. HPLC fingerprint of the active ethyl acetate extract of *C.quadrifidus*.

Table 7. Retention times (minutes) of the ethyl acetate fractions of the different *Carpobrotus* species.

Compound	*C. mellei*	*C. muirii*	*C. quadrifidus*
1	5.650	5.683	5.633
2	6.933	6.967	6.900
3	8.900	8.900	8.883
4	11.033	11.050	11.0725
5	14.517	14.546	14.500
6	15.417	15.467	15.400
7	15.967	15.950	16.050

Previously phyotchemical tests had indicated the presence of flavanoids. Flavanoids could be regarded as falling under the broader class of compounds of the phenolics type. HPLC, with its high speed and sensitivity, offers a promising system in analyzing plant substances such as phenolics. There have been reports in the literature (Andersen.et al., 1983; Vande Casteele et al., 1983; Lattanzio, 1982; Stutte et al., 1982) where phenolics have showed revised HPLC methods.

The HPLC method in this investigation was developed for qualitative "fingerprinting" of each *Carpobrotus* species. The chromatographic profiles on figures 9, 10 and 11 together with the retention times in table 6. show no major differences except for the quantitative differences, which can be noticed with respect to selected peaks. The peaks listed in table 6 are common in all three species with characteristic UV absorbance at 280 nm. Components 5 to 7 absorbed more strongly at 360 nm, whereas compounds 1 to 4 absorbed rather weakly at this wavelength, but at a small scale.

General Discussion and Conclusion

Despite the use of herbal medicines over many centuries, only a relatively small number of plant species has been scientifically studied for possible medical applications. Safety and efficacy data are available for an even smaller number of plants, their extracts and active ingredients and preparations containing them.

The isolation and use of natural products such as digoxin, morphine and quinine has resulted in replacing the plant extracts used with single chemical entities. There is a basic supposition that any plant possessing clinical effectiveness must contain an active principle, which can completely replace the plant extract (Phillipson, 2001). However prescription drugs can cause nutrition depletion and can lead to, among others, osteoporosis, heart and blood pressure problems, tooth decay and birth defects (Von Geusau, 2001).

There is a lack of knowledge about alternative therapies and herbal medications. The results obtained meet the goal of this study and the scientific information that is now documented can be used to enhance the overall knowledge of these species and their role in patient care, also to healthcare professionals to improve human health (Nice, 2002). It is advised to patients and healthcare professionals by Nice (2002), to approach herbal medication use cautiously. Scientific validation is essential in all herbal medication, to put more confidence to the claims made by the public and traditional healers.

The agar diffusion methods were reported to be ideal for water-soluble antimicrobial compounds (Vanden Berghe et al, 1991). This method was beneficial because Venden Berghe and Vlietinck (1991) noted that the filter paper disc method is an ideal method for assaying water-soluble antibiotics, while the dilution methods, where the minimum inhibition concentration values (MIC-values) are determined are more suitable for non- polar extracts (Vanden Berghe and Vlietinck., 1991; Rios et al., 1988).

In the agar diffusion technique inhibition zones of the samples were compared with those of a standard antibiotic, ciprofloxacin (Fig. 4 and 5). This is useful in establishing the sensitivity of the test organism and the standard antibiotic also serves as control. However, it was not used as a comparison of the antimicrobial potencies of the samples and the antibiotic. The diffusion method is well suited for preliminary screening and has the advantage of allowing the use of accommodating the small sample size (Rios et al., 1988).

In comparison to bioautography, other techniques provide at best, information on antimicrobial activity, they; however, do not give any qualitative information about the active components. By separating bioactive extracts on thin layer chromatography, it is possible to get information on the compounds present in the mixture and as indicated by figures 6, 7 and 8 all of the three species show chemical compounds responsible for the antimicrobial activity.

Figure 6. indicates the presence of six major inhibiting compounds present in the *C. mellei* tannin-free ethyl acetate extract, which are active against *S. aureus*, and only three compounds that are

active against *M.smegmatis*. The compounds as examined by TLC were visible under UV-366 nm and in some cases, there was direct correlation between UV activity and antimicrobial activity with respect to the R_f values measured. Figure 7. shows the presence of four distinct major inhibiting compounds in the *C.muirii* tannin-free ethyl acetate extract, which are active against *S. aureus* and an overlapping band of compounds, that are active against *M.smegmatis*. Table 4. and fig. 7. indicate a correlation between the active compounds relative to their distance travelled on the plate. The TLC separation of UV 366 nm shows several compounds but not all show similar R_f values as the active compounds with antimicrobial activity. Figure 8. indicates five major inhibiting compounds present in the *C. quadrifidus* tannin-free ethyl acetate extract active against *S. aureus* and three compounds active against *M.smegmatis*. The TLC separation shows the compounds present in the extract under UV-366 nm and in some cases, there were compounds present with R_f values similar to the antibacterial compounds visible on the bioautograms. This may suggest that such compounds may be implicated in antimicrobial activity. From the above results, it is evident that bioautography is a method that facilitates the localization of antimicrobial activity on a chromatogram. The main feature of the direct assay is the elimination of the diffusion step (Hamburger et al., 1987) .

The R_f values showing the activity do not all correlate between the species. It can be ascribed to the very high tannin content that was observed in the leaves of the studied *Carpobrotus* species, which made extraction procedures difficult, because of occasional precipitation of the tannins. This investigation also confirms the assertion by Hagerman (1998) that a high tannin content in the plant

In conclusion, this study provides new leads regarding ongoing search for novel antimicrobial drugs. This investigation confirmed that *C. mellei, C. muirii and C. quadrifidus* has scientific merit as an anti-infective indigenous medicine and can substitute one of the better-known *Carpobrotus* species for antimicrobial use-age.

Further studies on the isolation and characterization of the active compounds in this medicinal herbs, may well provide us with novel antimicrobial therapeutics for chronic infections, however the contribution of all the active compounds can provide more potent therapeutic effects and not necessarily only one active compound (Phillipson, 1995). It is confirmed that the studied *Carpobrotus* species can be used interchangeably, depending on their habitats, by local folks.

References:

Andersen, J. M., Pedersen, W. B. (1983). Analysis of plant phenolics by High performance liquid chromatography. Journal of Chromatography, 259, 131- 139.

Beugue, W.J., Kline, R.M. (1972). The use of tetrazolium salts in bioautographic procedures. Journal of Chromatography 64, 182-184.

Borris, R. P. (1996). Natural product research: perspective from a major pharmaceutical company. Journal of Chromatography 51, 29-38.

Eloff, J. N. (1998a). A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Medica 64, 711-713.

Eloff, J.N. (1998b) Which extractant should be used for screening and isolation of antimicrobial components from plants. Journal of Ethnopharmacology 60, 1-8.

Emboden W. (1997). Narcotic plants. Hallucinogens, stimulants, inebriants, and hynotics, their origins and uses., Macmillan Publishin Co.,Inc., Canada

Hagerman, A.E. (1998). " Tannin Handbook" Oxford USA: Miami university, p.1-30.

Hamburger, M. O., Cordell, G. A. (1987). A direct bioautographic tlc assay for compounds possessing antibacterial activity. Journal of Natural Products, vol.50, 1, 19-22.

King, S. R., Carlson, T. J., Moran, K. (1996). Biological diversity, indigenous knowledge, drug discovery and intellectual property rights: creating reciprocity and maintaining relationships. Journal of Ethnopharmacology, vol.51, p. 45-57.

Lattanzio, V. (1982). High performance liquid chromatography of free and bound phenolic acids in the egg-plant (*Solanum melongena* L.). Journal of Chromatography, 250, 143- 148.

Latte, K.P. (1999). Phytochemische und pharmakologische untersuctunger an Pelargonium reniforme curt., PhD thesis, University of Berlin.

Medicapharma.(2000). URL: http://www.medicapharma.com/Antibiotic/antibiotic.html.

On-line Medical Dictionary. (2002). URL: http://cancerweb.ncl.ac.uk/cgi-bin/omd?action=Home&query=.

Porter, L. J. (1989). Tannins. In Dey P.M., Harborne J.B. (series eds) Methods in plant biochemistry, volume 1. Academic Press limited, London.

Phillipson, J. D. (2001). Phytochemistry and medicinal plants. Phytochemistry 56, 237-243

Phillipson, J.D. (1995). A matter of some sensitivity. Phytochemistry 38, 1319-1343.

Rang, H.P., Dale, M. M., Ritter, J. M. (1999). Pharmacology, 4th edition. Churchhill Livingstone, Edinburgh.

Rios, J. L., Recio, M. C., Villar, A. (1988). Screening methods for natural products with antimicrobial activity: a review of the literature. Journal of Ethnopharmacology, vol23, 127-149.

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

Schnitzler, A. C., Nolan, L. L., Labbe, R. (1996), Screening of medicinal plants for antileishmanial and antimicrobial activity, International Symposium on Medicinal and Aromatic plants, Acta Hort.426, 235-241.

Sneader, W. (1986). Atibiotics: In: Drug discover: the evolution of modern medicines: John Wiley & Sons, Chichester: 296- 330.

Soejarto, D. D. (1996). Biodiversity prospecting and benefit- sharing: perspectives from the field. Journal of Ethnopharmacology, vol.51, p. 1-15.

South African Traditional Medicines Research Group. (1999). Pharmacopoeia Monograph Project. School of Pharmacy, University of the Western Cape, Bellville, South Africa.

Stutte, C. A., Hardin, J. M. (1982). Phenolic acid analysis comparing two high performance liquid chromatographic techniques. Journal of Chromatography, 248, 446- 450.

Turner, D. M. (1996). Natural product source material use in the pharmaceutical industry: the Glaxo experience. Journal of Ethnopharmacology 51, 39-44

Vande Casteele, K., Gieger, H., Van Sumere, C. F. (1983). Seperation of phenolics (Benzoic acids, cinnamic acids, phenylacetic acids, quinic acid esters, benzaldehydes and acetophenones, miscellaneous phenolics) and coumarins by reversed-phase HPLC. Journal of Chromatography, 258, 111- 124.

Vanden Berghe, D. A., Vlietinck, A. J. (1991). Screening methods for antibacterial and antiviral agents from higher plants. In Dey P.M., Harborne J.B. (series eds) Methods in plant biochemistry, volume 6. Academic Press limited, London: 47- 67.

Van der Watt. E., Pretorius. J. C. (2001). Purification and identification of active antibacterial components in *Carpobrotus edulis* L. Journal of Ethnopharmacology 76, 87-91

Van Wyk, B., van Oudshoorn, B., Gericke,N. (1997). Medicinal plants of South Africa. Briza Publications, Pretoria, South Africa.

Vogel, H.G. (1991). Similarities between various systems of traditional medicine. Considerations for the future of ethnopharmacology. Journal of Ethnopharmacology 35, 179-190

Von Geusau, R. (2001). Prescription drug danger. SHAPE, December, page 81.

Watt, J.M. and Breyer-Brandwijk, B.N. (1962). The Medicinal and Poisonous Plants of Southern and Eastern Africa., 2nd edition., Livingstone, London,47-69

Wisura, W., Glen, H.F. (1993). The South African species of Carpobrotus (Mesembryanthema, Aizoaceae) Contribution, Boletim Herbage 15: 76-107.

WHO. (1998). Regulatory situation of Herbal Medicine. A worldwide review. WHO/TRM/98.1