Antimicrobial activity of *Melianthus villosus*

by

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Contents

Preface

Structure of the dissertation

This dissertation consists of six chapters. Chapter 1 deals with the literature review of the genus *Melianthus* in terms of its medicinal properties. Chapter 2 provides a detailed description of *Melianthus* and *M. villosus*, as well as its distribution in Southern Africa. Chapters 3 and 4 describe the antibacterial and antifungal activities respectively, of the acetone crude extract of *M. villosus*. In Chapter 5 attempts to isolate, purify and identify the antibacterial compound(s) are described. Chapter 6 consists of the general discussion and conclusions.

CHAPTER 1

Literature Review

Literature Review

1.1 Introduction

Medicinal plants used by traditional healers are proving to be an important source of potentially therapeutic drugs (Cox & Balick, 1994). Since time immemorial indigenous people have used plants for therapeutic purposes and now medicinal plants are increasingly becoming an alternative form of health care, for example in homeopathy and ethnopharmacology. In South Africa alone, up to 80% of the population consult traditional healers in addition to using western medicine (Jäger et al., 1996).

Medicinal plants also play an important role in conventional western medicine. In 1984, at least 25% of the prescription drugs issued in the USA and Canada were derived from or modelled after natural products from plants (Farnsworth, 1984). Farnsworth & Soejarto (1991) identified 119 secondary plant metabolites that are used globally as drugs. They estimated that 14-28% of higher plant species are used medicinally, but that only 15% of

all angiosperms have been investigated chemically and that 74% of pharmacologically-active plant-derived compounds were discovered after following up on the ethnomedical use of the plant. Most of the plant-derived compounds are antimicrobials.

The antimicrobial compounds from plants may inhibit bacteria by a different mechanism than the presently used antibiotics and may therefore have clinical value for the treatment of resistant microbial strains (Eloff, 1998). The numbers of resistant strains of microbial pathogens are increasing locally. Penicillin-resistant and multi-resistant pneumococci are of major concern in hospitals worldwide (Eloff, 1998). Leggiadro (1995) stated that effective regimens might soon not be available to treat some enterococcal isolates and that it is critically important to develop new antimicrobial compounds for these and other organisms before we enter the post-antibiotic era. Recently, new compounds inhibiting microorganisms, such as benzoin and emetine have been isolated from plants (Cox, 1994).

For a variety of reasons more individuals are nowadays preferring to take control over their health, not only in the prevention of diseases but also in treating them. This is particularly true for a wide variety of chronic or incurable illnesses readily treated at home (common cold etc.) (Kincheloe, 1997). In this respect many individuals have become disenchanted with the worth of allopathic treatments, and the adverse effects that can be anticipated. They are seemingly unaware of the potential problems associated with herbal use or the fact that their limited diagnostic skills, or of those prescribing them, may prevent detection of serious underlying conditions like malignancies (Saxe, 1987; Winslow & Kroll, 1988; Donaldson, 1998; Shaw *et al.*, 1999; Stewart *et al.*, 1999;). This raises the need for a scientific evaluation of the methods used by some traditional healers. It is also necessary to establish the efficacy and safety of traditional treatments (Kelmanson *et al.*, 2000).

Amongst other things, three factors limiting rational use of herbal medicine are:

a) Variation in quality of product,

b) Uncertainty on safety and

c) No unambiguous proof of effectivity.

Indigenous people have used many herbal medicines over many centuries; hence the safety is frequently not such a big concern. The fact that they also have been used over so many years indicates that they may be effective. With more and more people collecting and distributing herbal medicine, the offered product is, however, frequently not what the label or the seller indicates either through a genuine mistake, but also through greed. This is especially the case where expensive herbal medicine is concerned. In some cases wrong identification has even led to death (Watt & Breyer-Brandwijk, 1962).

1.2 Traditional uses of the genus *Melianthus*

According to Sim (1907) four species of *Melianthus* (Melianthaceae) viz., *M. comosus* Vahl, *M. dregeanus* Sond. subp. *insignis* (Kuntze) Tansley, *M. major* L., and *M. minor* L., are used in the following ways: the leaves

are used as a poultice for cold sores, the bark is taken internally in small doses as a tonic and a poultice of the leaf is applied locally for snakebite. The root bark of all species is thought to be poisonous and deaths have been caused by it (Smith, 1888).

M. comosus, is widely used in traditional medicine. A decoction of the plant is applied to wounds, which are healing slowly, and a leaf is pasted as a dressing for sores and to reduce swelling of bruises. The Xhosa use the root prophylactically and therapeutically for snakebite by chewing the root and by applying a paste of the leaf or a tincture of the root bark or of the leaf to the wounds. The Xhosa sometimes administer a small amount of the root bark in water to wounds, in addition to the local treatment (Smith, 1888). They also regard the root bark as very poisonous but none the less take it in very small doses as a general tonic, especially for dyspepsia. It is said to be strongly emetic, the vomit being foamy. A bath medicated with the plant is used to promote sweating and a hot decoction to bathe rheumatic limbs. Whites also drink a decoction of the

leaf and the stem for the relief of rheumatism. In Queenstown district the African populace use the decoction to relieve foot troubles (Watt & Breyer-Brandwijk, 1962).

In the Mpumalanga Province, the Zulu and Swati drink a decoction of the leaf of *M. dregeanus* subsp *insignis*, made with the addition of the bulb of another plant, during the night and morning "to clean the system and keep the blood clean" (Watt & Breyer-Brandwijk, 1962).

A decoction of *M. major* is used as a lotion for sores. The root is regarded as a dangerous poison. The Xhosa use it as a snakebite remedy and as an emetic, with the vomit being foamy. Pappe (1857) regarded a decoction of the leaf as excellent in the treatment of *tinea capitis*, "crusta serpiginosa," necroses and foul ulcers, as a gargle for sore throats and in diseases of the gums. The bruised leaves, he states, promote granulation in ulcers. In the former Transvaal, Europeans used to apply to boils a poultice of flour and the plant, boiled in water (Pappe, 1857). It has proved fatally toxic to

animals with symptoms and *post-mortem* findings suggesting an irritant poison (Stein, 1929). The toxicity is of the same order as that of *M. comosus* and showed no diminution after sixteen months of storage (Watt & Breyer-Brandwijk, 1962). The plant is said to possibly contain saponins (Van Wyk *et al.*, 1997). The nectar yields toxic honey (Watt & Breyer-Brandijk, 1962).

Apart from *M. villosus*, which has not been used traditionally or tested medicinally, the other species of *Melianthus* are also highly toxic and are known to have caused human deaths (Watt & Beyer-Brandwijk, 1962). Nothing is known about the wound healing properties of these plants, and the perception that the medicinal value of *Melianthus* is partly due to triterpenoids (Van Wyk *et al.*, 1997), is a possibility that should be investigated.

1.3 Justification for, and objectives of the study

The literature survey showed that there is no record of *M. villosus* having been tested for antibacterial and antifungal properties, although species from the same genus are known to have antibacterial properties (Jäger et al., 1996; Van Wyk *et al.*, 1997). In contrast with *M. major* and *M. comosus*, *M. villosus* is not used locally as a medicinal plant, a factor possibly attributed to its secluded habitat alongside patches of forest in the Drakensberg foothills.

The lack of recorded information on the antimicrobial properties of *M. villosus* has led to the following objectives of this study:

- Evaluation of the antibacterial and antifungal properties of the crude extract of *M. villosus*
- Isolation and identification of the active compound(s) of *M. villosus*.
- To test the antibacterial activity of isolated compound(s).

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CHAPTER 2

Description and distribution of *Melianthus villosus*

2.1 Introduction

"*Melianthus, du botanicus cognitus, jam ab iniyio crux fuit systematicorum*" [Melianthus has been known by botanists for a long time but from the onset it has been a burden to the systematists] (Agardh, 1858).

Although the taxonomic position of *Melianthus* was problematical in the past, it has been resolved and two genera are now recognized in the Melianthaceae, viz., *Bersama* Fren. with 8 species of trees and shrubs in tropical and southern Africa, and *Melianthus* L., with 6 species of shrubs and undershrubs in southern Africa (Archer, 2000).

All six species of *Melianthus* are evergreen shrubs or undershrubs with unpleasant smelling foliage. The leaves are compound and have prominent stipules. The flowers are in erect racemes, irregular and profusely nectariferous.

The various species are widely distributed in both drier and moister parts of the country.

M. major has become an important garden plant and has become naturalised in India (Griffiths, 1994).

The name comes from the Greek 'Meli' - honey, and 'anthos' - flower, and refers to the copious amount of nectar secreted by the flowers. The English common name is honey flower, whereas in Afrikaans they are known as "kruidjie-roer-my-nie", literally translated as "herb brush me not", alluding to the foetid odour given off by the leaves when touched (Smith, 1966).

2.2 *Melianthus villosus*

2.2.1 Detailed description

M. villosus is a soft woody shrub up to 2m in height. Branches are tomentose and the leaves are compound imparipinnate, up to 26cm long and villous (stellate hairs) on both sides; leaflets 3-7 pairs, up to 14cm long and 4cm broad, elliptic-ovate, margins deeply serrate, lamina foetid when bruised; stipules are conspicuous, 20-25mm long, obliquely ovate-lanceolate.

Flowers pendulous in whorls of 2-4, borne in upper parts of erect racemes. Bracts 20mm long, ovate-acuminate. Calyx 5-partite; sepals brownish-purple with green veins, pubescent, asymmetric, odd sepal up to 20mm long. Corolla 4-partite; petals 12-16mm long, rose to purplish-black or brown, mainly glabrous in lower half with glandular hairs on face. Nectary unilateral with two lateral wings, exuding dark nectar. Stamens 4, 2 inserted on the inner side of the nectary, 2 in adaxial position. Ovary 4-angled, pubescent, 3 ovules in each locule. Style rigid, curved, and acute. Fruit inflated capsule, 4-angled, up to 40mm long. Seed hard, black, and shiny. Fig 2.1 and 2.2 show the descriptive properties (Phillips & Hofmeyer, 1927; Dyer, 1952; Ross, 1972; Killick, 1990).

The specific epithet of *M. villosus*, refers to the hairy leaflets, by which it is separated from the closely related and glabrous-leaved Cape species, *M. major* L.

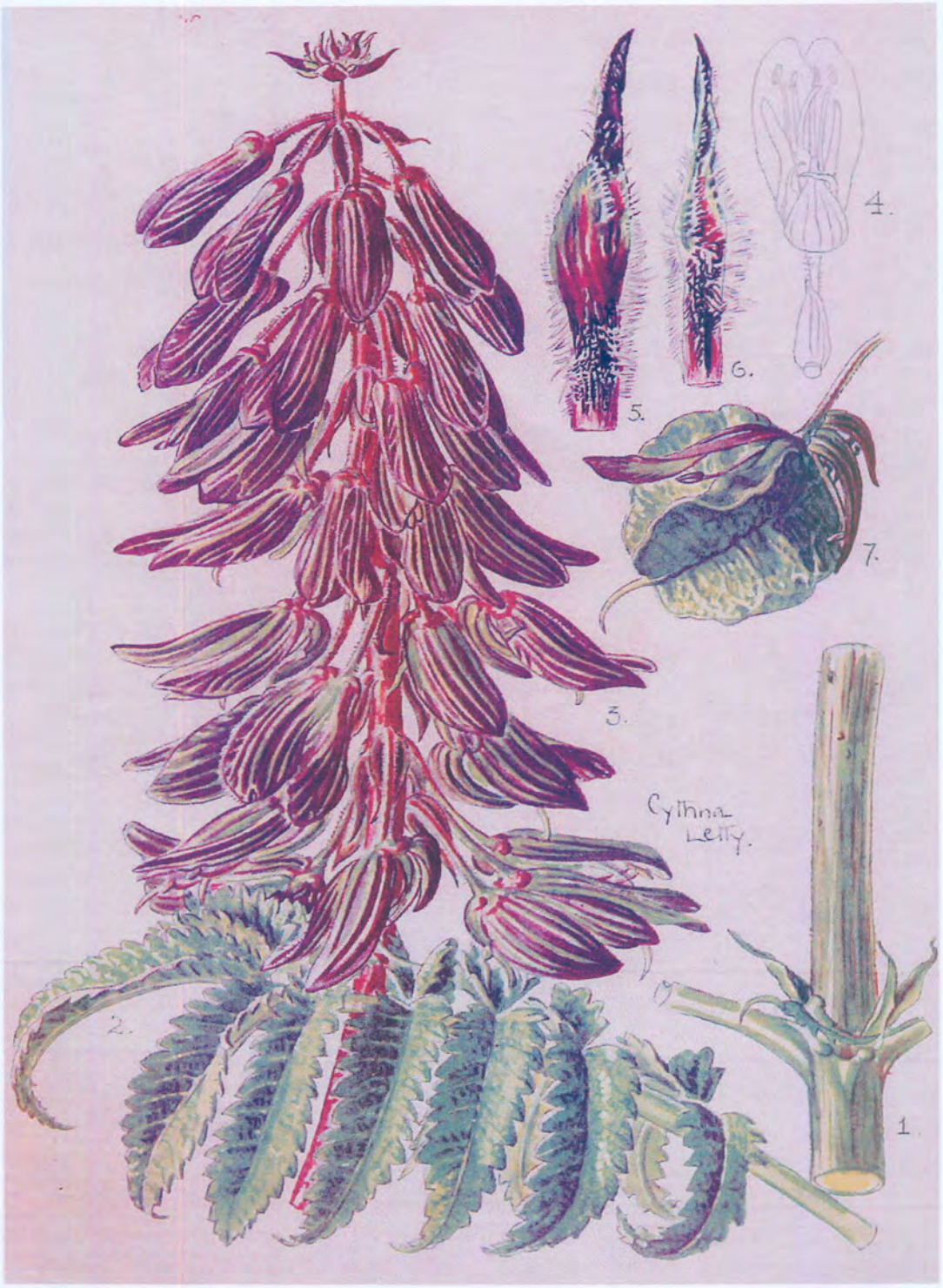

Fig 2.1 1, Uppermost stem node and base of peduncle of *M. villosus*; 2, leaf; 3, top of inflorescence; 4, flower placed erect, with bract; all natural size; 5, lateral petal, x 5; 6, on of the petals subtended by odd sepal, x 5; 7, fruit, natural size. (Dyer, 1952)

From the two other summer-rainfall species, *M. comosus* Vahl and *M. dregeana* Sond. subsp. *insignis* (Kuntze) S.A.Tansley, *M. villosus* is separated by having 2-4 flowers at each node, unlike the single flowers of those species (Phillips & Hofmeyer, 1927).

M. villosus is known in English as the maroon honey flower and in isiZulu as ibhonya (Pooley, 1998).

Fig. 2.2 *M. villosus* in its natural habitat, near Metsi-matso dam, 30km south-west of Harrismith.

2.2.2 Distribution

M. villosus occurs in moist grassland at the edge of scrub forest in the foothills of the Kwazulu/Natal Drakensberg and in the adjoining Free State and Lesotho. It ranges from the Loteni Valley near Himeville, through the Royal Natal National Park near Bergville and reaches across the low escarpment near Phuthaditjhaba (fig.2.3). It is usually found growing at an altitude of between 1 600m and 1 950m (Hilliard and Burtt, 1987).

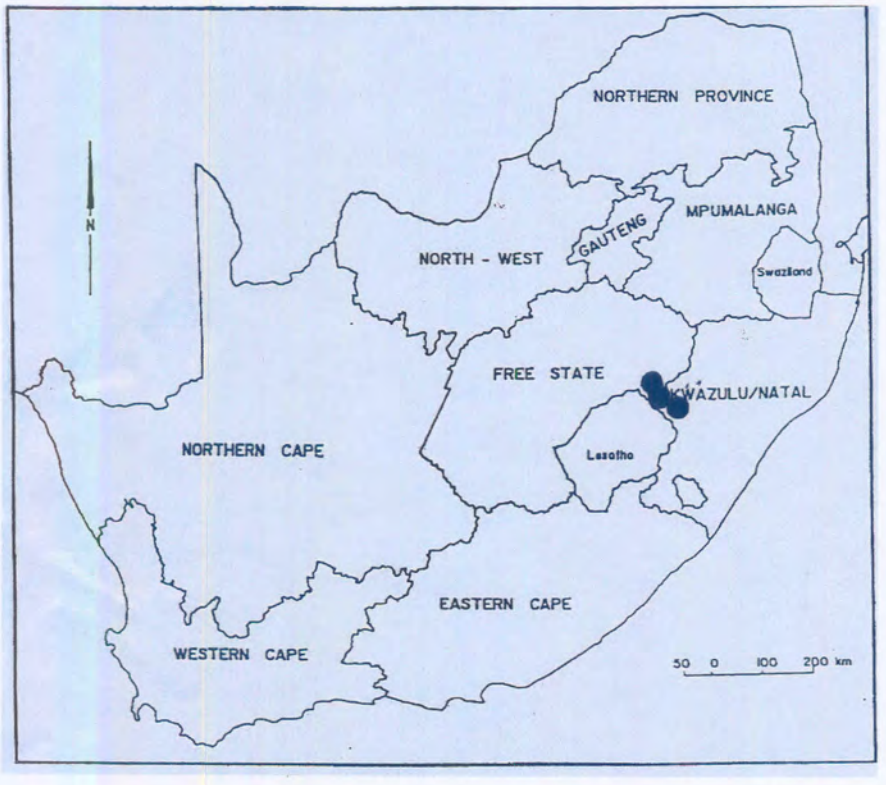

Fig. 2.3. Distribution (•) of *M. villosus* in South Africa.

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Chapter 3

Antibacterial activity of *Melianthus villosus* extracts

Antibacterial activity of *Melianthus villosus* extracts

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Abstract

Ethanol extracts of the leaves and roots of *Melianthus villosus* Bolus were investigated for their antibacterial properties. Most of the activity detected appeared to be against Gram-positive bacteria. Both extracts inhibited the growth of *Bacillus cereus, B. subtilis, B. pumilis, Staphylococcus aureus and Escherichia coli*. The lowest inhibitory concentration was found in the root extract with a minimum inhibitory concentration of 0.1mg/ml.

Keywords: antibacterial activity; *Melianthus villosus*; plant extract

3.1. Introduction

Different techniques and methods are employed in the antimicrobial study of medicinal plants and principles obtained from them. These methods can be classified into three groups, namely; diffusion, dilution and bio-autographic methods.

A great number of factors can influence the antibacterial results - namely,

- ❖ the extraction method (Nadir *et al.*, 1986),
- ❖ inocula volume (Bauer *et al.*, 1966; Hamburger and Cordell, 1987),
- ❖ culture medium composition (Bauer *et al.*, 1966) and pH (Leven *et al.*, 1979; Gutkind *et al.*, 1981; Bauer *et al.*, 1966),
- ❖ incubation temperature (Bauer *et al.*, 1966).

The dilution method was used in this study of the antibacterial activity. Dilution techniques are those, which require a homogenous dispersion of the sample in water. They are used to determine principally, the MIC values of an extract or pure substance and are generally used in the preliminary screening for antimicrobial

activity. A fixed amount of an extract dissolved in nutrient agar is allowed to set. The advantages of this method are its simplicity and speed and the possibility of using it in the microbial study of water soluble or insoluble samples such as essential oils (Rios *et al.*, 1988). Up to ten microorganisms can be inoculated in a petri dish and when these microbes do not grow, it can be said that there is antimicrobial activity.

3.2. Material and methods

3.2.1 Plant material

Leaves and roots of *M. villosus* were collected from plants in their natural habitat in the foothills of the KwaZulu-Natal Drakensberg mountains. A voucher specimen (Moffett & Lentsoane 5340) of the shrub was deposited at the herbarium of the University of Free State (QWA), Qwaqwa campus, in the Free State Province.

3.2.2 Preparation of the plant extract

Leaves

Air-dried leaves (600 g) were homogenised in ethanol and left on a shaker for a week before filtration. The resultant filtrate was concentrated to dryness and redissolved in acetone to a concentration of 100 mg/ml.

Roots

Fresh root material (1 kg) was homogenised in ethanol and left on a shaker for a week before filtration. The resultant filtrate was concentrated to semi-dryness, as it contained fatty material. It was then dissolved in ethyl acetate and bi-partitioned in a separating funnel using methanol. The aqueous methanol layer was then washed three times using ethyl acetate and the washings were collected and concentrated to dryness using a rota-evaporator. The resulting extract was redissolved in acetone to a final concentration of 100mg/ml.

3.2.3. Antibacterial testing

A total of ten selected bacteria, five Gram-positive: *Bacillus cereus, B. pumilis, B. subtilis, Staphylococcus aureus* and *S. pyogenes* as well as five Gram-negative bacteria: *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonae, Enterobacter cloacae* and *Serratia marscescens;* were obtained and cultured as described by Meyer & Afolayan, 1995. Each organism was maintained on nutrient agar slant (Biolab) and recovered for testing by growth in nutrient broth No.2 (Biolab) for 24 hours at 37°C. Before streaking, each culture was diluted 1:100 with fresh sterile nutrient broth.

Nutrient agar was prepared by dissolving it in distilled water and autoclaving before the extract was added. The *M. villosus* extracts (leaf and root) were filtered through sterile syringe-fitted filters (0.22µm). To test at 1.0 mg/ml, a 10 mg of the dried extract was dissolved in 0.1 ml of acetone and added to 9.9 ml of molten nutrient medium. This was poured into a petri dish, swirled carefully until the agar began to set and

left overnight for the acetone to evaporate. Acetone was chosen as a solvent because in addition to dissolving the extract completely it showed no inhibition of the microbes (Eloff, 1998), at the final concentration.

The organisms were streaked in radial patterns on agar plates (Mitscher *et al.*, 1972), incubated at 30°C and examined after 24 and 48 hours. Complete suppression of bacterial growth by a specific extract concentration was required for it to be declared active. The extract was tested at 1.0, 0.1 and 0.01 mg/ml (three replicates). Blank plates containing only nutrient agar and 1% acetone without the plant extract served as controls.

3.3. Results

Evaluation of the antibacterial activity of the ten randomly selected bacteria indicated significant activity against all the Gram-positive bacteria (Table 3.1). The minimum inhibitory concentration of the leaf extract was 1.0 mg/ml for all Gram-positive bacteria but

S. pyogenes. The root extract showed higher inhibition than the leaves with the minimum inhibition at 0.1 mg/ml for all the Gram-positive bacteria including *E. coli*, which is a Gram-negative bacterium.

Table 3.1 Antibacterial activities (MIC[a] mg/ml) of the *M. villosus* extracts

Bacterial species	Gram +/-	Leaves	Roots
B. cereus	+	1.0	0.1
B. pumilis	+	1.0	0.1
B. subtilis	+	1.0	0.1
S. aureus	+	1.0	0.1
S. pyogenes	+	na[b]	1.0
E. coli	-	1.0	1.0
P. aeruginosa	-	na	na
K. pneumonae	-	na	na
E. cloacae	-	na	na
S. marscescens	-	na	na

[a]Minimum Inhibitory Concentration;
[b]Not active

3.4 Discussion

The plant extract of both the leaves and roots of *M. villosus* were tested and found to have antibacterial activity mainly on Gram-positive bacteria. Of the five Gram-positive bacteria tested, only *S. pyogenes* could not be inhibited by the leaf extract. The plant extracts were not active to all Gram-negative bacteria except *E. coli*, which was inhibited by both the leaf and root extracts.

The results reported here are in line with those from previous screenings of medicinal plants for antibacterial activity, which also reported that the Gram-negative bacteria showed resistance against most of the plant extracts which were active against Gram-positive bacteria (Martin, 1995; Vlietinck *et al.*, 1995).

However, on comparison of the leaf and root extracts, the root extract had the lowest MIC values. This might explain to a certain degree, why it is the root part of

the plant, which is utilized among the other members of the family Melianthaceae (chapter 1, traditional uses of the genus Melianthus). The results obtained by Kelmanson *et al.* (2000), of the antibacterial activity of *M. comosus* also showed that the activity was more pronounced on Gram-positive bacteria than Gram-negative bacteria. These results can only be relatively compared as these are not the same plant, but rather belong to the same genus.

As no pharmacological studies have previously been reported on other members of this genus, these results indicate that they might also have antibacterial properties.

3.5 Acknowledgements

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CHAPTER 4

Antifungal activity of

Melianthus villosus

Antifungal activity of *Melianthus villosus*

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Abstract

Antifungal activity of the leaf extract of *Melianthus villosus* was examined against six fungal species. The effect of the plant extract on fungi was tested by the disc diffusion method using *Aspergillus flavus*, *A. niger*, *Cladosporium cladosporoides*, *C. cucumerinum*, *C. sphaerospermum* and *Phytophthora capsici*. The crude acetone extract was active against *C. cladosporoides*, *C. cucumerinum and C. sphaerospermum* at the minimum inhibitory concentration of 1.0 mg/ml.

Keywords: *Melianthus villosus*, antifungal and minimum inhibitory concentration

4.1 Introduction

Antifungal drugs are amongst the most expensive antibiotics (Collee, 1976). However, only a limited number of antifungals are currently available for the treatment of life-threatening infections. Furthermore, the prevalence of systemic fungal infections has increased significantly during the past decade (Vincete, et al., 2003), thus making the cost of the newer and more effective antimicrobial drugs to be high, and the search for less expensive, alternative substances more imperative (Salie *et al.*, 1996). Most fungi are completely resistant to the action of antimicrobial drugs. Only a few substances have been discovered that exert an inhibitory effect on human pathogenic fungi. A number of these are relatively toxic and the few non-toxic ones give rise to psychic disturbances in high or cumulative dosages (Collee, 1976). Their inappropriate and non-discriminatory use has put humans and animals at a health risk. In an attempt to improve this situation some alternative methods of control have to be investigated. Within

this context is the utilisation of medicinal plants, which are natural sources of antimicrobial substances and whose fungitoxic potentials have been described in several studies (Fiori *et al.*, 2000).

A great variety of secondary metabolites synthesised by plants play an important role in the defensive mechanisms of plants against pathogens, herbivores and in competition with other plants (Picman & Schneider, 1993). These secondary metabolites are defined as naturally produced substances, which do not play an explicit role in the internal economy of the organisms that, produce them (Vincete *et al.*, 2003).

Higher plants that contain low molecular weight secondary metabolites appear to be involved in protecting themselves from microbial infections. Disease resistance in plants is provided by a number of barriers, including defence proteins, lignification, physical appressoria, etc. (Harborne, 1993). Additionally, low molecular weight chemicals provide barriers to microbial invasion either by their presence

on the plant surfaces, where they inhibit spore germination or else by their occurrence in bound form within the leaf and their subsequent release in the free state during the infection process (Grayer & Harborne, 1994).

Although almost 20 000 microbial metabolites and approximately 100 000 plant products have been described so far, secondary metabolites still appear to be an inexhaustible source of lead structures for new antimicrobials, antivirals, antitumour drugs, and agricultural and pharmacological agents. In addition, numerous secondary metabolites, such as erythromycin were lead structures that later became the basis for synthetic and semi-synthetic derivatives with improved pharmacological properties (Vincete *et al.*, 2003).

Until the 1970's fungal infections were considered largely treatable and the demand for new medicines to treat them was very small. Thus only a limited number of new antifungals are currently available for the treatment of life threatening fungal infections. These

antifungal agents show some limitations and resistance to the azoles (Georgopapadokou & Walsh, 1994), despite several recent improvements (Vincete *et al.*, 2003). The research and development of new naturally occurring antifungal agents, with novel mechanisms of action is therefore needed.

In this part of the study, the acetone extract of *M. villosus* was tested for its antifungal activity.

4.2 Materials and Methods

4.2.1 Plant extract

The acetone leaf extract was prepared as described in chapter 3, page 29. The crude extract was tested at final concentrations of 5.0, 1.0 and 0.1 mg/ml for its antifungal effect.

4.2.2 Fungal cultures

The following fungal cultures: *Aspergillus flavus, A. niger, Clasdosporium cladosporioides, C. cucumerinum, C. sphaerospermum* and *Phythophthora capsici*, were used in the antifungal tests. The fungal cultures were

maintained on Potato Dextrose Agar (PDA) (Biolab). One loop of inoculum was applied to the centre of a 10 ml petri dish with PDA medium and then incubated at 25°C in the dark for 48 to 72 hours (Adedayo *et al.*, 1999; Picman & Schneider, 1993; Ohashi *et al*, 1994).

4.2.3 Antifungal bioassay

Under aseptic conditions, using sterile filters (0.22µm), 0.05 ml of leaf extract was added to 5 ml of PDA maintained at 45°C. The mixture was swirled and poured into petri dishes, which were then set aside to solidify overnight. A petri dish containing 1.0% acetone and PDA and another petri dish with only PDA served as controls. Small circular disks containing fungi were cut from the growing edge of young fungi mycelia and placed in the centre of the petri dishes (Adeyayo et al., 1999; Grosvenor et al., 1995). The plates were then sealed with parafilm to prevent desiccation, and incubated at 25°C for 48 to 72 hours and observed.

4.3 Results and Discussion

Inhibition of fungal growth was evaluated by comparing the size of colonies of the controls with those of the treatments (fig 4.1). The minimum inhibitory concentration (MIC) values are given in Table 4.1.

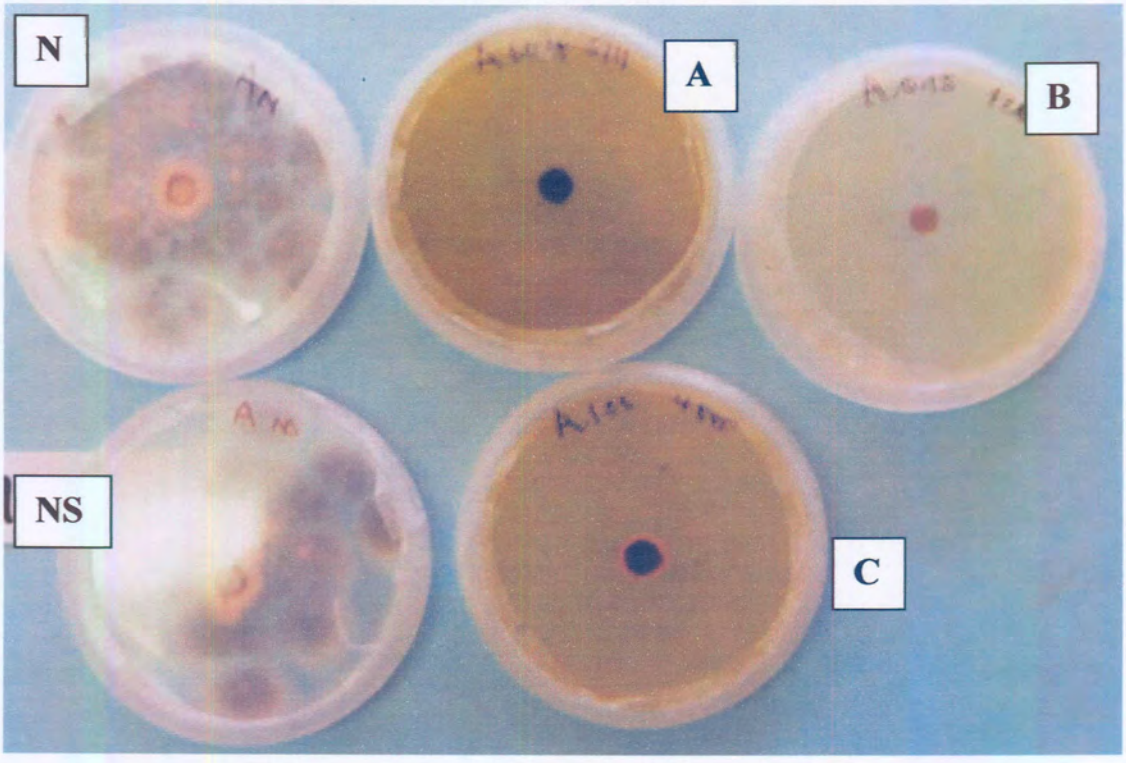

Fig. 4.1. A photograph showing the inhibition of fungi by the plant extract of *M. villosus* (Control N - nutrient agar only; Control NS - nutrient agar and solvent; A, B & C nutrient agar, solvent and extract)

Table 4.1. The antifungal activity of the acetone leaves extract of *M. villosus*.

Fungal species	[a]MIC (mg/ml)
Aspergillus flavus	na[b]
A. niger	na
Cladosporium cladosporoides	1.0
C. cucumerinum	1.0
C. sphaerospermum	1.0
Phytophthora capsici	na

[a]Minimum Inhibition Concentration
[b]Not active

Cladosporium cladosporoides, *C. cucumerinum* and *C. sphaerospermum* were inhibited at the minimum inhibitory concentration of 1.0 mg/ml of the *M. villosus* extract. No growth of these cultures was observed at this concentration. The acetone solvent also proved to have had no effect on the inhibition of the fungal cultures as the control plates showed significant growth.

There are no pharmacological studies reported thus far on the antifungal activity of the species of the genus *Melianthus*. This study however shows that the genus might have antifungal properties.

There is no doubt that *M. villosus* has *in vitro* antifungal activity. However, purification and identification of the antifungal agents will be of importance if these compounds are to be used for the benefit of humanity.

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Chapter 5

Isolation and purification of antibacterial compounds from *Melianthus villosus*

Isolation and purification of antibacterial compound from *Melianthus villosus*

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Abstract

The leaf and root extracts of *M. villosus* were subjected to chromatographic isolation and purification techniques, which resulted in two compounds whose activity was investigated by direct bioassay with thin layer chromatography. Ultraviolet/visible spectral identification of the antibacterial active flavonoid compound indicated that it was a flavonol, quercetin, and the second compound an unidentified triterpenoid.

Keywords: *Melianthus villosus*, flavonol, quercetin, triterpenoid.

5.1 Introduction

Natural products form an important category of test compounds in the continued search for new drugs. The rich diversity of structural types provided by nature also adds to their attractiveness as bioactive compounds (Lednicer & Narayan, 1993; Cordell, 1995).

The availability of suitable separation methods for the isolation of pure products is the key to any study of material from natural sources. Chromatography, the separation process in which the compound is distributed between two phases in the chromatographic bed (which can either be a column or a plate) was used to separate and purify the crude extract. One phase is stationary whilst the other passes through the chromatographic bed. The best mobile phase for specific separation is usually determined by thin layer chromatography (TLC) (Meyer, 1994).

According to Betina (1973), bio-autography is the most important detection method for new or unidentified

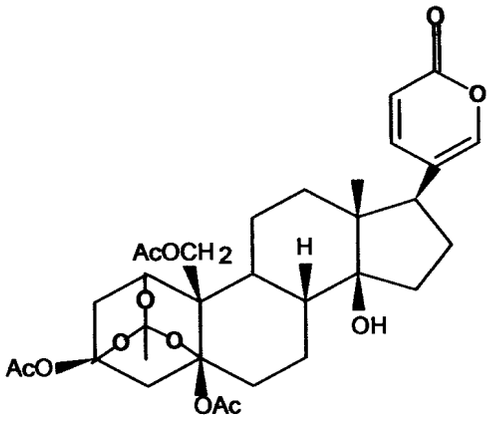
antimicrobial compounds. It is based on the biological (antibacterial, antiprotozoal, antitumoral, etc.) effects of the substances under study. The typical bio-autography is based on the so-called agar-diffusion technique, whereby the antibacterial compound is transferred from the chromatographic layer to an inoculated agar plate (Rios *et al.*, 1988).

In direct bioautography, a microorganism suspension in liquid medium is sprayed on a developed chromatoplaque after removing the solvents. It is then incubatedand inhibition zones are visualised with tetrazolium salts (Hamburger & Cordell, 1987).

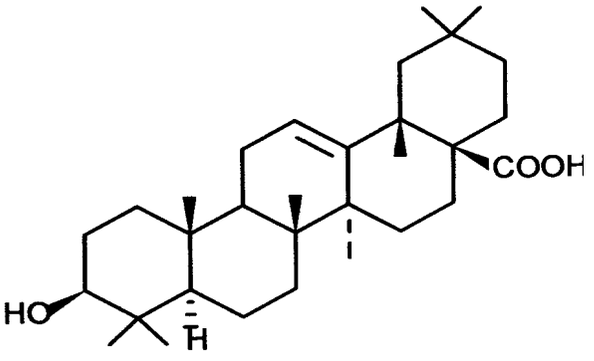
In this study, *M. villosus* was extracted with ethanol and separated into fractions through the use of column chromatography and preparative thin layer paper chromatography. The antibacterial compound was tested by direct bioassay and identified by the use of UV/VIS spectroscopy and nuclear magnetic resonance spectroscopy.

5.2 Previously isolated compounds from *Melianthus comosus*

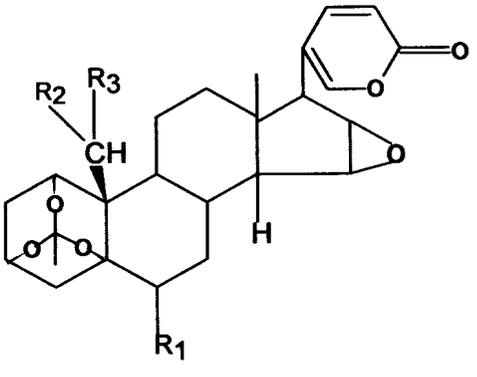
Anderson and Koekemoer (1968) described the isolation of hellebrigenin-3-acetate and other unidentified bufadienolides from the rootbark of *M. comosus*, and in 1969 they described the toxic bufadienolide, melianthusigenin isolated from *M. comosus* (Anderson & Koekemoer, 1969)(fig.5.1). The isolation of two novel bufadienolides from *M. comosus*, 14-deoxy-15β,16β-epoxy-melianthugenin and 6β-acetoxy-14-deoxy-15β,16β-epoxy-melianthugenin was reported by Koekemoer *et al.*, (1971)(fig.5.1). Van Wyk *et al.*, (1997) reported on the isolation of a triterpenoid and oleanolic acid (fig.5.1).



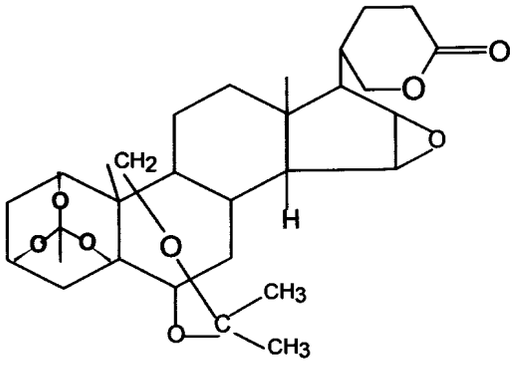
Melianthusigenin
(Van Wyk, *et al.*, 1997)



Oleanolic Acid
(Van Wyk, *et al.*, 1997)



14-deoxy-15β,
16β-epoxymelianthugenin
(Anderson, *et al.*, 1971)



6β-acetoxy-14-deoxy-15β,
16β-epoxymelianthugenin
(Koekemoer, *et al.*, 1971)

Fig. 5.1 Previously isolated compounds from *Melianthus comosus*

5.3 Materials and methods

5.3.1. Extract preparation

Leaves (1.0 kg) and roots (1.0 kg) of *Melianthus villosus* were homogenised separately and left to stand for a week submerged in ethanol, thereafter the extracts were filtered and concentrated to almost dryness under reduced pressure. The crude extract was re-dissolved in ethyl acetate and then extracted three times using aqueous methanol. The aqueous methanolic supernatant was decanted, and the ethyl acetate layer was then concentrated to dryness under reduced pressure resulting in dry matter of 5.030g from the leaves and 7.095 g from the roots (fig 5.2).

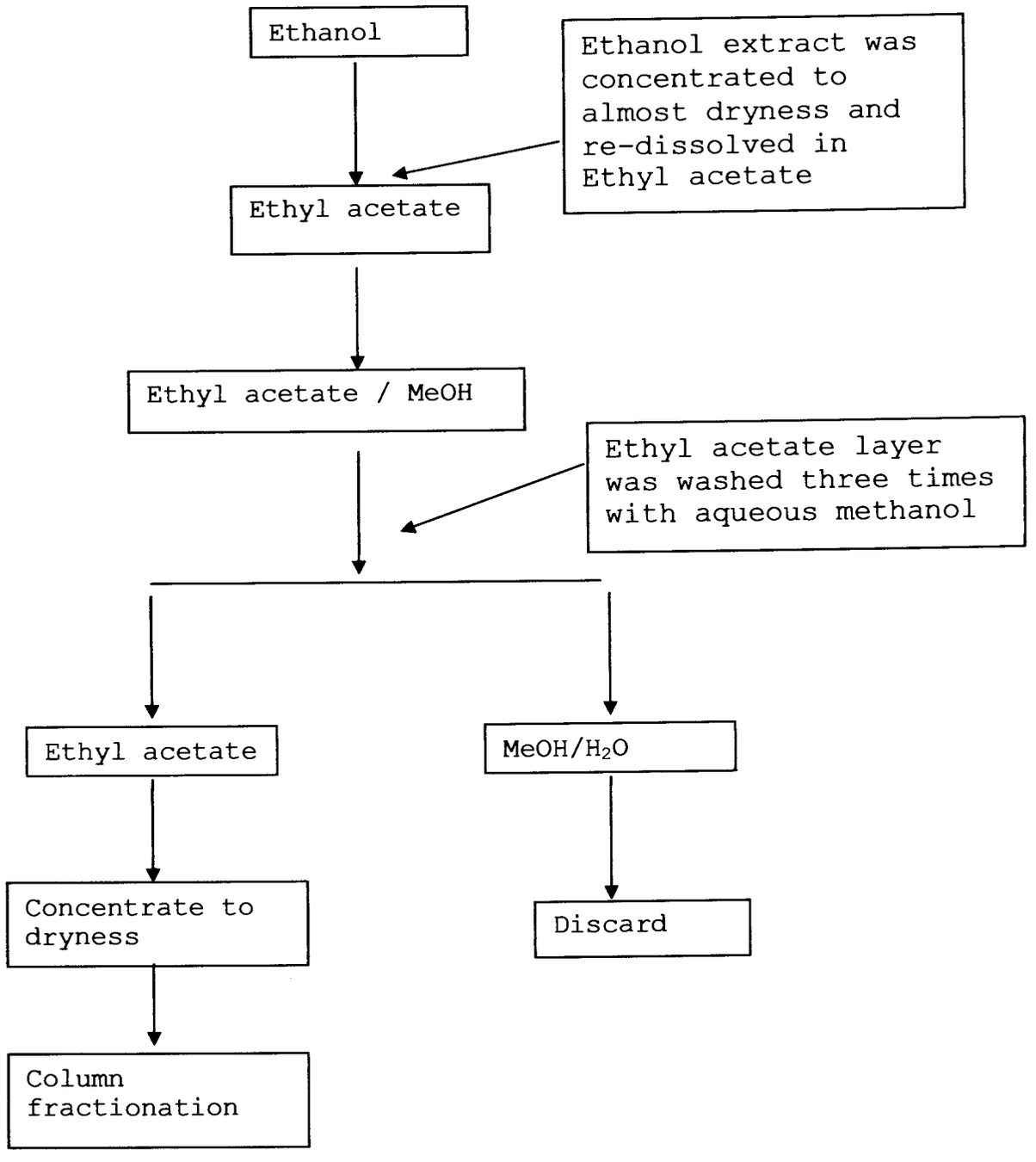


Fig. 5.2 A schematic drawing showing the preparation of the extract

5.3.2 Development of solvent systems (mobile phase) for separation of extract

The *M. villosus* extract was spotted on TLC plates and eluted individually with mobile phases consisting of;

Hexane : Ethyl acetate (1: 1)

Hexane : Ethyl acetate (8 : 2)

Hexane : Ethyl acetate (2 : 8)

Chloroform : Methanol (9 : 1)

Chloroform : Methanol (1 : 1)

Chloroform : Methanol (1 : 9)

The solvent that yielded maximum separation of the leaf extract (fig 5.3) proved to be hexane/ethyl acetate (8:2) whereas chloroform/methanol (9:1) gave the best separation of the root extract (fig 5.4).

Fig. 5.3 TLC plate showing the extract separation using hexane/ethyl acetate (8:2) mobile phase. (Left: leaf extract; right: root extract)

Fig. 5.4 TLC plate showing the extract separation using chloroform/methanol (9:1) mobile phase. (Left: leaf extract; right: root extract)

5.3.3 Column Preparation

A glass column (75cm X 8.0cm); was loaded with up to a third of its volume with about 20g of silica gel (silica gel 60, size 0.015 - 0.040 mm, Merck). The crude root extract was dissolved in a small amount of ethyl acetate and then mixed with a small amount of silica gel and allowed to dry. The silica gel containing the extract was then introduced to the glass column and topped up with pure silica gel. The column was prepared in this manner, so as to ensure that on introduction of the solvent the compounds within the extract would elute in narrow bands.

The following elution programme was then followed for the fractionation of the *M. villosus* crude extract:

Hexane : Ethyl acetate (70 : 30)

Hexane : Ethyl acetate (50 : 50)

Hexane : Ethyl acetate (30 : 70)

Ethyl acetate (100%)

The eluted fractions were collected and then concentrated to dryness under reduced pressure. The dried fractions were redissolved in a small volume of ethyl acetate then

spotted on a TLC plate and developed with hexane : ethyl acetate (8:2) for the initial fractions collected. A system of hexane : ethyl acetate (1:1) followed by that of (2:8) for previous fractions up to the last fractions. After development of the plates, they were air dried, sprayed with a sulphuric/acetic acid spray reagent ((sulphuric acid: acetic acid : water) (1 : 20 : 4)), and then blow dried with a heat gun.

Fractions containing similar compounds were combined and re-chromatographed on a new column in the same manner as described above, resulting in fractions containing fewer and better separated compounds.

5.3.4 Antibacterial activity determination by direct bioassay

10µl of each fraction was applied to duplicate silica gel 60 plates (Merck) and developed with 5% methanol in chloroform. A 24-hour-old *Bacillus cereus* culture, which was centrifuged at 3000 rpm for 20 minutes, of which the supernatant was discarded and the sedimentary bacteria resuspended in fresh nutrient broth, was sprayed onto the

TLC plates, and then incubated overnight (Meyer & Dilika, 1996). Thereafter the plates were spayed with an aqueous solution of 2.0 mg/ml p-iodonitrotetrazolium violet. The plates were then reincubated at 25°C overnight (Meyer & Dilika, 1996).

The fractions that exhibited antibacterial activity were chosen for further isolation and purification of the compounds. The results were observed after a period of incubation (fig.5.5).

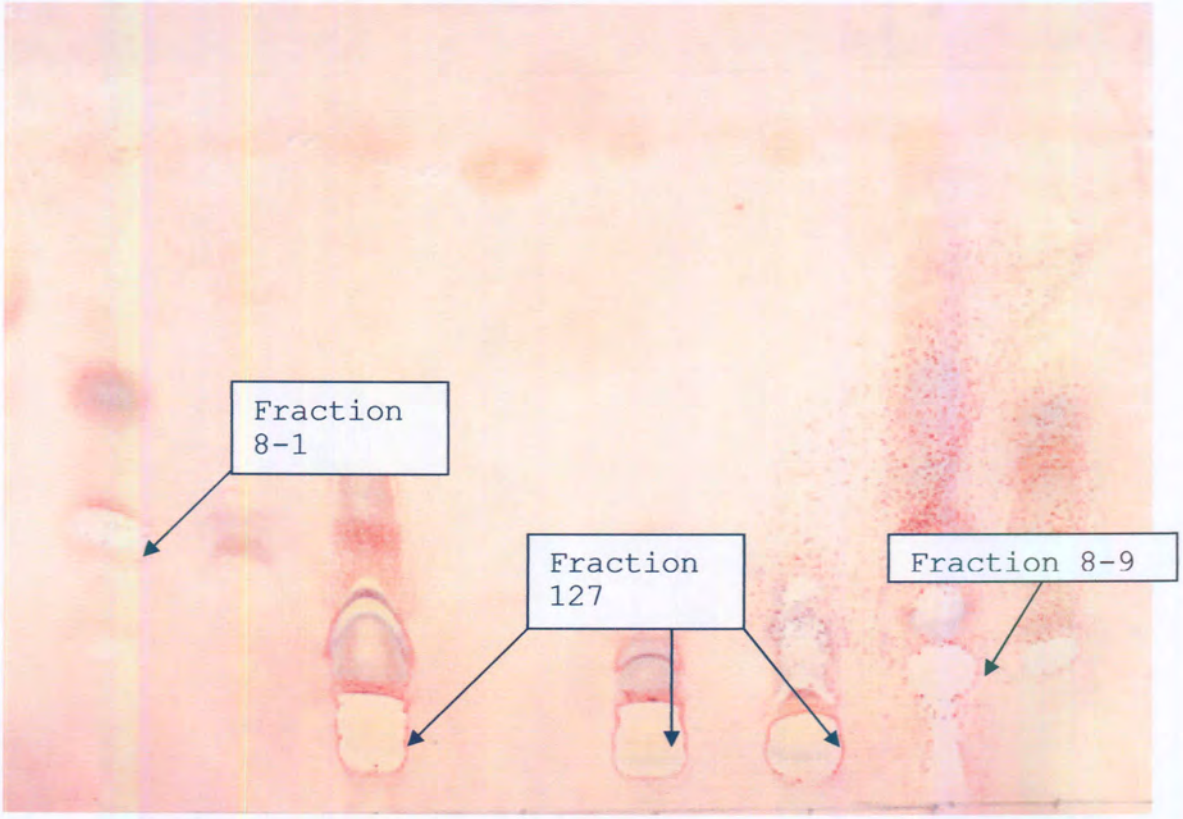


Fig. 5.5 TLC plate showing antibacterial activity using the direct bioassay

5.3.5 Final purification of compound

Fraction 8-9 was spotted on a TLC plate (silica gel 60) and eluted with 20% methanol in chloroform. The resultant traces of a flavonoid were visualised by means of bright yellow-orange spots, which were fluorescent blue under ultraviolet light. A paper chromatographic method of isolation was then implemented. The collected fraction was evenly spread on the paper chromatograph (57 cm x 47 cm) and eluted with 15% acetic acid. The resulting bands were viewed under ultraviolet light, and the bands that exhibited flavonoid characteristics of fluorescent blue colour were cut out. These bands on paper from the chromatograph were soaked in methanol to extract the flavonoid. An ultraviolet absorbance determination of the bandwidth shifts was performed and the ensuing spectra are described under results and discussion (figures 5.7 to 5.12). In addition to the UV/VIS spectroscopy, the extracted compound was then analysed by nuclear magnetic resonance (NMR) spectroscopy.

5.4 Results and Discussion

Fraction/compound 8-9 exhibited characteristics of a flavonoid observed under ultraviolet whilst exposed to ammonia. A fluorescent yellow-green colour was exhibited on the paper chromatograph where the flavonoid was located. The fluorescent yellow-green colour is exhibited by flavones and flavonols with (and sometimes without) a free 5-OH (e.g. 5 - O - glycosides)(Markham, 1982).

5.4.1 UV/VIS Absorbance spectra

The absorbance spectra (fig. 5.7) of the flavonoid/fraction 8-9 dissolved in methanol had band II is between 250 and 280nm, which characteristic of flavones and flavonols, but since band I is between 330 and 360nm it shows that it is a flavonol that has a 3-OH substituted group.

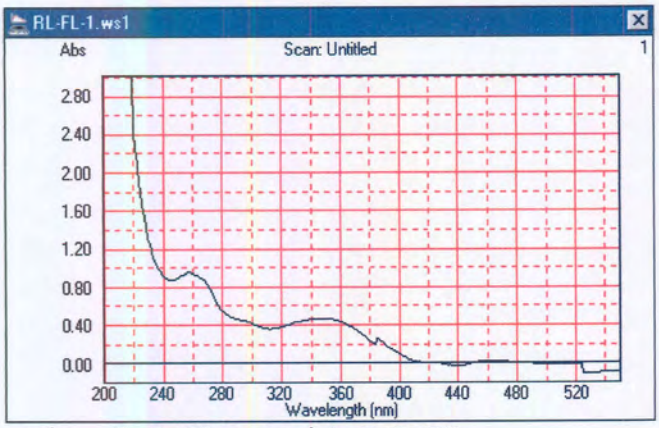


Fig. 5.7 Flavonoid + MeOH

When the flavonoid/fraction 8-9 was added to sodium methoxide (fig. 5.8) band I showed reduced intensity (i.e. decomposition) as compared to the spectra where methanol was added. This means that there is a 3,4'-OH substitution; an A-ring; an O-diOH and a B-ring with an -OH group (this is characteristic of flavones and flavonols) (Mabry *et al.*, 1970).

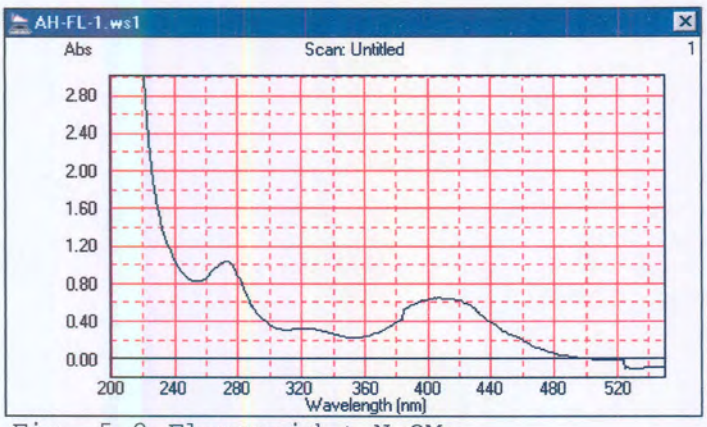


Fig. 5.8 Flavonoid + NaOMe

When the flavonoid fraction (8-9) was added to aluminium chloride (fig. 5.9) band II shifted by 30 to 40nm when compared to the spectra in methanol, showing that there is a B-ring with O-diOH. (And this is characteristic of flavones and flavonols) (Mabry *et al.*, 1970).

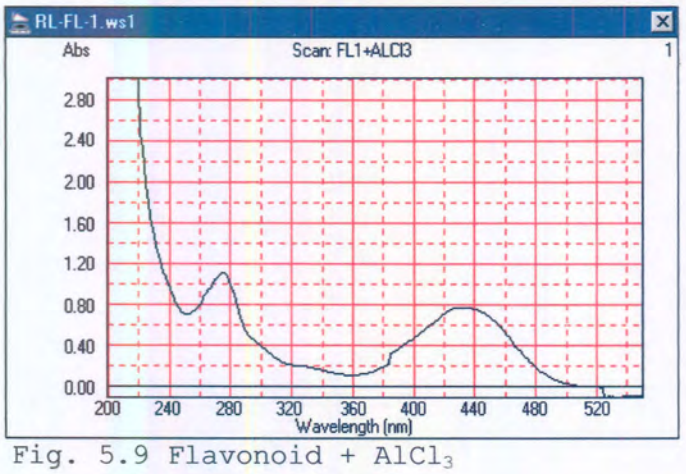


Fig. 5.9 Flavonoid + $AlCl_3$

The flavonoid fraction (8-9) mixed with aluminium chloride and hydrochloric acid (fig. 5.10), shifted band I by 50 to 60 nm when compared to the spectra in methanol, meaning that a 3-OH substitution is possible (and it may be with or without 5-OH) (Mabry *et al.*, 1970).

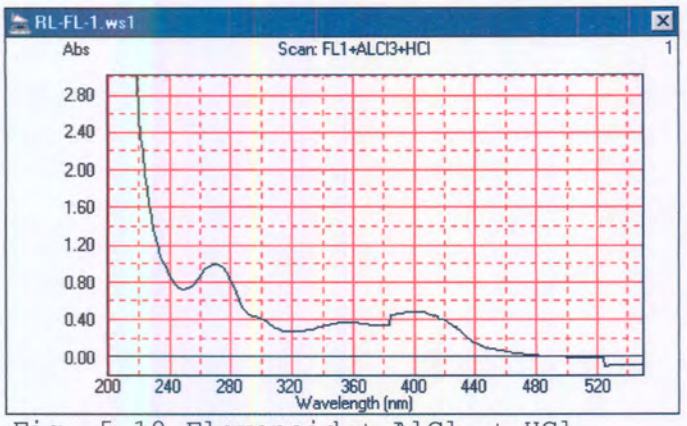


Fig. 5.10 Flavonoid + $AlCl_3$ + HCl

The flavonoid fraction (8-9) mixed with sodium acetate and boric acid (fig. 5.11), shifted the bands between 12 and 36 nm when compared to the spectra with methanol, suggesting a B-ring with O-diOH (a characteristic of flavonols) (Mabry *et al.*, 1970).

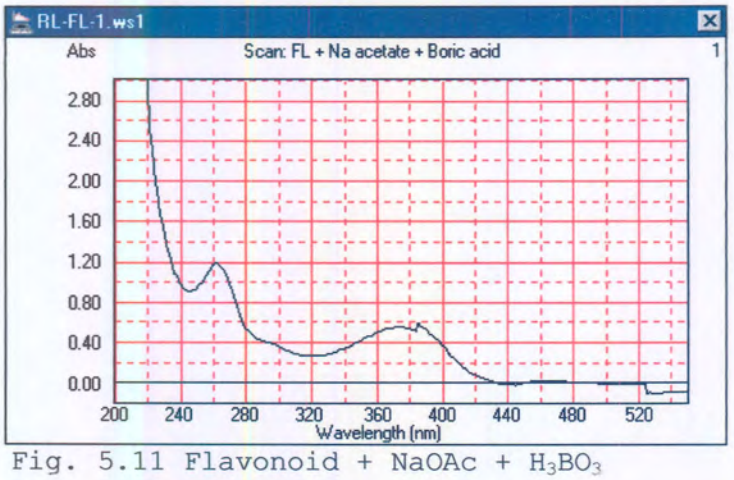


Fig. 5.11 Flavonoid + NaOAc + H_3BO_3

The flavonoid fraction (8-9) with sodium acetate only (fig. 5.12), shifted band II by +5 to 20 nm when compared to the spectra in methanol, suggesting there is a 7-OH substitution (reduced if 6- or 8- oxygenation present) (a characteristic of flavonols) (Mabry *et al.*, 1970).

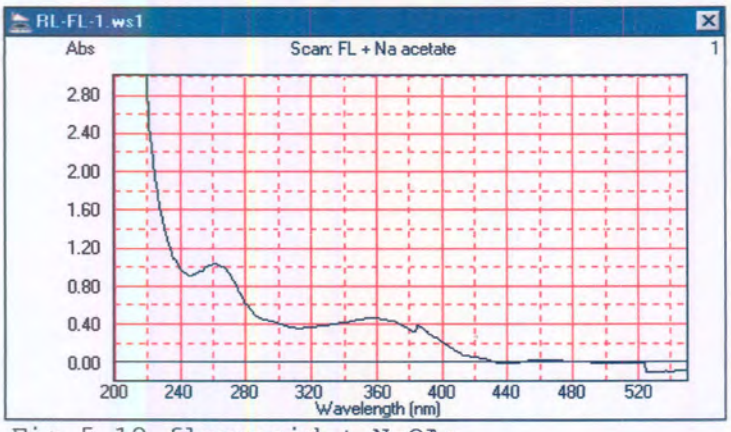


Fig.5.12 flavonoid + NaOAc

These results and interpretations show to a certain degree (Table 5.1) that the flavonoid is a flavonol, probably quercetin as it also has an -OH substitution at 3′, 4′ positions, but certainty can only be achieved if this flavonoid fraction were compared to a quercetin standard.

Table. 5.1 Comparison of chromatographic data of the flavonoid fraction and the Quercetin standard

Treatment	Standard Quercetin	Isolated compound
UV Spectral data (λ_{max}.nm)		
MeOH	255, 269sh, 301sh, 370	255, 270sh, 289sh, 350
NaOME	247sh, 321 (dec.)	265, 321 dec.
$AlCl_3$	272, 304sh, 333, 458	272, 320sh, 382 - 430
$AlCl_3$/HCl	265, 301sh, 359, 428	265, 301sh, 360, 420
NaOAc	257sh, 274, 329, 390(dec)	257sh, 274, 330, 390 dec.
NaOAc/H_3BO_3	261, 303sh, 388	261, 303sh, 388

These results could not be confirmed by the ^{1}H-nmr, as the flavonoid/fraction 8-9 showed to contain impurities. The fraction was then spotted on a TLC plate alongside a standard Quercetin solution (Sigma-Aldrich). The results depicted in figure 5.13 show that the plant extract has a comparable compound in terms of R_f values and or position. This in essence means that the isolated flavonol from *M. villosus* is most probably quercetin. Quercetin was previously isolated from *M. major* (Harborne, 1988).

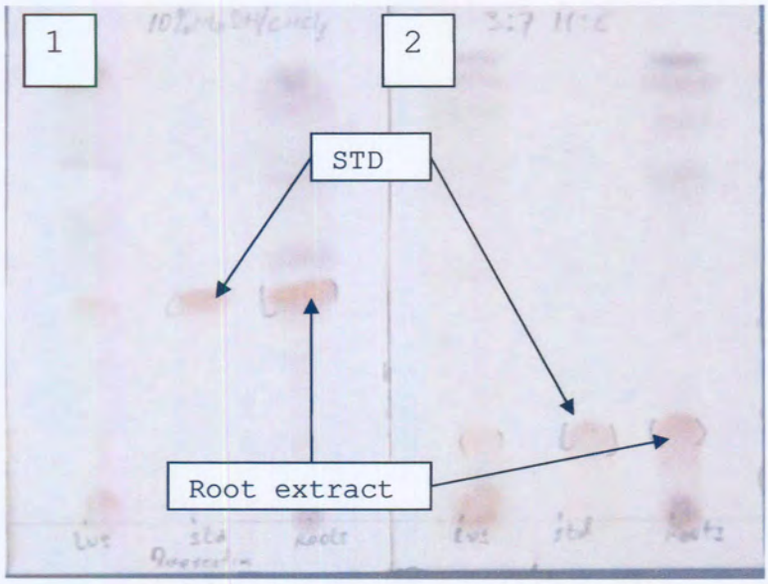


Fig.5.13 TLC plate 1: The separation using 10% methanol in chloroform and TLC plate 2: The separation using hexane and ethyl acetate (3:7). Also shown are the compared fractions of the standard quercetin (STD) and the relevant extract fractions (root extract).

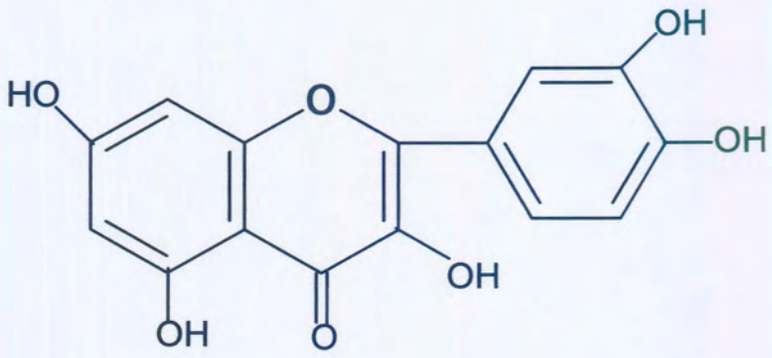


Fig. 5.14 Structure of the flavonol, *Quercetin*

5.4.2 Antibacterial activity of the isolated compound

The preparative paper chromatography resulted in good separation of the fractionated extract to yield the isolated flavonoid fraction. By using the bioassay method on TLC plates, the growth of *B. cereus* was inhibited by the isolated fraction of the flavonoid. Since only less than 10 mg was yielded as the final product, the complete test for antibacterial activity could not be performed.

Flavonols found among the members of the Melianthaceae family are quercetin and or kaempferol (Harborne, 1988), Cottiglia *et al.*, 2001, showed that the flavonol quercetin has antibacterial activity.

5.4.3 Nuclear Magnetic Resonance spectra

Fraction 127 was selected on its purity and eluted with Sephadex (LH-20) as the stationary phase and methanol as the solvent. A relatively pure compound was isolated, and then analysed by proton and carbon nuclear magnetic resonance (^{1}H & ^{13}C NMR) (figures 5.15 and 5.16). The

resultant analysis showed that the compound is probably a triterpenoid, which at this stage could not be identified.

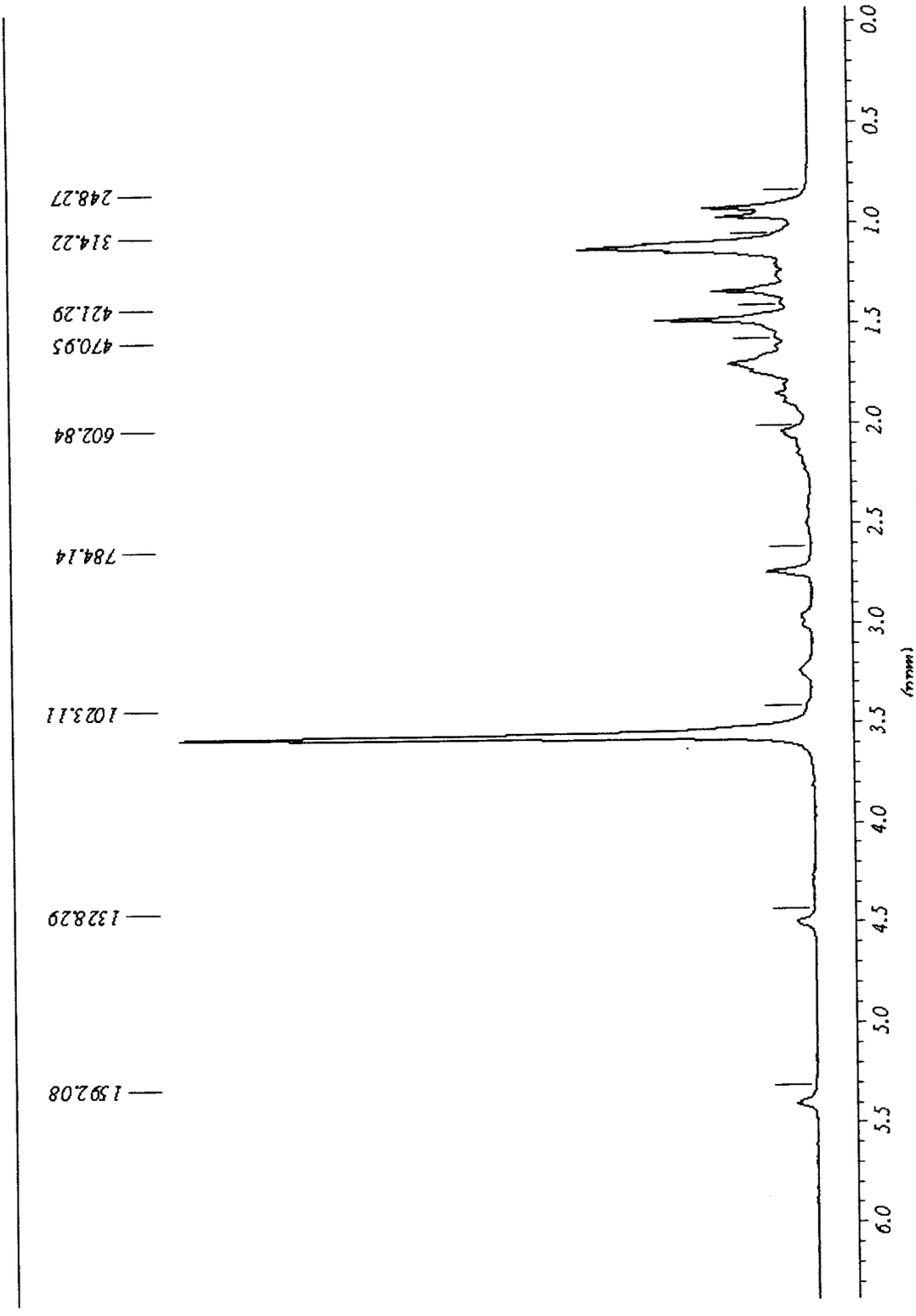


Fig. 5.15 1H NMR spectrum of the compound isolated from M. villosus

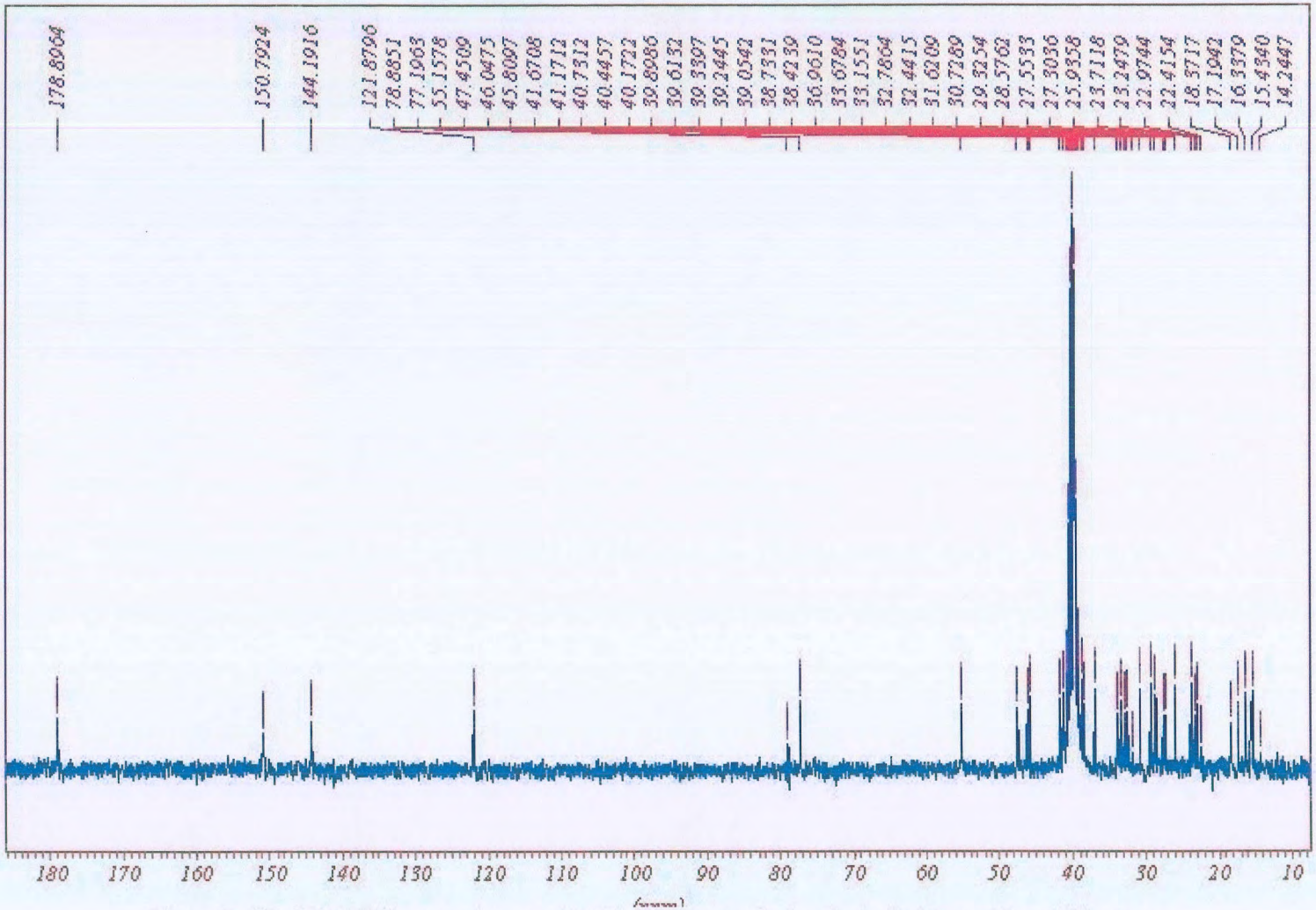


Fig. 5.16 13C NMR spectrum of the compound isolated from M. villosus

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Chapter 6

General Discussion

6.1 Introduction

Medicinal plants used by traditional healers are proving to be an important source of potentially therapeutic drugs (Cox & Balick, 1994) and are thus increasingly becoming an alternative form of health care.

The purpose of this study was to investigate the *in vitro* antimicrobial activity of *M. villosus*. The study was undertaken because according to literature the plant has never been tested for antimicrobial properties, though species from the same genus are known to have antibacterial properties. In contrast with *M. major* and *M. comosus*, *M. villosus* is not used locally as a medicinal plant, and in actuality very few of the traditional healers consulted are aware of its existence.

The lack of ethnobotanic knowledge of *M. villosus* was a further stimulus to investigate its microbial properties, and to isolate the active principles.

6.2 Antibacterial activity of *M. villosus*

The antibacterial activity of the plant extract was found mainly on Gram-positive bacteria and also against *E. coli*. This confirmed the suspicion that some members of the genus *Melianthus* contain active antibacterial agents (Jäger, *et al.*, 2000). Leaves and roots from *M. villosus* both showed activity, but it was the root extract which showed higher activity in terms of MIC, and this may explain why may people use the roots of other species of the genus of *Melianthus* in traditional medicine.

6.3 Antifungal activity

Antifungal activity was observed at the MIC 1.0 mg/ml level for the following fungal cultures pitted against the *M. villosus* leaf extract: *Cladosporium cladosporoides, C. cucumerinum* and *C. sphaerospermum*.

6.4 Isolation of antibacterial compounds

The main antibacterial principle from *M. villosus* was isolated. Using column chromatography, and monitoring

by thin layer chromatography several fractions were collected. The fraction that showed activity against *B. cereus,* as determined by direct bioautography on TLC plates, was then subjected to nuclear magnetic resonance (NMR) analysis for ^{1}H NMR and ^{13}C NMR.

Upon further investigation of the bioactive fraction using UV/VIS spectroscopy, the active compound was identified as the flavonol, quercetin, and this was further confirmed by the comparison made of the plant extract against the standard of Quercetin on thin layer chromatography plates.

The primary objective of this study was achieved by showing that *M. villosus* has microbial properties, which in essence means that traditional healers could possibly use *M. villosus* like other members of this genus. It must, however, be remembered that this study only investigated *in vitro* activity, and toxicity was not evaluated at all.

Further studies on *M. villosus* are required but as with the other members of the genus, the active principles are in very small quantities in the plants. This was evident during the study, because from an initial amount of 1.0 kg of plant material a yield of less than 10 mg was achieved. Anderson *et al.*, (1971), started with 11 kg of plant material and their yields varied between 190 mg and 200 mg of the compounds isolated (chapter 5 section 5.2) from the extract of *M. comosus*.

Further studies on the plant will therefore have to take cognisance of the fact that vast amounts of plant material will be required in order to achieve a substantial amount of end products. This makes it an unattractive study to undertake as the plant will be exposed to over-reaping and this might lead to extinction. A possible solution is to create sustainable populations by means of biotechnolocal propagation such as tissue culture.

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Chapter 7

Summary

Antimicrobial activity of *Melianthus villosus*

by

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Many South Africans continue to use traditional medicine in their daily lives as an alternative form of health care, also as part of their cultural

heritage. Medicinal plants are proving to be an important source of novel drugs, and the knowledge provided by traditional healers is a useful tool in the search for antimicrobials.

The antimicrobial activity of *M. villosus* was investigated against ten bacteria and six fungi. The antibacterial assay showed that the root extract had the highest inhibition against the Gram-positive bacteria at the minimum inhibition concentration of 0.1 mg/ml, as well as against the Gram-negative, *E. coli*, at the MIC of 1.0 mg/ml.

Antifungal activity was witnessed against *Cladosporium cladosporoide*, *C. cucumerinum* & *C. sphaerospermum* all at the minimum inhibitory concentration of 1.0 mg/ml.

An attempt was made to isolate and identify the active antimicrobial compounds. A flavonol, *quercetin* was isolated and identified by means of UV spectral graphs, and TLC comparison of the plant extract and standard. However, a second isolated

antibacterial compound could not be identified fully but it can be said that it is a triterpenoid.