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ANTIMICROBIAL ACTIVITY OF *HELICHRYSUM PEDUNCULATUM* USED IN CIRCUMCISION RITES

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Antimicrobial activity of *Helichrysum pedunculatum* used in circumcision rites

by

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CONTENTS

CHAPTER 1

INTRODUCTION

INTRODUCTION

Background

The practice of traditional healing plays an important role in the health care of millions of people in developing countries (WHO, 1978; Dimayuga & Garcia, 1991). For a long time prior to the development of phytochemical extraction, herbal remedies played a major part in the treatment of diseases. In countless societies throughout the world today, plants feature significantly in local cures for common ailments (Stafford, 1991; Martin, 1995).

In South Africa, people use plants especially for their medicinal, religious and cultural needs. Herbal remedies can also be applied to strategic points for protection against evil spirits and healing from diseases believed to have been inflicted by witches.

The indigenous people of South Africa especially the Xhosas, Pondos, Zulus and Sothos still practise their cultural traditions. These include initiation of girls into womanhood and the traditional method of male circumcision. Male circumcision entails a surgical operation, seclusion (both performed in the wild) and a "coming out" ceremony. The surgical operation involves severing of the foreskin of the male reproductive organ with a sharp knife (Bryk, 1934). The surgery is followed by a

period of seclusion for several weeks in a hut specially made for that purpose, during which the wounds are treated with herbs (Wallerstein, 1990).

Circumcision performed in the wild has a high risk of infection (Green, 1994). The numbers of patients admitted to the Umtata General Hospital as a result of infection after traditional circumcision, for the months of November 1992, December 1992 and January 1993 were 135, 127 and 105 respectively (Dilika *et al.*, 1994). Irrespective of the high incidence of infection, a large sector of the society still believes in the traditional method of circumcision. The use of antibiotics, antiseptics and pain inhibitors by the circumcised youth at the hospital, reduce the respect of the community for them (Bryk, 1934; Dilika *et al.*, 1994).

In Transkei, the Xhosas use *Helichrysum pedunculatum* Hilliard & Burtt, *H. appendiculatum* Hilliard & Burtt or *H. longifolium* DC, depending on the locality and the availability, as bandages for the wounds. The dry outer covering of the bulb of *Boophane disticha* is used as an outer covering of the wound for rapid healing. This prevents inflammation and further prevents the wound from becoming septic (Watt & Breyer-Brandwijk, 1962; Battern & Bokelmann, 1966; Bolofo & Jonson, 1988). *H. pedunculatum* is the most commonly used and this may probably be due to the fact that the herb grows over a wide range of habitats and is hence easily available.

H. pedunculatum is a perennial herb of up to 60 cm in height and grows in grasslands and on hillsides. It is widely distributed (Figure 1), ranging from southern Lesotho to the Eastern Cape province, with isolated records from Graaff-Reinet, Great Swartberg, Uitenhage and Riversdale (Hilliard, 1983). Its leaves are dark green above and pale and woolly below with the capitula arranged in a dense flat head. Involucral bracts are brown (Hilliard, 1983; Battern & Bokelmann, 1966). No specific antimicrobial properties of this plant have been reported. However, it has been reported from folklore to possess medicinal values, which include its ability to cure stomach ailments, anti-inflammatory properties and activity against coughs and colds (Watt & Breyer-Brandwijk, 1962; Bolofo and Johnson, 1988).

A number of *Helichrysum* species have been found to have antimicrobial properties (Tomas-Barberan *et al.*, 1990). These include extracts from *H. stoechas*, which showed antibacterial activity against *Staphylococcus aureus, Mycobacterium phlei* and *Candida albicanus* (Rios & Villar, 1991); *H. decumbens* showed antifungal activity against *Cladosporium herbarum* (Tomas-Barberan *et al.* 1988); *H. nitens* inhibited the growth of *C. cucumerinum* (Tomas-Barberan *et al.*, 1988). *H. odoratissimum* was reported to be active against *Escherichia coli, Klebsiellla pnuemoniae, Pseudomonus aeruginosa, Serratia marcescens, Bacillus subtilis, Staphylococcus aureus* and *Aspergillus flavus* (Van Puyvelde *et al.*, 1989). The South African species, *Helichrysum aureonitens* showed antibacterial activity against *Micrococcus kristinae,*

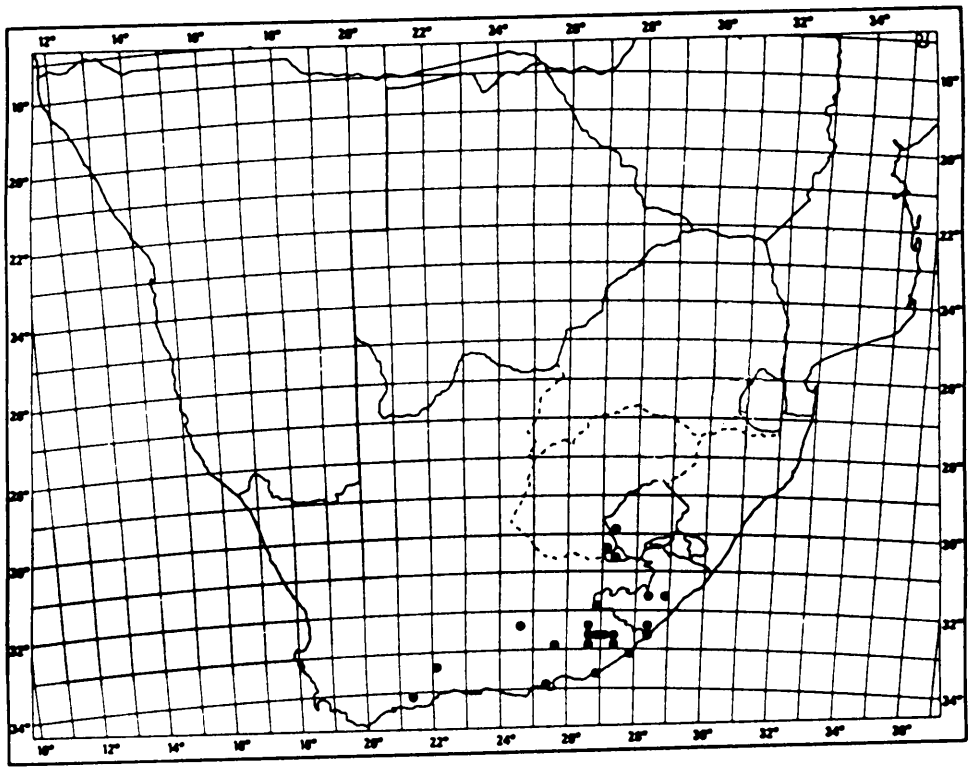

Figure 1. Distribution map of *Helichrysum pedunculatum* (Hilliard, 1983)

Bacillus cereus, B. pumilus and Staphylococcus aureus (Meyer & Afolayan, 1995). *H. caespitium*, another South African species, inhibited the growth of *S. aureus, Streptococcus pyogenes* and *Cryptococcus neoformans* (Dekker *et al.*, 1983).

Objectives of the study

The main objective of this project was to determine if the leaf extracts of *Helichrysum pedunculatum* have activity against some selected microorganisms to verify its claimed medicinal properties. The specific objectives were:

1. To evaluate the bioactivity of the crude dichloromethane, methanol and water extracts against some bacteria.
2. To test the dichloromethane extract against some fungi.
3. To compare the efficacy of *H. pedunculatum* and *H. longifolium* against *Staphylococcus aureus*.
4. To induce callus formation of *H. pedunculatum* and determine if the callus has antibacterial activity.
5. To attempt to isolate, purify and identify the antimicrobial compound(s).

Scope of the dissertation

Antimicrobial compounds from *Helichrysum* species have been shown to be externally deposited (Tomas-Barberan *et al.*, 1988, Tomas-Lorente *et al.*, 1989, Meyer & Afolayan, 1995). The leaves of *Helichrysum pedunculatum* were therefore shaken in dichloromethane, methanol, and water while another portion was

propagating medicinally useful plants is by tissue culture. A large number of investigations to study the nutritional requirements of plant tissue cultures of different origin have been conducted. The concentration of each component of the medium is evaluated by studying its effect on tissue growth mainly determined by fresh weight increase (Basile *et al.*, 1993; Sacchi *et al.*, 1995; Tadera *et al.*, 1995; Zagorska & Dimitrov, 1995). For maintenance of tissue viability and growth, successive subcultures on fresh medium are necessary. This is mainly to avoid any effect of spent culture medium in terms of both nutrient availability and presence and accumulation of metabolites excreted by the callus (Hussain *et al.*, 1994; Sacchi *et al.*, 1995). Another objective of this study was to induce callus formation from *H. pedunculatum*. The callus produced was homogenised in dichloromethane and the extract tested for antibacterial activity using direct bioautography on thin layer chromatography plates.

Bioactivity directed fractionation is a common method of isolating medicinal compounds from plants (Glinski *et al.*, 1990, Cordell, 1995). Isolation of pure active compounds is essential for structure elucidation. In an attempt to isolate and identify biologically active compounds from this plant, numerous techniques were used including centrifugal partition chromatography (CPC), counter-current chromatography (CCC), medium pressure chromatography (MPLC), thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC).

Structure:

This dissertation consists of contributions in the form of a reprint of a published paper (Chapter 2), two manuscripts sent for publication (Chapters 4 and 5) and two manuscripts under consideration for publication (Chapters 3 and 6).

The antibacterial activity of extracts prepared from *Helichrysum pedunculatum* by shaking and homogenising the leaves in water methanol and dichloromethane, is described in Chapter 2. Chapter 3 describes the antifungal activity of leaves shaken in dichloromethane. Antibacterial activities of *H. pedunculatum* and *H. longifolium* leaf extracts were compared in Chapter 4. The effect of temperature on the antibacterial activity of the latter was also dealt with in this chapter. Initiation of the callus from the leaves of the plant is described in Chapter 5. This chapter also deals with the antibacterial activity of the callus produced. In the sixth chapter, all attempts to separate, purify and identify the antibacterial compound(s) from dichloromethane extracts are described, while Chapter 7 consists of the general discussion and conclusions.

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CHAPTER 2

ANTIBACTERIAL ACTIVITY OF *HELICHRYSUM PEDUNCULATUM* USED IN CIRCUMCISION RITES

ELSEVIER

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Short communication

Antibacterial activity of *Helichrysum pedunculatum* used in circumcision rites

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Abstract

Antibacterial assays of *Helichrysum pedunculatum* showed that dichloromethane extracts are active against all the gram positive bacteria tested, as well as two gram negative bacteria, *Enterobacter cloacae* and *Serratia marcescens*. A water extract was effective against *Staphylococcus aureus* and *Micrococcus kristinae*, while a methanol extract showed no activity against any of the tested organisms. The antibacterial activity of dichloromethane extract was also investigated by direct bioassay on TLC plates against *S. aureus*.

Keywords: Helichrysum pedunculatum; Antibacterial; Anti-inflammatory; Asteraceae

1. Introduction

A number of *Helichrysum* (Asteraceae) species have been reported to have antimicrobial properties (Tomas-Barberan et al., 1990). These include extracts from *H. stoechas*, which showed antimicrobial activity against *Staphylococcus aureus*, *Mycobacterium phlei* and *Candida albicans* (Rios and Villar, 1991). The southern African species, *H. aureonitens* showed activity against *Micrococcus kristinae*, *Bacillus cereus*, *B. pumilus* and *Staphylococcus aureus* (Meyer and Afolayan, 1995).

The *Helichrysum* genus consists of about 500 species which are mainly found in Africa. The 245 South African *Helichrysum* species are morphologically heterogeneous and are therefore classified into 30 groups (Hilliard, 1983). *H. pedunculatum* Hilliard and Burtt (group 23) is a perennial herb with a wide distribution, ranging from southern Lesotho to the Eastern Cape province, with isolated records from Graaff-Reinet, Great Swartberg, Uitenhage and Riversdale (Hilliard, 1983). No specific antimicrobial properties of this plant have been reported. Bolofo and Johnson (1988) and Watt and Breyer-Brandwijk (1962) have however reported on its medicinal values, which included its ability to cure stomach ailments and its anti-inflammatory properties, respectively. The Xhosas of Transkei (South Africa) commonly use the plant to dress wounds especially after cir-

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cumcision. No scientific basis has however been established for its effectiveness. To investigate its claimed medicinal properties, the antibacterial activity of *H. pedunculatum* was examined on nine selected bacterial species.

2. Materials and methods

2.1. Plant material

Leaves of *H. pedunculatum* were collected from Transkei, a region in the Eastern Cape province of South Africa, during May 1995. A voucher specimen (Dilika 299) was prepared and deposited at the H.G.W.J. Schweickerdt Herbarium (PRU), Pretoria.

2.2. Preparation of extracts

Leaves of *H. pedunculatum*, 20 g and 60 g, were homogenized in water and dichloromethane (CH_2Cl_2) respectively, and 40 g, 60 g and 80 g were shaken in water, methanol (CH_3OH) and CH_2Cl_2 respectively. The five extracts were filtered and concentrated to dryness under reduced pressure to determine the mass of extracted material. The dry CH_2Cl_2 extract was redissolved in CH_3OH because of the toxicity of CH_2Cl_2 towards bacteria. CH_3OH showed no inhibition to the growth of the bacterial cultures at a final concentration of 2% (Meyer and Afolayan, 1995)

2.3. Bacteria

Nine selected bacterial species (Table 1) were collected from the Department of Microbiology and Plant Pathology, University of Pretoria. Each organism was maintained on nutrient agar slant and was recovered for testing by growth in nutrient broth (No. 2, Biolab) for 48 h at 37°C. Before streaking, each culture was diluted 1:10 with fresh sterile nutrient broth.

2.4. Antibacterial testing

Plant extracts were sterilized by filtering through 0.22 μm syringe fitted filters and then added to autoclaved nutrient agar (Biolab). Before congealing, 10 ml of nutrient agar medium containing the plant extract was added aseptically to each Petri dish and swirled carefully until the agar began to set. The organisms were streaked in radial patterns on agar plates containing plant extracts, incubated at 37°C and observed after 24 h (Mitscher et al., 1972). Complete inhibition of bacterial growth was expected for an extract to be declared active. The five extracts were tested at 10, 20, 33, 50 and 100 mg/ml. A blank plate containing

Table 1
Antibacterial activity of homogenized (H) and shaken (S) dichloromethane, water and methanol extracts from the leaves of *H. pedunculatum*

Bacterial species	Gram +/−	MIC (mg/ml)				
		CH_2Cl_2		H_2O		CH_3OH
		H	S	H	S	(S)
Bacillus cereus	+	10	10	100	na	na
B. pumilus	+	10	33	na	na	na
B. subtilis	+	10	33	na	na	na
Micrococcus kristinae	+	10	10	100	na	na
Staphylococcus aureus	+	20	33	100	na	na
Enterobacter cloacae	−	50	33	na	na	na
Escherichia coli	−	na	na	na	na	na
Klebsiella pneumoniae	−	na	na	na	na	na
Serratia marcescens	−	na	33	na	na	na

MIC, minimum inhibitory concentration; na, not active.

only nutrient agar and another containing nutrient agar and 2% CH_3OH were used as controls.

For direct bioassay on thin layer chromatography (TLC) plates, 18 μl CH_2Cl_2 extract (20 mg/ml) was applied to silica gel 60 plates (Merck) and developed with chloroform–ethyl acetate (60:40). A 48 h old *Staphylococcus aureus* culture in nutrient broth was centrifuged at 3000 rev./min for 20 min, the supernatant discarded and the sedimented bacteria resuspended in fresh nutrient broth to an absorbance of 0.84 at 560 nm. A fine spray was used to spray the bacterial suspension onto the TLC plates. These plates were then dried until they appeared transluscent and incubated at 25°C for 48 h in humid conditions. After incubation, the plates were sprayed with an aqueous solution of 2.0 mg/ml *p*-iodonitrotetrazolium violet. The plates were then reincubated at 25°C for 24 h (Lund and Lyon, 1975). Each experiment was performed three times.

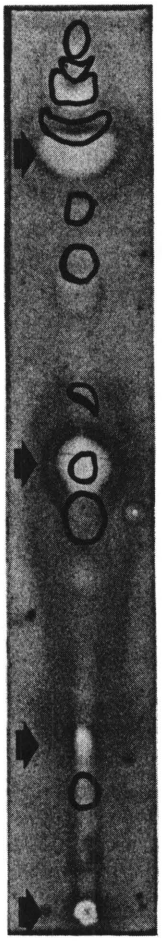

Fig. 1. Zones of inhibition (arrows) of *Staphylococcus aureus* produced after chromatography of a dichloromethane extract of the leaves of *H. pendunculatum* on silica gel 60 plates developed in chloroform–ethyl acetate (60:40). Compounds that absorbed UV light (366 nm) are circled in black.

3. Results and discussion

Klebsiella pneumoniae and *Escherichia coli* showed resistance against all the extracts tested (Table 1). The extracts shaken in CH_3OH and water did not inhibit the growth of any of the tested bacterial species. All the other extracts inhibited the growth of *Bacillus cereus*, *Micrococcus kristinae* and *Staphylococcus aureus* at varying concentrations. Both CH_2Cl_2 extracts were active against all the gram positive bacteria tested as well as the gram negative bacterium, *Enterobacter cloacae*. Only the CH_2Cl_2 shaken extract was active against *Serratia marcescens*. These results are consistent with the pattern of in vitro inhibition emerging from other studies in which it was also found that plant extracts readily inhibit gram positive rather than gram negative bacteria (Grosvenor et al., 1995).

Four zones of bacterial growth inhibition could be seen on TLC plates sprayed with *Staphylococcus aureus* (Fig. 1). Only one of these compounds could be detected by UV light at 366 nm. We are currently attempting to identify these antibacterial compounds.

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CHAPTER 3

ANTIFUNGAL ACTIVITY OF *HELICHRYSUM PEDUNCULATUM*

ANTIFUNGAL ACTIVITY OF *HELICHRYSUM PEDUNCULATUM*.

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ABSTRACT

Antifungal assays of *Helichrysum pedunculatum* proved that dichloromethane extract is active against *Aspergillus flavus*, *A. niger*, *Cladosprorium cladosporioides*, *C. cucumerinum*, *C. sphaerospermum* and *Phythophthora capsici*. The growth of *C. cucumerinum* was inhibited at a concentration of only 0.1 mg/ml and *C. cladosporoides* and *C. sphaerospermum* were inhibited at 1 mg/ml.

Keywords: Helichrysum pedunculatum, antifungal

INTRODUCTION

Antifungal drugs are amongst the most expensive antibiotics (Collee, 1976). The high cost of the newer and more effective antimicrobial drugs makes 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 pathogenic fungi of humans. 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). Pathogenic fungi are biochemically similar to their human hosts and this similarity may cause some difficulty in the development of a drug that is effective against an invading fungus, but safe to the host (Erol *et al.*, 1995).

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). As part of the continued search for antifungal compounds from plants, several Spanish *Helichrysum* species have been investigated. Various compounds isolated from *H. decumbens, H. italicum and H. stoechas* showed antifungal activity against *Phythophthora capsici, Aspergillus flavus and A. niger*. (Tomas-Barberan *et. al.* 1990). A number of compounds isolated from *H. nitens* showed some antifungal activity against *Cladosporium cucumerinum* (Tomas-Barberan *et al*,

1988). Caespitin isolated from *H. caespititium* showed antifungal activity against *Microsporum canis, Trichophyton rubrum and T. mentagrophytes* (Dekker *et al.*, 1983).

In this study, the dichloromethane extract of *Helichrysum pedunculatum* was tested for its antifungal activity.

MATERIALS AND METHODS

Extract preparation.

Leaves of *Helichrysum pedunculatum* (300 g) were shaken for 5 minutes in dichloromethane (CH_2Cl_2) without being homogenised. This was repeated two times. The extract was then filtered and concentrated to dryness under reduced pressure. The dry weight of the residue was 2.683 g, and this was dissolved in 50 ml of CH_2Cl_2. Final concentrations of 10 mg/ml, 1 mg/ml, 0.1 mg/ml and 0.01 mg/ml were prepared and tested for their antifungal effect.

Fungal cultures

The extract was tested against the following fungi, *Aspergillus flavus, A. niger, Cladosporium cladosporioides, C. cucumerinum, C. sphaerospermum* and *Phythophthora capsici*. Fungi were obtained from the Institute of Plant Protection, Pretoria. The fungal cultures were maintained on Potato Dextrose Agar (PDA) (Biolab). One loop of inoculum was applied to the centre of 10ml PDA medium in a petri dish and it was then incubated at 25^0C in the dark for 72 hours (Picman & Schneider, 1993; Ohashi *et al.*, 1994).

The antifungal bioassay

Under aseptic conditions, 0.05 ml of each plant extract was added to 5 ml of PDA maintained at 45 ^{o}C. The mixture was swirled and poured into petri dishes (65 mm diameter) which were then set aside to solidify overnight. Most of the CH_2Cl_2 should have evaporated during this time. All tests were run in three replicates. A petri dish with 0.05 ml (1 %) CH_2Cl_2 and PDA and another one with with only PDA, served as controls. Small circular disks containing fungi were cut from the actively growing margins of the subcultures and placed in the centre of petri dishes containing the extract (San Fransisco & Cooper-Driver, 1984; Picman *et al.*, 1990; Grosvenor *et al.*, 1995). The plates were then sealed with parafilm to prevent desiccation. After incubation for 72 hours at 25^{o}C, the plates were examined.

RESULTS AND DISCUSSION

Inhibition of fungal growth was evaluated by comparing the size of colonies of the controls with those of the treatments. The minimum inhibitory concentration (MIC) of the fungal growth was determined (Table 1).

Table 1. The antifungal activity of the dichloromethane extract of *Helichrysum pedunculatum* .

Fungal species	MIC (mg/ml)
Aspergillus flavus	not active
A. niger	not active
Cladosporium cladosporoides	1.0
C. cucumerinum	0.1
C. sphaerospermum	1.0
Phytophtora capsici	10.0

Cladosporium cucumerinum was the most sensitive fungus to the CH_2Cl_2 extract (0.1 mg/ml). A number of compounds in the CH_2Cl_2 rinses of soaked *Helichrysum nitens* leaves also showed antifungal activity against *C. cucumerinum* (Tomas-Barberan *et al.*, 1988). Three antifungal phloroglucinol derivatives identified from *H. decumbens* prevented the growth of *C. herbarum* in a bioassay (Tomas-Lorente *et al.*, 1989). Compounds isolated from *H. decumbes and H. italicum* investigated by Tomas-Barberan *et al.* (1990) were also active against *Phytophthora capsici*. The concentrations tested in this study had no effect on the growth of *Aspergillus flavus and A. niger*. These fungal species also showed resistance against the various compounds separated from *H. decumbes and H. italicum* (Tomas-Barberan *et al.*, 1990). However, 3-0-methylquercetin isolated from *H. odoratissimum* was active against *A. flavus* at 100 µg/ml (Van Puyvelde *et al.*, 1989)

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CHAPTER 4

*COMPARATIVE ANTIBACTERIAL ACTIVITY OF TWO *HELICHRYSUM* SPECIES USED IN MALE CIRCUMCISION IN SOUTH AFRICA

* Written in the format of a short communication for South African Journal of Botany

SHORT COMMUNICATION

Comparative antibacterial activity of two *Helichrysum* species used in male circumcision in South Africa.

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Abstract

The leaves of *Helichrysum pedunculatum* and *H. longifolium* are used for the treatment of wounds arising from circumcision in Transkei, South Africa. The antibacterial activity of these herbs were compared by direct bioautography using *Staphylococcus aureus*. Extracts from the leaves of *H. pedunculatum* showed more activity against the bacterium than those from *H. longifolium*. Heating the extracts from the latter, further reduced their activity against *S. aureus*. The traditonal heating of the leaves of this plant over hot ash before use is, therefore, likely to reduce their activity against infection.

Keywords: Antibacterial, circumcision, *Helichrysum*, medicinal, Pondos, Xhosas.

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Traditional male circumcision is a common practice among the indigenous people of South Africa especially the Xhosas and the Pondos. The practice involves the surgical removal of the foreskin of the male reproductive organ with a sharp knife followed by a period of seclusion for several weeks during which the wounds are treated with herbs. Usually, the whole ceremony takes place in the bush and the patients who are mainly teenagers are kept far away from families and friends throughout the period.

Circumcision performed in the wild has a high risk of infection. Information obtained from various local communities has revealed high incidence of complications arising from wound contaminations. Cases of complications requiring hospital admission ranged from 105 to 135 during a single ceremony, depending on the community. While reports of contamination leading to complications are common among the Pondos which, in most cases, have resulted in the death of many patients, such reports are rare among the Xhosas.

The authors observed that different species of *Helichrysum* are used by the different communities for the treatment of the wounds after circumcision. The Xhosas use *H. pedunculatum* Hilliard & Burtt while the Pondos use *H. longifolium* DC for the treatment of the wounds. Both species are perennial herbs and are widely distributed in southern Africa (Hilliard 1983). Traditionally, the Pondos heat the leaves of *H. longifolium* over very hot ash

before using them as a bandage for the treatment of wounds after circumcision. Extracts from the shoots of *H. pedunculatum* have been reported to be active against a number of bacteria species (Meyer and Dilika 1996). However, no information is available on the antimicrobial property of *H. longifolium*.

The aim of this study was to compare the antibacterial activities of *H. pedunculatum* and *H. longifolium* by direct bioautography using *Staphylococcus aureus*. *S. aureus* is one of the common organisms usually isolated from such wounds. The effect of temperature on the antibacterial activity of *H. longifolium* was also examined.

Leaves of *H. pedunculatum* and *H. longifolium* were collected from the Eastern Cape province of South Africa in September 1996. Voucher specimens (Dilika 299) of the former was prepared and deposited in the H.G.W.J. Schweickerdt Herbarium (PRU), Pretoria, while the latter was compared and confirmed with specimens at the same herbarium.

Leaves from *H. pedunculatum* and *H. longifolium* (8 g each) were shaken in acetone for 5 min and the resultant extracts were filtered separately and concentrated to dryness under reduced pressure giving 92.4 and 18.7 mg respectively. The remaining leaf material was then homogenised in acetone, filtered and concentrated to dryness yielding 97.9 and 70.0 mg of dry extract respectively. The four dry extracts were each dissolved in acetone to a final concentration of 20 mg/ml.

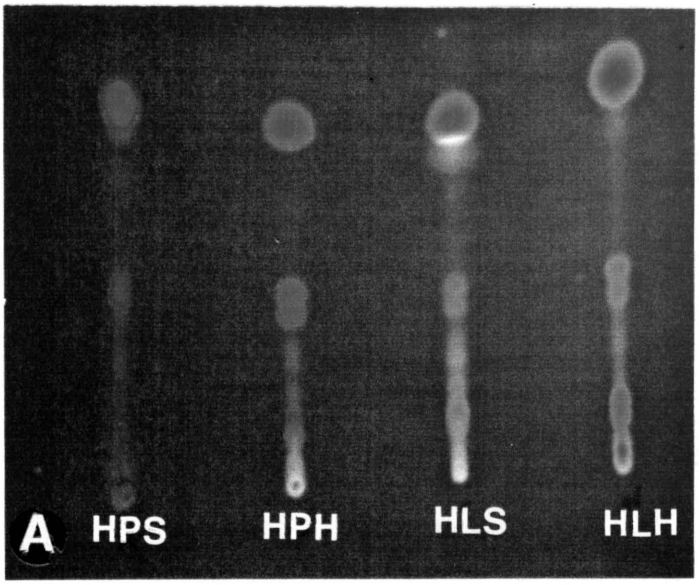
For direct bioassay on thin layer chromatography (TLC), 10 µl of each extract was spotted on silica gel 60 plates (Merck) and developed in chloroform-benzene (60:40). A suspension of 24 h old *S. aureus* cultured in nutrient agar was sprayed onto the TLC plates (Meyer and Dilika 1996), and incubated at 37°C for 24 h. After incubation, the plates were sprayed with an aqueous solution of *p*-iodonitrotetrazolium violet and reincubated at 37°C for 3 h (Lund and Lyon 1975).

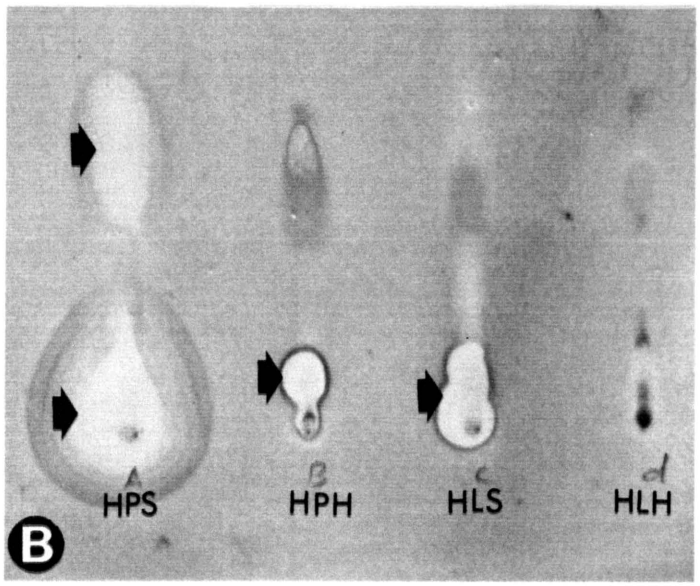
The extract obtained from the leaves of *H. longifolium* shaken in acetone was divided into four parts. Three portions were heated at 60°C, 80°C and 100°C respectively in closed glass tubes for 15 min, while the fourth part was left unheated and served as control. The four treatments were then subjected to direct bioautography as described above.

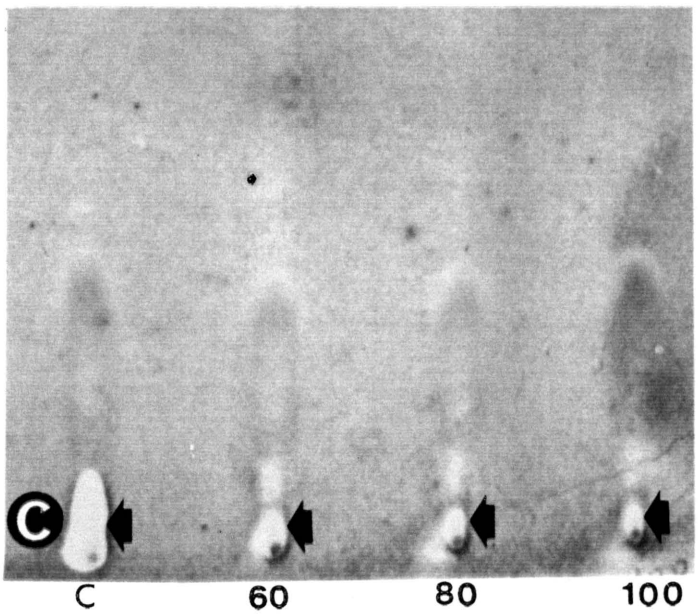
Considering their positions and colours on the TLC plates under UV light at 254 and 366 nm, many similar compounds were detected in the extracts obtained from shaken and homogenised leaves of *H. pedunculatum* and *H. longifolium* (Figure 1A). This might be of taxonomic interest since Hilliard (1983) placed these species in different groups. According to Gershenzon and Mabry (1983), individual or whole classes of secondary compounds are frequently restricted to groups of plant taxa that are considered to be related on many grounds.

Caption for figure

Figure 1 Direct bioautographic assay of extracts from *Helichrysum pedunculatum* and *H. longifolium* using *Staphylococcus aureus*. **A.** TLC plate of extracts from leaves of both plants under UV light at 366 nm. (HPS, extract from leaves of *H. pedunculatum* shaken in acetone; HPH, extract from the homogenised leaves of the same plant; HLS, extract from leaves of *H. longifolium* shaken in acetone; HLH, extract from the homgenised leaves of the same plant). Note the similarity in colour and positions of the compounds. **B.** Activity of the extracts against *Staphylococcus aureus* shown as clear zones (arrowed) after spraying with *p*-iodonitrotetrazolium violet. **C.** Effect of temperature on the activity of the shaken extract of *H. longifolium* against *S. aureus*. Extracts were heated at 60, 80 and 100°C for 15 min. The control (C) was not heated.







Clear (white) zones on the TLC plates indicated antibacterial activity of the extracts. *H. pedunculatum* extracts from both shaken and homogenised plant material exhibited activity against *S. aureus*, whereas only the shaken extract from *H. longifolium* showed activity using direct bioassay (Figure 1B). Although direct bioassay on TLC plates is not an ideal method for the quantification of bioactivity, it could be safely stated that the acetone extract from the leaves of *H. pedunculatum* has more activity against *S. aureus* than *H. longifolium*. Meyer and Dilika (1996) have found the MIC of the dichloromethane extract from *H. pedunculatum* against *S. aureus* to be 35 mg/ml.

The antibacterial activity of the shaken extract from *H. longifolium* decreased with increase in temperature as indicated by the relative areas of the clear zones on the TLC plate (Figure 1C). The highest activity was observed in the control (unheated) extract, while the extract heated at 100°C showed little activity.

Although many factors may be responsible for the occurrence of complications following male circumcision among the Pondos, it is plausible to assume that the lower antibacterial activity of *H. longifolium*, coupled with the traditional method of heating the leaves over hot ash before use, may be some of the factors. Since *H. pedunculatum* also grows in the area where the Pondos live, it is recommended for use rather than *H. longifolium*. Heating of the plant should be discouraged as it appears to reduce the activity of the leaf extract against *S. aureus*.

Acknowledgement

We thank Mary-Ann Njeje of Mvenyane for the collection of the plants and the Foundation for Research Development of South Africa for financial assistance.

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CHAPTER 5

ANTIBACTERIAL ACTIVITY OF *HELICHRYSUM PEDUNCULATUM CALLUS*

Antibacterial activity of *Helichrysum pedunculatum* callus.

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ABSTRACT

Helichrysum pedunculatum (Asteraceae) is used by the Xhosas of Transkei in South Africa to dress wounds after circumcision. Calli of this plant were established in half strength Murashige and Skoog enriched with naphtalene acetic acid and kinetin. The homogenised dichloromethane extract of this callus was evaluated for its antibacterial activity by direct bioautography on TLC. The extract inhibited the growth of the gram positive bacteria *Bacillus cereus, B. pumilus, B. subtilis* and *Staphylococcus aureus* as well as the gram negative bacterium *Serratia marcescens*.

Keywords: Antibacterial; Callus; *Helichrysum pedunculatum*; Medicinal; Tissue culture

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1. INTRODUCTION

The antimicrobial properties of a number of *Helichrysum* (Asteraceae) species have been described (Dekker et al., 1983; Boily and Van Puyvelde, 1986; Tomas-Barberan et al., 1990; Rios and Villar, 1991; Meyer and Afolayan, 1995; Meyer and Dilika 1996). A South African species, *Helichrysum aureonitens* showed antibacterial activity against *Micrococus kristinae, Bacillus cereus, B. pumilus* and *Staphylococcus aureus* (Meyer and Afolayan, 1995). Although work has been done on the antimicrobial activity of *Helichrysum* species, very little has been published on callus induction and the testing of these cultures for antibacterial activity. Leeuwner and Meyer (1995) induced callus from *H. aureonitens* and showed that it was active against *B. subtilis, S. aureus, Klebsiella pneumoniae* and *Escherichia coli*.

Helichrysum pedunculatum Hilliard and Burrt has been reported in folklore to have medicinal value. These include its ability to cure stomach ailments and its anti-inflammatory properties (Watt and Breyer-Brandwijk, 1962; Hilliard, 1983; Bolofo and Johnson, 1988). The plant is used by Xhosas in Transkei (South Africa), to dress wounds especially after circumcision. The claimed medicinal property of the plant was substantiated by Meyer and Dilika (1996) who reported the antibacterial activity of the extract against *B. cereus, B. subtilis, B. pumilus, M. kristinae, S. aureus, Enterobacter cloacae* and *Serratia marcescens*.

In this paper, we describe the establishment of *H. pedunculatum* callus and present the results of its antibacterial properties against ten bacterial species.

2. MATERIALS AND METHODS

2.1 Callus induction and maintenance

Young leaves from *Helichrysum pedunculatum* were surface sterilised for 30 seconds in an aqueous solution of 0.35 % sodium hypochlorite (NaOCl) and rinsed 5 times in sterile distilled water. Sterile leaf material was excised into 5 x 5 mm pieces and incubated on half strength Murashige and Skoog (MS) basal medium (ICN Biomedicals Inc., UK) supplemented with 3 % sucrose, 1 mg/l naphthalene acetic acid (NAA) and 0.05 mg/l kinetin. The medium was solidified with 0.5 % agar (Biolab, SA).

Homogenous calli appeared within three weeks and were maintained by subculturing every six weeks onto fresh medium as described above but solidified with 0.3 % agar, (Adkins, 1992; Ross et al., 1995). The soft friable callus was carefully spread cautiously on the surface of the medium. The spreading was necessary to secure a sufficiently large area for secondary metabolite production which was mainly restricted to the upper cell layers (Dodds and Roberts, 1986). Friable callus was then selected and used for antibacterial testing.

2.2 Preparation of callus extracts

Callus material was agitated in cold water in order to remove the growth medium from the tissue without damaging the callus, blottted dry and weighed (6.885 g). Metabolites were extracted by homogenising the sample in dichloromethane (CH_2Cl_2). The homogenate was filtered and the sediment re-extracted by stirring three times in CH_2Cl_2 for 30 minutes each. The filtrate was concentrated to dryness under reduced pressure, the mass determined (39 mg) and the sample redissolved in 1 ml CH_2Cl_2.

2.3 Bacteria

The callus extract was tested against the following gram positive bacterial species; *Bacillus cereus, B. pumilus, B. subtilis, Micrococcus kristinae, Staphylococcus aureus* and the gram negative species, *Enterobacter cloacae, Escherichia coli, Klebsiella pnuemoniae, Pseudomonas aeruginosa and Serratia marcescens*. Each organism was maintained on nutrient agar slant and recovered for testing by growth in nutrient broth (No. 2, Biolab, SA) for 24 hours at 37^{o}C except *Pseudomonas aeruginosa* which was harvested only after 72 hours.

2.4 Antibacterial testing

The callus extract was tested for antibacterial activity by direct bioautography on thin layer chromatography (TLC) plates. 4 μl from the callus extract (39 mg/ml) was applied to silica gel

60 plates (Merck, Germany) using a calibrated syringe. The plant extract (Meyer and Dilika, 1996) was also applied to the same TLC plates and developed in benzene:chloroform (40:60 v:v). It was observed under uv light (254 and 366nm) after development, left overnight for the solvent to evaporate completely and sprayed with the bacterial suspension.

The 24 h (and 72 h *Pseudomonas aeruginosa*) old bacterial cultures in nutrient broth were centrifuged at 3000 rpm for 20 min. The supernatant was discarded and the sedimented bacteria resuspended in fresh nutrient broth to an absorbance of 0.84 at 560 nm with a spectrophotometer (Lund and Lyon, 1975). The bacterium suspension was sprayed with a fine spray onto the TLC plates. These plates were then dried for a few minutes until they appeared transluscent and incubated at 25^{o}C for 24 h (and 72 h for *P. aeruginosa*) in humid conditions. The plates were then sprayed with an aqueous solution of 2.0 mg/ml *p*-iodonitrotetrazolium violet and reincubated at 25^{o}C for 3 to 72 h depending on the bacterial species. Any inhibition of bacterial growth could clearly be seen as white spots on a red background.

3. RESULTS AND DISCUSSION

The development of friable callus was observed three weeks after the experiment commenced. Faster callus growth was obtained after changing the agar content in the growth medium from 0.5 to 0.3 %.

A difference in the compounds produced by the plant and callus was observed on the TLC plates. This confirms the findings by Das and Law (1990), Thorpe (1990) and Missaleva *et al.* (1993), that secondary metabolites in a source plant may not be present in its callus.

Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Micrococcus kristinae and Enterobacter cloacae showed resistance against all the compounds of the callus extract. Certain compounds in the callus extract were however active against *Bacillus cereus, B. pumilus, B. subtilis, Staphylococcus aureus and Serratia marcescens.* When the plant extract was tested with the agar dilution method (Meyer and Dilika, 1996), all the above mentioned gram positive bacteria were inhibited as well as the gram negative bacteria, *S. marcescens, E. cloacae* and *M. kristinae.* This might be due to the synergistic effect of the combined antibacterial compounds in the extract.

Although not all the compounds produced by the plant were found in the callus extract, they both contained compounds that had more activity on gram positive bacteria than on gram negative ones. These results are consistent with other studies which also showed that plant extracts inhibit gram positive bacteria more than gram negative ones (Grosvenor et al., 1995; Meyer and Dilika, 1996).

4. ACKNOWLEDGEMENTS

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CHAPTER 6

ISOLATION OF ANTIBACTERIAL COMPOUNDS FROM *HELICHRYSUM PEDUNCULATUM*

ISOLATION OF ANTIBACTERIAL COMPOUNDS FROM *HELICHRYSUM PEDUNCULATUM*

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ABSTRACT

Leaves of *Helichrysum pedunculatum* were shaken in dichloromethane and the resulting extract was subjected to different purification techniques including, medium pressure liquid chromatography, high performance thin layer chromatography, counter current chromatography, high perfomance liquid chromatography and thin layer chromatography. The antibacterial activity was investigated by direct bioassay on thin layer chromatography against *Bacillus subtilis* and *Staphylococcus aureus*. The extract contained four antibacterial compounds and had more activity against *S. aureus* than *B. subtilis*. A compound containing about 32 carbons and another one, containing an aromatic ring and a number of CH, CH_2, and CH_3 groups, were isolated from the extract. Both showed activity against *S. aureus*.

Key words: Helichrysum pedunculatum, antibacterial

INTRODUCTION

Various approaches for identifying new compounds that might serve as therapeutic agents, have been fruitful since the inception of medicinal chemistry. Natural products form an important category of test compounds in the continued search for new drugs. The rich diversity of structural types provided by natural products also add to their attractiveness as bioactive compounds (Lednicer and Narayanan, 1993; Cordell, 1995). This class of compounds is of particular interest because it has been proven to be a rich source of biologically active compounds.

The availability of suitable separation methods for the isolation of pure products is the key to any study of material from natural sources. The impact of modern counter-current chromatography and pressure liquid chromatography is beginning to be noticed in this regard (Marston, *et al.*, 1988; Marston and Hostettmann, 1994). Following the determination that an extract is indeed active, and a strategic decision has been made to pursue the fractionation of the extract for the active principle(s), attention must be focused on the most effective methods for the purification so that bioactivity-directed fractionation can be used to monitor the isolation process. Activity monitoring is usually accomplished with the same biological material as was used for the determination of activity in the original extract. There are exceptions, one of which occurs when multiple tests are run on the original extract and the materials show activity in more than

one system. In such cases, only the assay in which the extract shows the highest activity is usually used for the fractionation (Glinski *et al.*, 1990; Vanden Berghe and Vlietinck, 1991; Ghisalberti, 1993).

In this study, *Helichrysum pedunculatum* was extracted with dichloromethane (CH_2Cl_2) separated into fractions through the application of medium pressure liquid chromatography, counter-current chromatography, high performance liquid chromatography and preparative thin layer chromatography and its purified antibacterial compounds investigated by nuclear magnetic resonance spectroscopy.

MATERIALS AND METHODS

1. Extract preparation

Leaves of *Helichrysum pedunculatum* were shaken in (CH_2Cl_2), filtered and concentrated to dryness under reduced pressure and the dry weight of the extracted material determined (2.724 g). The crude extract was thoroughly mixed with 27.243 g of silica, concentrated to dryness and subjected to different purifications techniques for the identification of antibacterial compounds.

2. Separation and purification.

The separation of one or more substances from a crude extract, can be a long and expensive process. Obtaining a pure compound

from a crude extract often requires a number of separation steps involving different techniques. This might be due to the target compound(s) being present in only small quantities.

Chromatography, the separation process in which the sample mixture 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 a specific separation is usually determined by thin layer chromatography (TLC) (Meyer, 1994).

2.1. MPLC (Medium Pressure Liquid Chromatography).

This method involves the use of longer columns with larger internal diameters than in conventional chromatography. These columns require the higher pressures delivered by a pump for a sufficiently high flow rate. This has a number of advantages over the open column or flash chromatography in terms of sample load, higher resolution and shorter separation time. The sample is loaded separately first into a small column through which the solvent is allowed to run through.

After separation, care should be excercised since a complex fraction may contain more than one compound with the same chromatographic retention characteristics (Calton *et al.*, 1986).

2.1.1. Column packing

A silica column (45cm length and 4.6cm diameter) was loaded with dry silica (silica gel 60, size 0.015-0.040 mm, Merck, Darmstadt) under vacuum after which nitrogen gas (8 bars) was introduced to fill any air spaces in the system that may in turn interfere with the elution.

2.1.2. Development of solvent system and chromatography of extract.

H. pedunculatum extract was applied as six spots on HPTLC plates (high performance thin layer chromatography) and eluted individually with mobile phases (presented as v:v) consisting of; hexane: CH_2Cl_2 (50:50), CH_2Cl_2 (100 %), CH_2Cl_2: MeOH (99:1), CH_2Cl_2: MeOH (98:2), CH_2Cl_2: MeOH (97:3) and CH_2Cl_2: MeOH (95:5). After development, the HPTLC plates were sprayed with 10 % sulphuric acid and heated at 121 ^{o}C for 10 minutes. The solvent gradient that would yield maximum elution of the sample in MPLC was then selected (Hasunuma *et al.*, 1991).

The sample was loaded into a small column and glass sand was used to fill the rest of the column. This prevents most of the air that might interfere with the elution from flowing through the system and ensures its stability when pressure is applied. This also retains the flow of solvent as the solvent system changes. All solvents were degassed prior to use.

The following programme was then followed on MPLC for the fractionation of *H. pedunculatum* extract;

Solvent	Time in minutes
1. Hexane (100 %)	30
2. Hexane: CH_2Cl_2 75:25	50
3. Hexane: CH_2Cl_2 50:50	40
4. CH_2Cl_2 (100 %)	50
5. CH_2Cl_2: MeOH 99:1	60
6. CH_2Cl_2: MeOH 98:2	50
7. CH_2Cl_2: MeOH 97:3	50

The flow rate was 19.8 ml/min and the eluent was monitored by a light scattering detector. Fractions containing the same compounds as determined by TLC, were combined into 22 fractions. These fractions were concentrated to dryness under reduced pressure, spotted on a TLC plate and then developed with hexane: EtOAc: MeOH: H_2O (10: 5: 5: 1.5). The plate was sprayed after development with 10 % H_2SO_4 and dried in an oven at 120^{o}C for 10 minutes (Hasunuma et al., 1991; Alfatafta *et al.*, 1994).

For direct bioassay on thin layer chromatography (TLC), 10 μl of each fraction was applied to silica gel 60 plates (Merck) and developed with hexane: EtOAc: MeOH: water (10:5:5:1.5). 48 Hour old *Bacillus subtilis* and *Staphylococcus aureus* cultures were centrifuged at 3000 rpm for 20 min, the supernatant discarded

and the sedimented bacteria resuspended in fresh nutrient broth to an absorbance of 0.84 at 560 nm. This was then sprayed onto the TLC plates. After incubation, the plates were sprayed with an aqueous solution of 2.0 mg/ml *p*-iodonitrotetrazolium violet. The plates were then reincubated at 25^{o}C for 6 hours (Lund & Lyon, 1975; Meyer and Dilika, 1996)

The fraction with highest antibacterial activity was chosen for subsequent countercurrent fractionation.

2.2. Counter-Current Chromatography (CCC)

Counter-current chromatography was originally developed in the early 1950's with the pioneering work done by Lyman Craig. Although this method provided high-resolution capabilities for separation, it never achieved widespread use. One of the reasons might be because it requires a lot of solvent. It is however, now widely used in the field of natural products because crude extracts and semi-pure fractions can be successfully chromatographed with this technique (Marston and Hostettmann, 1994).

CCC is a liquid-liquid partition chromatography technique employing two immiscible or partially miscible liquid phases, the upper phase (UP) and the lower phase (LP). One phase is designed as the stationary phase (SP) and retained as such by either gravitational or centrifugal forces while the mobile phase (MP) is passed through. Separation of the components of the extract

or any mixture is achieved by introducing the sample at one end of the stationary phase (Head-H) with the mobile phase. In all cases, solutes are more soluble in the stationary than in the mobile phase. Each component is then retained by the stationary phase to a degree dependent on its partition coefficient for the two-phase system. Individual components are eluted from the other end of the stationary phase (Tail-T) by the mobile phase. A compound with a favourable partition coefficient in the mobile phase will be eluted first as relatively sharp bands. The bands of the last compounds are usually broader (Zweig and Sherma, 1972; Belter, 1979; Macalpine and Hochlowski, 1989).

In the separation of a polar extract for example, a polar solvent is used as the stationary phase and a less polar or organic solvent which serves as the mobile phase, is employed as the developing solvent (Osterman, 1986).

When compounds of intermediate polarity are chromatographed, as is the case for many bioactive secondary metabolites, the system is reversed after some time so that the stationary phase then becomes the mobile phase. The time to reverse the system is normally determined by checking carefully the composition of the test tubes in which the separated fractions are collected in terms of solvent phases. The test tube in which two layers of the solvent system is observed thus determines the time to reverse the system.

The technique is capable of operating with both aqueous and non-aqueous solvent systems and with samples of a wide range of polarities. This versatility has led to an ever increasing number of applications in the field of natural products. The advantages of this system includes the ability to separate complex mixtures without material losses and shortened separation time due to the high flow velocity (Marston *et al.*, 1990; Okuda *et al.*, 1991; Knight, 1992; Lee, 1994).

2.2.1. Solvent system and sample preparation.

The solvent system, hexane: CH_3CN: MeOH (8: 5: 2 v:v:v) was prepared by mixing the required volumes in a large separatory funnel. After equilibration, the two phases were separated and each degassed using an ultrasonic bath. The CCC column was loaded after the coil was filled with the stationary phase.

The biphasic solvent was made from the upper and lower phases (50:50). 114.7 mg of fraction HP009 was dissolved in 8 ml of the solvent and injected into the system via a loop. This was chromatographed into 12 fractions under the following parameters: H-T, LP-MP, UP-SP, and at 301 minutes (tube 62) the system was changed to T-H, LP-SP, UP-MP, loop: 10 ml, volume : 350 ml, flow: 1 ml/min, tube: 5 ml, speed: 1050 rpm, pressure: <100 psi, equilibrium: 51 ml. The remaining sample was eluted after 485 min, at 4 ml/min. The volume of each fraction was 16 ml.

Similar compounds, as determined by TLC, were combined into 12 fractions (023-034). The combined fractions were evaporated to dryness at 45 ^{o}C under reduced pressure.

Fractions were analysed for their antibacterial activity by direct bioautography. The TLC plate was developed and sprayed with *S. aureus*.

2.3. PTLC

Fraction 031 had antibacterial activity and was further purified using preparative TLC (silica gel 60, Merck, 2mm) because it contained a mixture of compounds. The upper phase of hexane: EtoAc: MeOH: H_2O (10:5:5:1.5) was used as solvent and the plate was developed two times under the same conditions.

2.4. HPLC (High Performance Liquid Chromatography)

This gives much improved and more rapid separations than column chromatography and it is therefore finding increasing use for analytical separations. The best mobile phase for any separation is determined by TLC, using mainly organic solvents as the eluent (Trease and Evans, 1978; Berthod, 1991; Meyer, 1994).

Analytical HPLC analysis was performed using a 5 μm Spherisorb Si (8 mm x 250 mm) column at a flow rate of 2 ml/min. Fraction 026 isolated by CCC was further subjected to HPLC separation with

hexane: EtOAc 90: 10 for 10 min then changed to hexane: EtOAc 70: 30.

Purified antibacterial fractions were dissolved in chloroform from which approximately 1 μl was mixed with a drop of ($CDCl_3$). Samples were analysed using proton as well as carbon nuclear magnetic resonance (nmr)

RESULTS AND DICUSSION

The dichloromethane extract of *H. pedunculatum* leaves contained four antibacterial compounds. Preliminary fractionation by MPLC, together with a bio-autographic TLC assays for antibacterial activity enabled the attribution of activity to a certain number of defined components of the mixture.

The use of MPLC, CCC, preparative TLC and HPLC resulted in good separation of the crude extract and the isolated antibacterial fractions. From the bioassay on TLC plates, the growth of *S. aureus* was more inhibited than *B. subtilis* and hence only *S. aureus* was used in the subsequent assays. The assays indicated activity in all fractions from MPLC with fraction 009 being the most active. In order to accommodate limited solubility of some components, fraction 009 was dissolved in a mixture of both phases and loaded into a 10 ml loop in CPC. From CPC, all fractions were active and fraction 031 showed the highest activity.

Changing the elution mode resulted in much more rapid elution of compounds than would otherwise have been possible. The elution mode was adjusted to ascending after 301 minutes (tube 62) with the upper phase as the mobile phase. This lead not only to time saving but also reduced the volume of the solvent required. The ^{1}H nmr and ^{13}C nmr spectra of compound HP055 (about 2 mg), separated and purified by HPLC, showed that it contains about 32 carbons which include $8xCH_3$, $10xCH_2$, 7xCH and 7xC groups (Figures 1 and 2). The coupling information of C-H and C-C was not clear and hence the structure of the compound was not fully elucidated.

About 26 mg of an oily liquid (compound HP057) was seperated from HP035 by HPLC (Figure 3). The information from the nmr spectra of the compound dissolved in $CDCl_3$ showed that the compound contains an aromatic ring and a number of CH, CH_2 and CH_3 groups (Figures 4 and 5).

A comparison of the ^{1}H nmr spectra of compounds HP028 and HP055 (Figures 2 and 6), reveals that the compounds might have a similar structure.

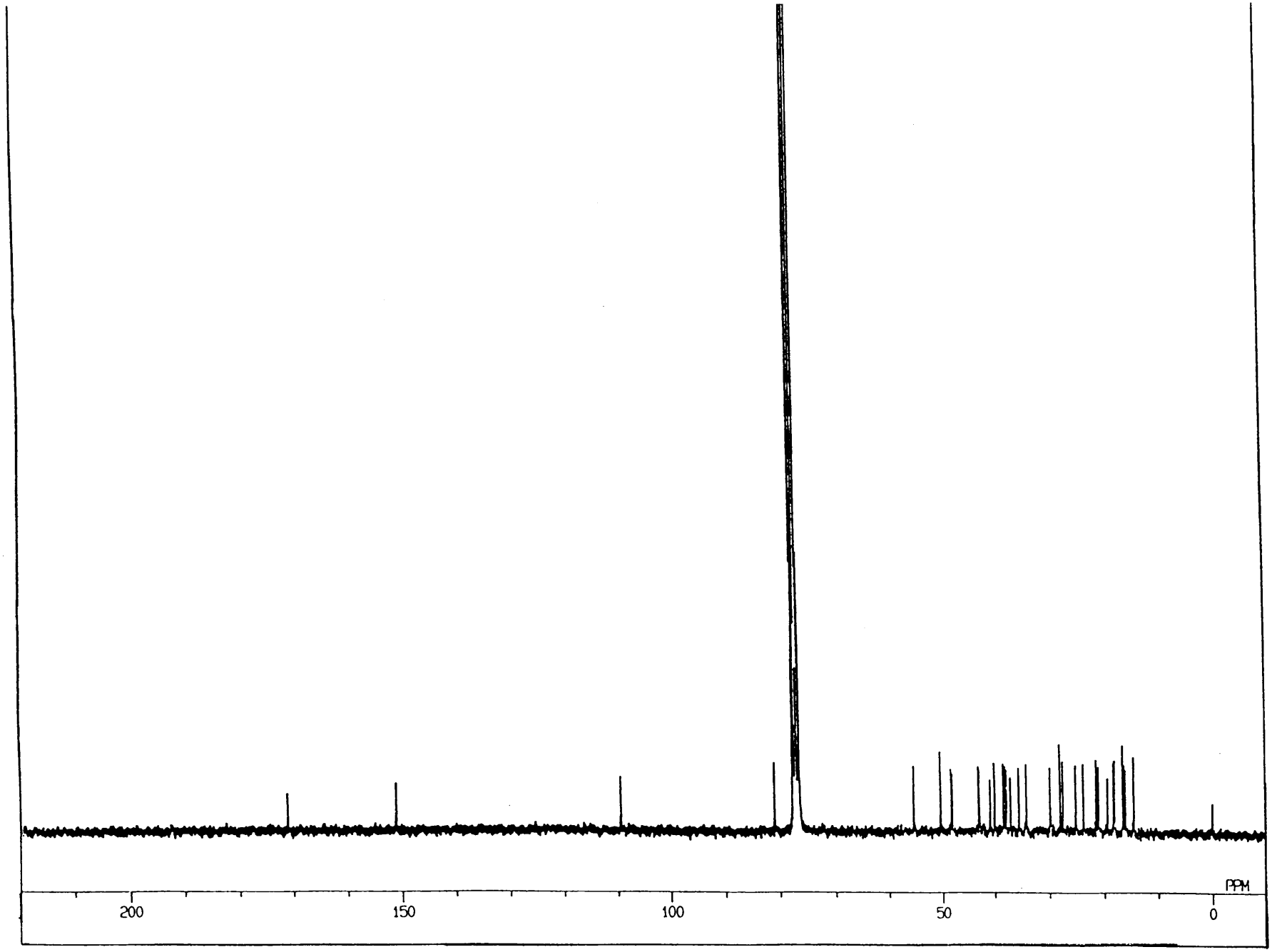

Figure 1. ^{13}C nmr spectrum of compound HP055

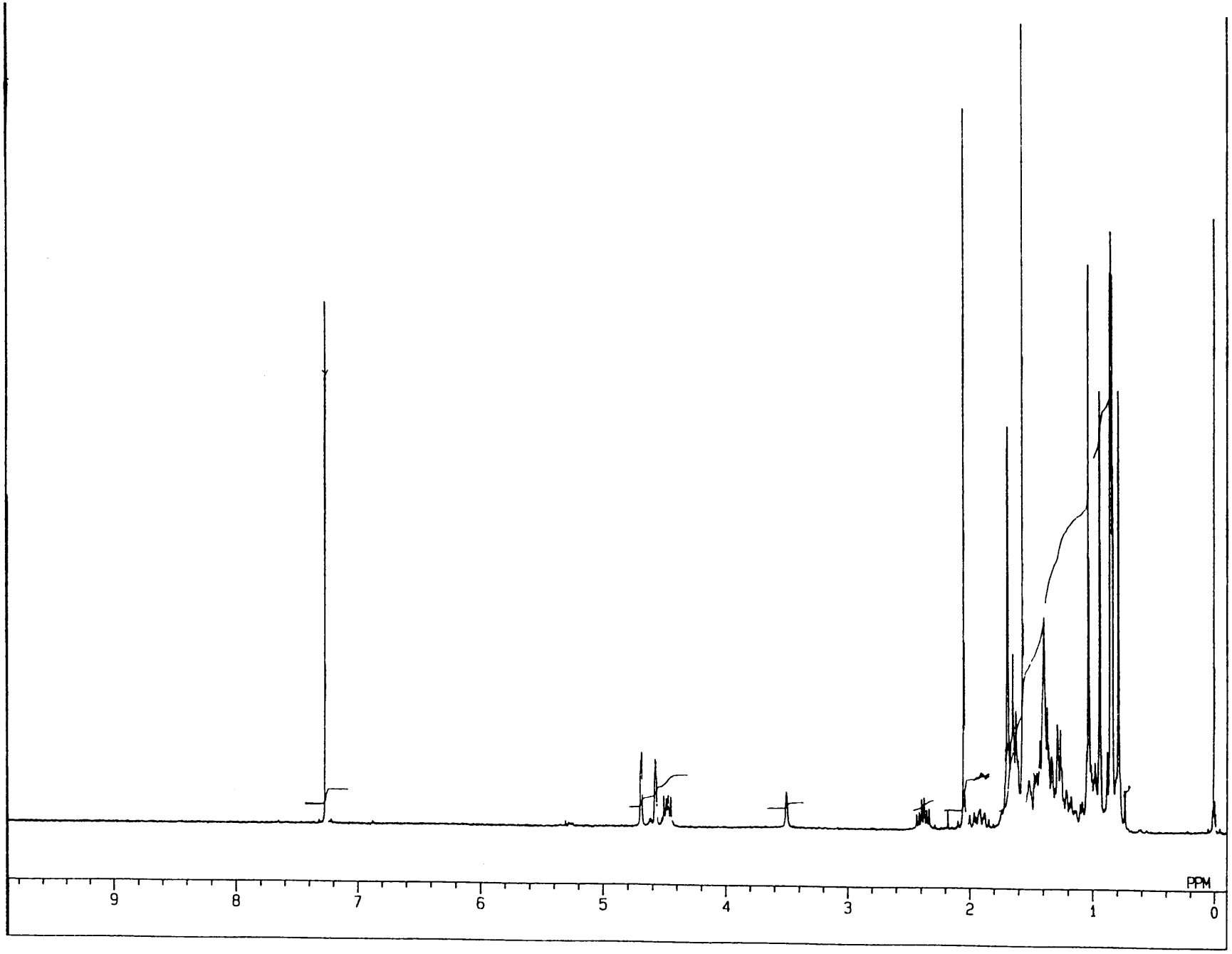

Figure 2. ^{1}H nmr spectrum of compound HP055

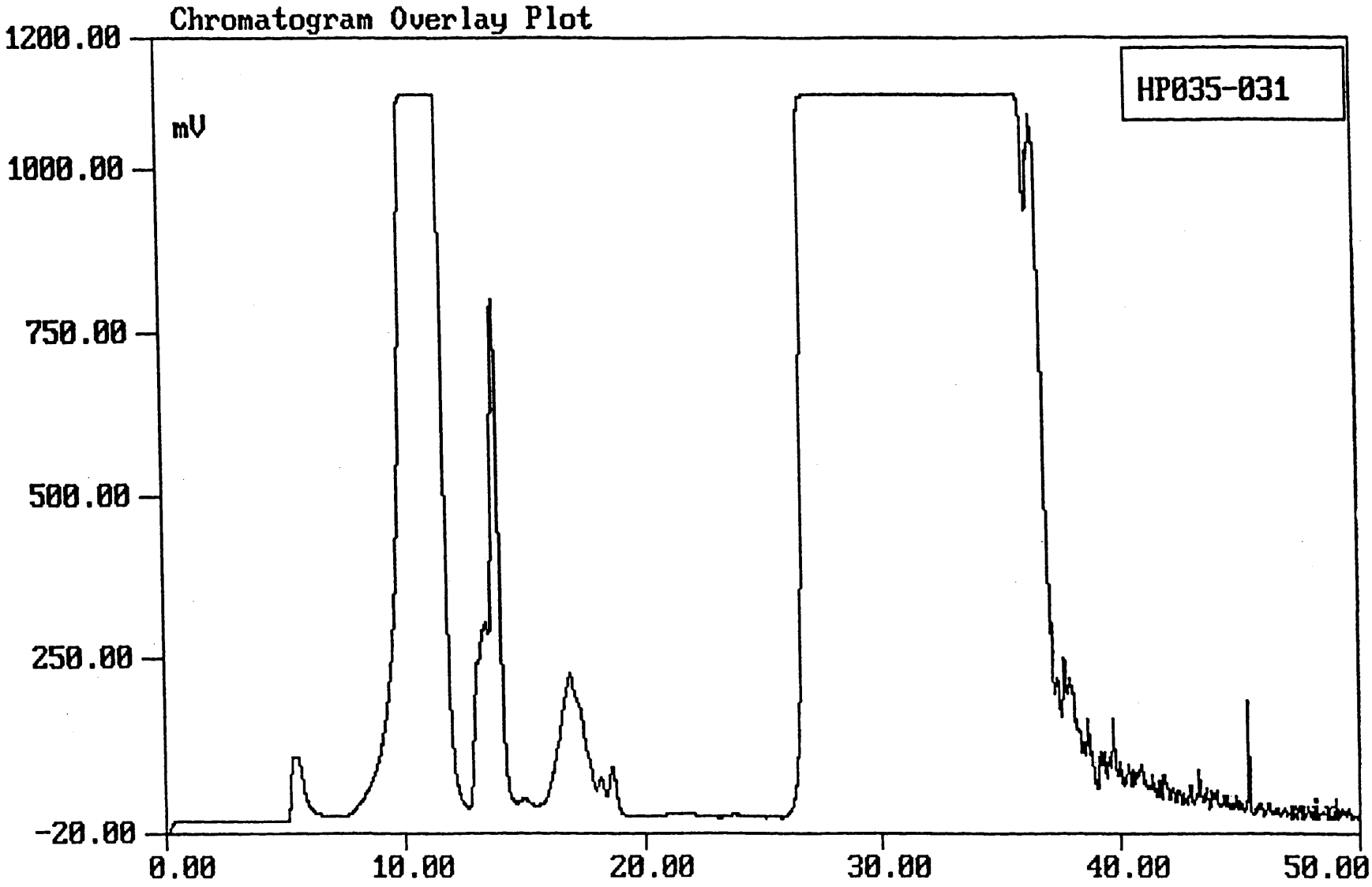

Figure 3. HPLC spectrum of compound HP057. Eluent: hexane: dichloromethane (30:70 v:v). Flow rate, 5 ml/min. Column: Lichrospher 100Si μm, 250X230mm, VDS-Optilab.

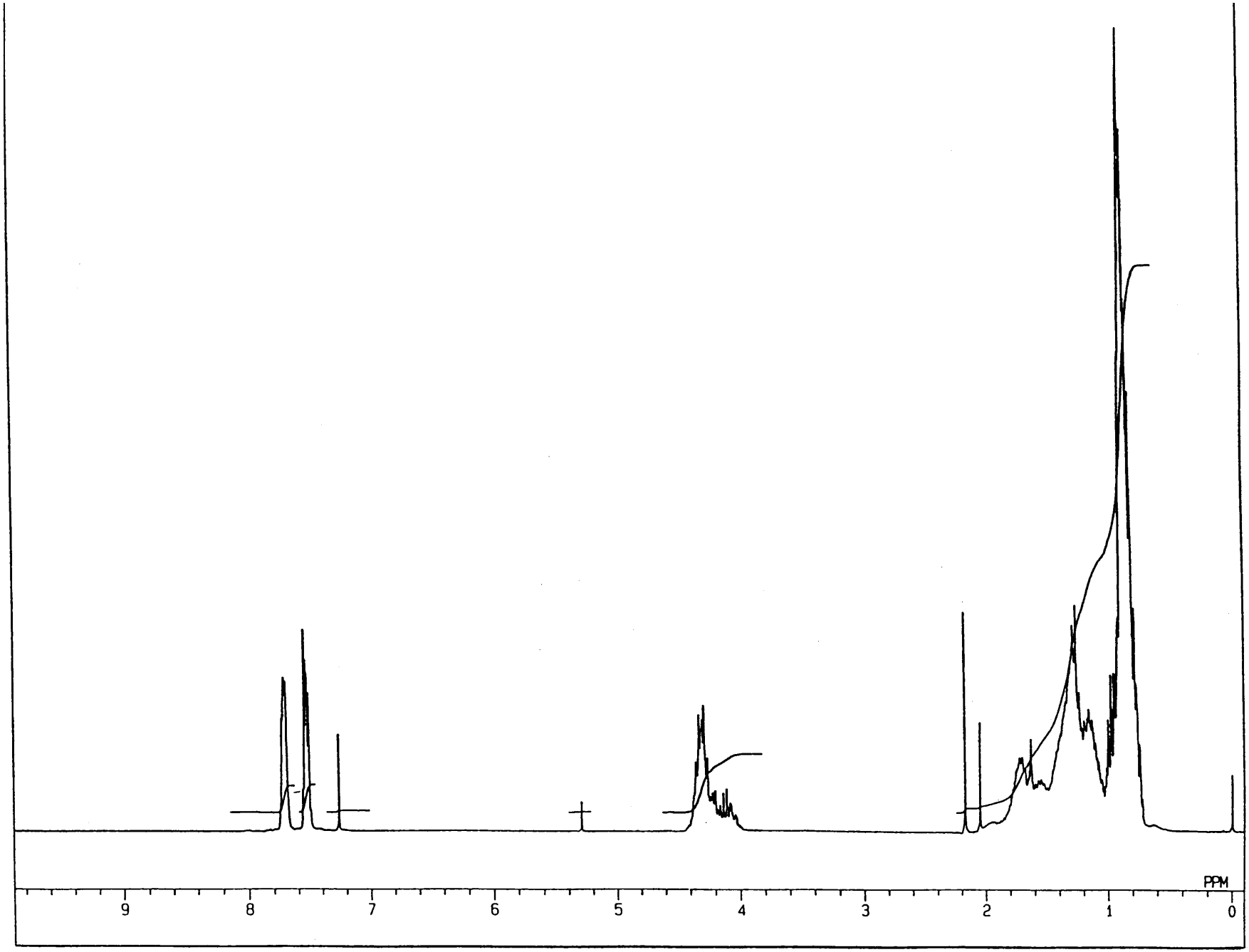

Figure 4. ^{1}H nmr spectrum of compound HP057

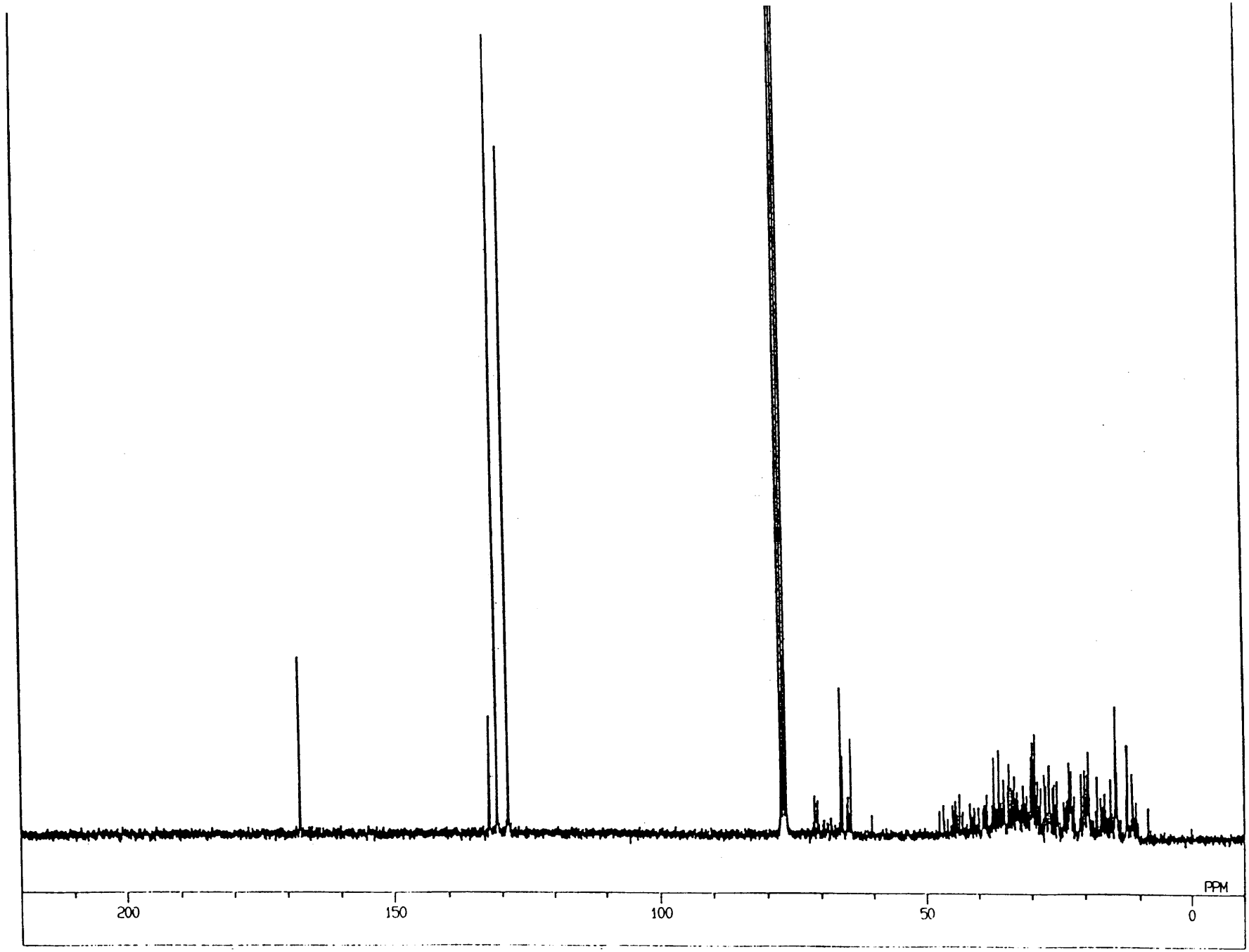

Figure 5. ^{13}C nmr spectrum of compound HP057

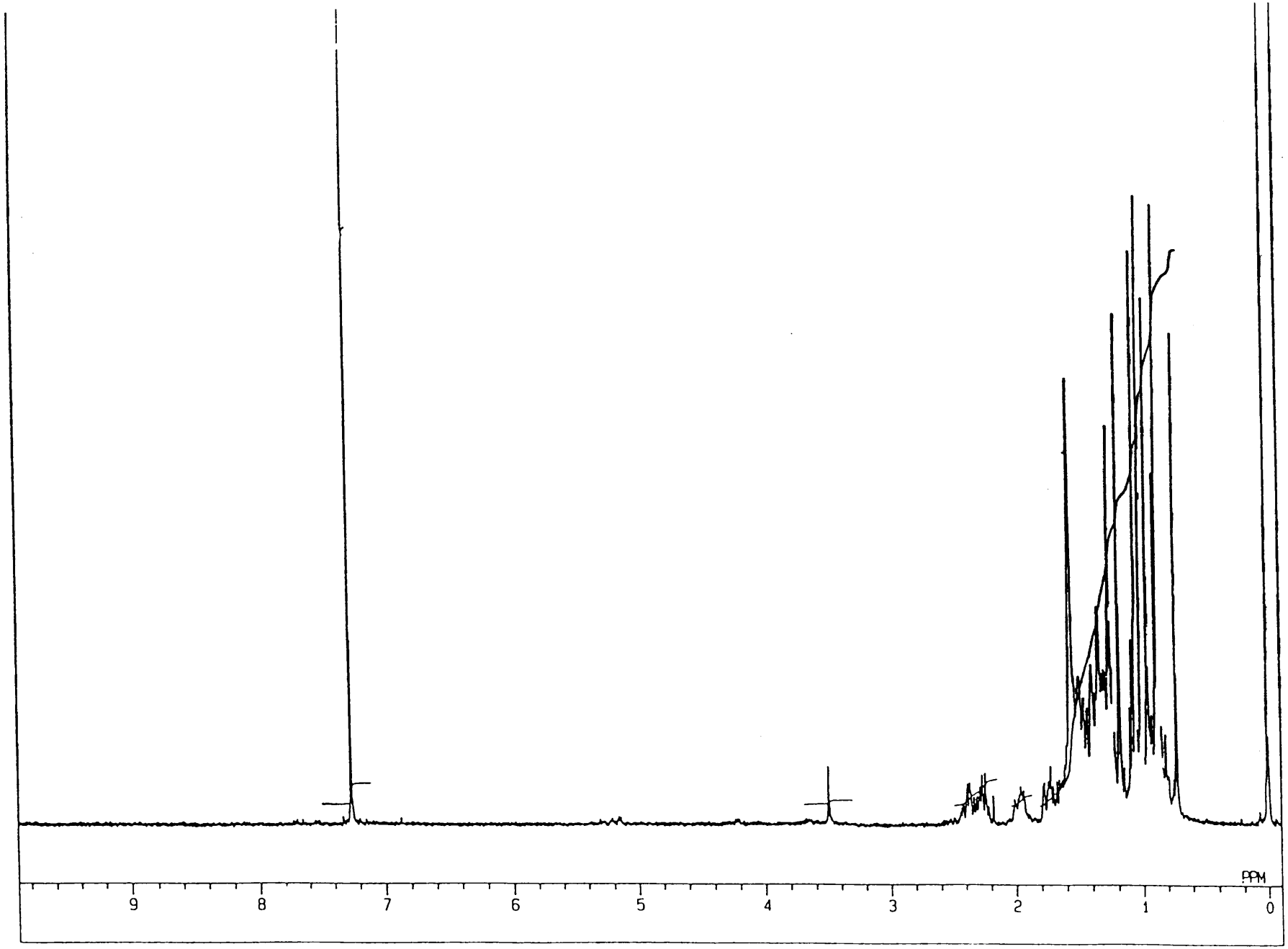

Figure 6. ^{1}H nmr spectrum of compound HP028

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

GENERAL DISCUSSION AND CONCLUSION

Most people in developing countries rely on plant derived medicine for their primary health care. Despite advances in the field of medicine and organic chemistry, plants are still a commercial source of medicinal compounds for more than 80% of the world's population use plants as their primary source of medicinal agents (Cordell, 1995). The contribution of plants in medicine is indisputable since 65% of the present drugs have their origin in plants (Simon & Very, 1988). 25% ethnobotanically guided screens for medicinal plants are more successful than screens of plants that have been randomly selected (Haslam, 1989; Richardson *et al.*, 1992; Ohashi *et al.*, 1994). It could be assumed that such information of herbal medicines compiled over centuries by trial and error, mainly using humans as experimental animals should contain some material worthy of further investigation (Cordell, 1995; Martin, 1995).

Most traditional medicines used by the indigenous communities of the western Cape are derived from plants belonging to the Asteraceae family (Salie, *et al.*, 1996). According to Tomas-Barberan *et.al.*, 1990, a number of *Helichrysum* species have been reported to have medicinal values.

The antibacterial activity of *Helichrysum pedunculatum* used in circumcision rites

Antibacterial assays of *H. pedunculatum* showed that the

epicuticular dichloromethane extract is active against all gram positive bacteria tested as well as two gram negative bacteria, *Enterobacter cloacae* and *Serratia marcescens*. A water extract was effective against *Staphylococcus aureus* and *Micrococcus kristinae*. Methanol extract showed no activity against any of the tested bacterial species. In a number of other studies (Dimayuga & Garcia, 1991, Meyer & Afolayan, 1995), plant extracts were found to readily inhibit gram positive rather than gram negative bacteria (Grosvenor *et al.*, 1995). This may be because of the lipopolysaccharide layer in the gram -ve bacteria acting as a selectively permeable barrier that keeps many large molecules away from the plasma membrane. This layer is absent in the the gram +ve bacteria (Grosvenor *et al.*, 1995; Mauseth, 1995).

The antibacterial activity of dichloromethane extract was also investigated by direct bioassay on TLC plates against *S. aureus*. Four zones of bacterial growth inhibition were observed on TLC plates sprayed with *S. aureus*. Only one of these compounds could be detected under UV light at 366 nm.

Comparative antibacterial activity of two *Helichrysum* species used in male circumcision in South Africa.

The leaves of *H. pedunculatum* and *H. longifolium*, both used in circumcision rites, were compared for their antibacterial activity by direct bioautography using *S. aureus*. Traditionally, the leaves of *H. longifolium* is heated over hot ash before use.

Leaves from both herbs were shaken and homegenised in acetone and the resultant extracts were tested. Extracts from *H. pedunculatum* showed more activity against *S. aureus* than those from *H. longifolium*. Heating the extracts from the latter reduced their activity against the bacterium. Heating of the plant should be discouraged as it appears to reduce the activity of the leaf extract against *S. aureus*.

Antifungal activity of *Helichrysum pedunculatum*

The antifungal activity of *H. pedunculatum* produced by shaking the leaves in dichloromethane was investigated. *Aspergillus flavus* and *A. niger* showed no reduction in growth rate when it was exposed to the extract. *Cladosporium cladosporoides, C. cucumerinum, C. sphaerospermum* and *Phytophtora capsici* were susceptible to the extract. *C. cucumerinum* was the most sensitive fungal species and was inhibited at a concentration of 0.1 mg/ml.

Antibacterial activity of *Helichrysum pedunculatum* callus

Young leaves of *Helichrysum pedunculatum* proved to be a good explant source. The callus extract inhibited the growth of the gram positive bacteria *Bacillus cereus, B. pumilus, B. subtilis* and *Staphylococcus aureus* as well as the gram negative bacterium *Serratia marcescens*.

The difference in compounds produced by the plant and callus as observed on TLC plates, confirms the findings by Das and Law (1990), Thorpe (1990) and Missaleva *et al.* (1993), that secondary metabolites in a source plant may not be present in its callus.

Isolation of antibacterial compounds from *Helichrysum pedunculatum*

Antibacterial activity of fractions and isolated compounds was mainly determined by the direct bioautography on TLC plates. This method is suitable for rapid screening of plants for the presence of antibacterial compounds in the extract. The location of the active compound is established by a direct correlation with a reference TLC plate (Rios *et al.*, 1988, Fuery & Magnolato, 1990).

Dichloromethane extract of *H. pedunculatum* leaves contained four antibacterial compounds. Fractionation by medium pressure liquid chromatography, eliminated many of the compounds with no antibacterial activity. All the fractions collected after counter-current chromatography were active against *S. aureus* with fraction HP031 showing the most activity.

The ^{1}H NMR and ^{13}C NMR spectra of compound HP055 showed that it contains about 32 carbons. Eight CH_3, ten CH_2, seven CH and seven C groups could be observed in the spectra. The coupling information of C-H and C-C was not clear and hence the structure of the compound was not fully elucidated.

The NMR spectrum of compound HP057 showed that the compound contains an aromatic ring and a number of CH, CH_2 and CH_3 groups. The ^1H NMR spectra of compounds HP055 and HP028 showed that the compounds might have a similar structure.

The ten bacterial species tested vary in terms of their susceptibility to the extract. Although the leaves from the plant are effective as a source of antimicrobial agents, the actual antimicrobial effect may be concealed by the growth of the bacterial species which are resistant to the plant extracts. This might explain why isolated cases of bacterial infection after circumcision still occur.

Helichrysum pedunculatum has been used as a folk medicine to bandage circumcision wounds for centuries and this usage suggested that the species might have antimicrobial activity. The antibacterial and antifungal activities of the leaf extracts as shown in this study, verify the folkloric use of this plant. Attempts will be made to isolate, characterise and structurally identify the antimicrobial compounds in the plant during further studies.

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CHAPTER 8

SUMMARY

THE ANTIMICROBIAL ACTIVITY OF *HELICHRYSUM PEDUNCULATUM* USED IN CIRCUMCISION RITES

by

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The indigenous people of South Africa still practise many cultural traditions especially the traditional method of male circumcision. This is mainly performed in the wild. *Helichyrsum pedunculatum* is the most commonly used herb in the treatment of wounds arising from circumcision.

The antimicrobial activity of *H. pedunculatum* leaves was investigated against ten bacteria and six fungi. The antibacterial assays showed that dichloromethane extracts were active against all the gram positive bacteria tested, as well as two gram negative bacteria, *Enterobacter cloacae* and *Serratia marcescens* (33 mg/ml). Antifungal assays of the dichloromethane extract showed activity against *Aspergillus flavus, A. niger,*

Cladosporium cladosporoides, *C. cucumerinum*, *C. sphearospermum* and *Phytophthora capsici*.

H. pedunculatum and *H. longifolium* leaves were compared for their antibacterial activity against *S. aureus*. Many similar compounds were detected on TLC plates in extracts obtained from shaken and homogenised leaves of both herbs. Acetone extracts from the leaves of *H. pedunculatum* showed more activity than those from *H. longifolium*. When the extracts of *H. longifolium* were subjected to high temperatures, their bioactivity decreased with an increase in temperature.

Callus was successfully produced from the leaves of *H. pedunculatum*. The homogenised dichloromethane extract from the callus was evaluated for antibacterial activity by direct bioautography on thin layer chromatography plates. The extract inhibited the growth of the gram positive bacteria; *Bacillus cereus*, *B. pumilus*, *B. subtilis* and *Staphylococcus aureus* as well as the gram negative bacterium, *Serratia marcescens*.

An attempt was made to isolate and identify the antibacterial compounds in the dichloromethane extract. The following techniques were used; medium pressure liquid chromatography, high performance thin layer chromatography, countercurrent chromatography, high performance liquid chromatography and nuclear magnetic resonance. Pure compounds could not be identified. The antibacterial property of the compounds was investigated against *B. subtilis* and *S. aureus* using direct

bioassay and two compounds were found to be active. The ^{1}H nmr and ^{13}C nmr spectra of compound HPO55 showed that it has about 32 carbons that include 8xCH_3, 10xCH_2, 7xCH and 7xC groups. The nmr spectrum of compound HPO57 showed that it contains an aromatic ring and a number of CH, CH_2 and CH_3 groups. The coupling information of C-C and C-H was not clear and hence the compound were not fully identified.

CHAPTER 9

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