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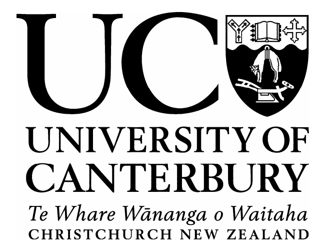

Application of a particle filtration method in the search for new bioactive natural products from fungi.

A thesis submitted in partial fulfilment of the requirements for the Degree of

Master of Science in Microbiology

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by

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Abstract

Fungi have been an important source for producing a wide range of secondary metabolites of widely differing chemical structures, as well as biological activities. Many of their metabolites now play a major role in pharmaceutical and agricultural industries.

A number of fungi were isolated from soil and leaf litter collected from Arthur's Pass, West Coast and Kaituna Valley using a particle filtration technique. Fungi were selected based on their unusual morphology or observed cytotoxicity and antimicrobial activity for large scale culture and extraction.

A pale yellow compound was isolated from cytotoxic extracts from the culture of *Aspergillus versicolor*. This compound was identified as sterigmatocystin and the identity confirmed by UV profile and mass spectrometry.

Five compounds were isolated from extracts prepared from two different species of *Penicillium* of which three were active against P388 cells (mycophenolic acid, cycloaspeptide A and mevastatin), one was active against dermatophytes (griseofulvin) and one was not active (3,4,6,8-tetrahydroxy-3-methyl-3,4-dihydroisocoumarin).

Two compounds were isolated from extracts prepared from two different species of *Phoma*. A dark red compound was found to be novel and showed activity against P388 cells and *Bacillus subtilis*. A second compound also showing cytotoxicity was identified as the known compound phomenone.

A further new compound was isolated from extracts of an identified dematiaceous fungus. This alkyl glucoside, however, was not bioactive.

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ABBREVIATIONS

ACN	Acetonitrile
BSA	Bovine Serum Albumin
CD_3OD	Deuterated methanol
$CDCl_3$	Deuterated chloroform
COSY	Correlation spectroscopy
d	doublet (in connection with NMR data)
DMDCS	Dimethyldichlorosilane
DNA	Deoxyribonucleic acid
ELSD	Evaporative Light Scattering Detector
HCl	Hydrochloric acid
HMBC	Heteronuclear multiple bond correlation spectroscopy
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HPLC	High Performance Liquid Chromatograpgy
HSQC	Heteronuclear single quantum coherence
Hz	Hertz
ITS	Intergenic Sequence
m	multiplet (in connection with NMR data)
m/z	mass to charge ratio
MEM	Minimal Essential Media
$MgSO_4$	Magnesium sulphate
min	minute(s)
$NaHCO_3$	Sodium carbonate
NaOCl	Sodium hypochlorite
NMR	Nuclear Magnetic Resonance
P388	Murine leukemia cell line
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
ppm	parts per million

PVC	Polyvinyl chloride
q	quartet (in connection with NMR data)
s	singlet (in connection with NMR data)
TFA	Trifluoroacetic acid
UV	Ultra-Violet

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Chapter 1

Introduction

1.1 Fungi

Fungi are a kingdom of eukaryotic organisms. They are distantly related to plants and more closely related to animals but rather different from either of those groups. About 70,000 species of fungi have been described, however, some estimates of total numbers suggest that 1.5 million species may exist (Hawksworth *et al.*, 1995). They are mainly multicellular organisms which composed of long and thread like cells connected end to end called hyphae which in mass are constitute the mycelium. Some fungal species also grow as single cells. Their cell wall is rigid and largely composed of chitin. Most fungal species are non-motile and reproduce by means of spores. Both sexual and asexual spores may be produced depending on the fungal species and the environment (Kendrick, 1992).

Fungi play an important role in maintaining the ecosystem. By breaking down dead organic material, they continue the cycle of nutrients through ecosystems. In addition, most vascular plants could not grow without the symbiotic fungi or mycorrhizae, which inhabit their roots and supply essential nutrients.

Fungi are also of great economic importance as they have both positive and negative effects on us. Some are used in the beer and champagne industry, provide numerous

drugs such as penicillin and other antibiotics, foods like mushrooms, truffles and morels. Some are also studied as model organisms that can be used to gain knowledge of basic processes such as genetics, physiology, biochemistry, and molecular biology which are applicable to many organisms (Taylor *et al.*, 1993). At the same time, fungi also contribute to the loss of millions of dollars in damage through food spoilage, destruction or degradation of materials used by humans, and diseases of plants as well as humans and animals (Mueller *et al.*, 2004).

1.2 Bioactive metabolites from fungi

Nature has proven and continues to be a promising source for the discovery of bioactive compounds, important for the development of new pharmaceuticals and agrochemicals. Microorganisms, especially fungi are significant in this respect (Schulz *et al.*, 2002). Fungi are of major interest because only a small percentage of them have been investigated for their role in producing novel bioactive compounds and hence offer huge potential. To date, soil fungi have yielded most of the compounds presently in commercial use. These bioactive compounds are mostly derived from fungal secondary metabolism.

Secondary metabolic pathways are not necessarily required for the organisms to survive. The induction of secondary metabolism is usually in response to external stimuli such as nutritional changes, infection and interspecies competition. The products of secondary metabolism are called secondary metabolites and many fungal secondary metabolites have found a place in medicine or agriculture.

Important examples of fungal secondary metabolites used in the pharmaceutical industry include the penicillins and cephalosporins. Penicillin was first isolated from *Penicillium notatum* in 1929 by Alexander Fleming and became the wonder drug of the age (Keller *et al.*, 2005). Cephalosporin was first isolated from *Cephalosporium acremonium* in 1948 by Giuseppe Brotzu (Weil *et al.*, 1995). Both penicillins and cephalosporins are β-lactam antibiotics, which are usually used for prevention or treatment of bacterial infection. They

act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. **Figure 1.1** shows the core structure of penicillin and cephalosporin which has been manipulated to give rise to different types of penicillin and cephalosporin with different properties.

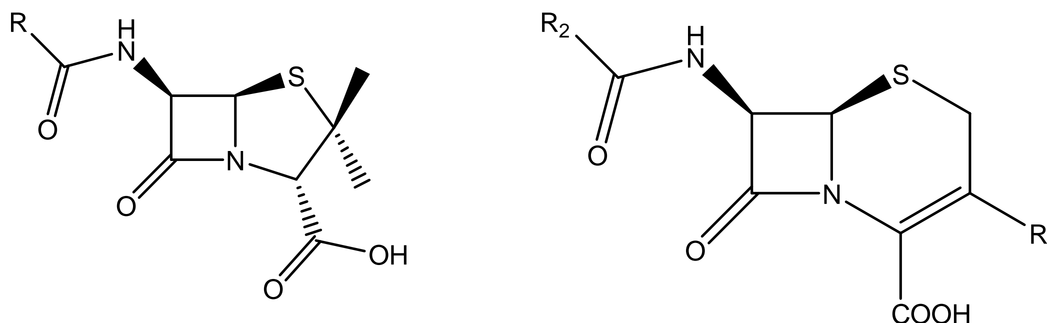


Figure 1.1 Core structure of penicillin (left) and cephalosporin (right).

Another important group of fungal secondary metabolite of pharmacological importance are the statins. An example of a statin that available commercially is lovastatin, which is used for lowering cholesterol in those in those with hypercholesterolemia and thus preventing heart disease. It was first isolated from *Aspergillus terreus*. Lovastatin lowers cholesterol level by acting as an inhibitor of HMG-CoA reductase, an enzyme that catalyzes the conversion of HMG-CoA to mevalonate. Mevalonate is a required building block in cholesterol synthesis (Tobert, 2003).

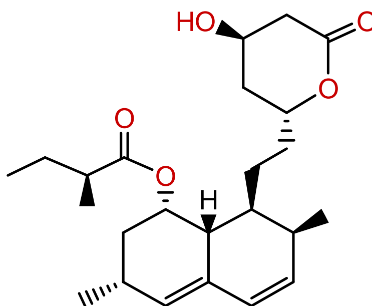


Figure 1.2 Structure of lovastatin.

Cyclosporin is another important one of the fungal secondary metabolites used in medicine and was first isolated from *Tolypocladium inflatum*. It is an immunosuppressant

drug used in post-allogeneic organ transplant to lower the risk of organ rejection. It does this by binding to cyclophilin of immunocompetent lymphocytes which is responsible for activating the transcription of interleukin-2. It also inhibits lymphokine production and interleukin release, thus reduces the function of effector T-cells. **Figure 1.3** shows the structure of cyclosporin A which is available commercially.

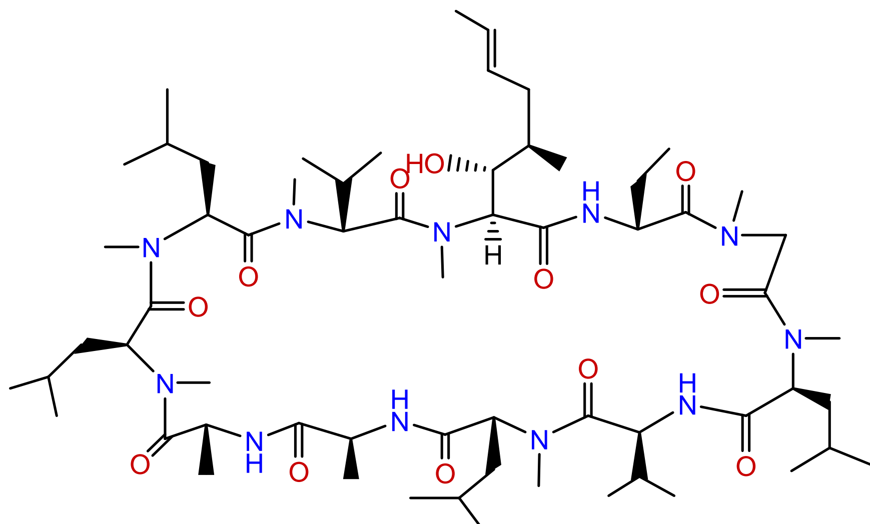


Figure 1.3 Structure of cyclosporin A.

Gibberellin is an example of a fungal secondary metabolite used in agriculture. It is a hormone that plays important roles in plant development such as stem elongation, germination, dormancy, flowering, sex expression, enzyme induction as well as leaf and fruit senescence. It was first isolated from *Gibberella fujikuroi* (Tudzynski and Holter, 1998). **Figure 1.4** shows several types of gibberellins that have been isolated from fungi.

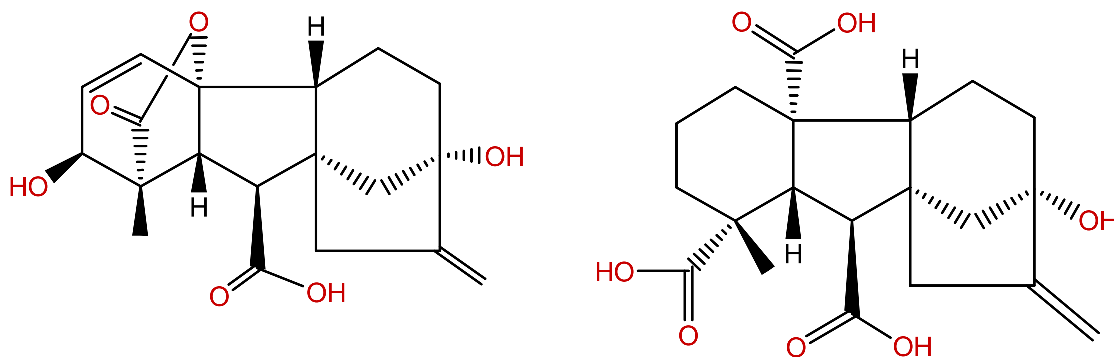


Figure 1.4 Structure of gibberellin GA3 (left) and gibberellin A17 (right)

1.2.1 Classification of secondary metabolites

Secondary metabolites can be divided into several chemical classes. Most fungal secondary metabolites fall into four main chemical classes; peptides, alkaloids, terpenes and polyketides.

Peptide metabolites can be either ribosomal peptides or nonribosomal peptides. Ribosomal peptides are synthesized by translation on mRNA. These kind of peptides usually undergo a post-translational modification after it is being translated. Fungal nonribosomal peptides, which are synthesized by specific enzymes rather than the ribosomes and are termed nonribosomal peptide synthetases. Examples of fungal peptides include penicillin, which has anti-bacterial properties and destruxin A, which has insecticidal as well as anti-viral properties.

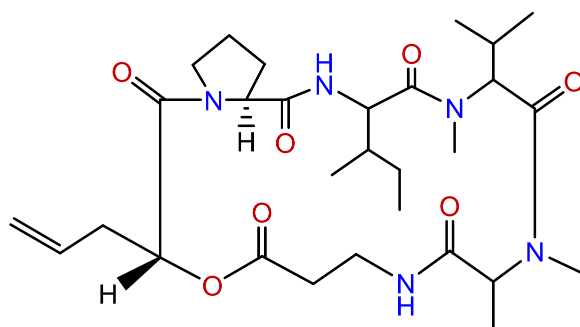


Figure 1.5 Structure of destruxin A

Alkaloids are derivatives of amino acids. Many alkaloids possess pharmacological effects on humans and animals. They can be further divided into 9 groups (pyridine, pyrrolydine, tropane, quinoline, isoquinoline, phenetylamine, indole, purine, terpenoid and vinca alkaloids) based on the metabolic pathway used to construct the molecule. Examples of fungal alkaloids are ergopeptides, used to treat migraine as well as Parkinson's disease and fumitremorgen C, a mycotoxin.

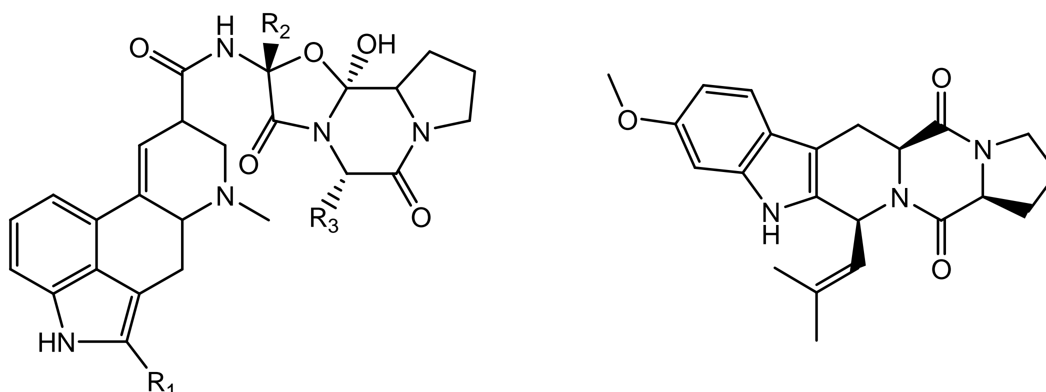


Figure 1.6 Structure of ergopeptides (left) and fumitremorgen C (right).

Terpenes are a large and varied class of hydrocarbons. They are derived from polymerization of isoprene subunits. The basic molecular formula for terpenes is $(C_5H_8)_n$, where n is the number of linked isoprene units. The terpene metabolites can be further divided into hemiterpenes, monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, triterpenes and tetraterpenes, depending on their size. Examples of fungal terpenes are vomitoxin and gibberellin GA3. Vomitoxin possesses mycotoxin properties while gibberellin is a plant growth regulator.

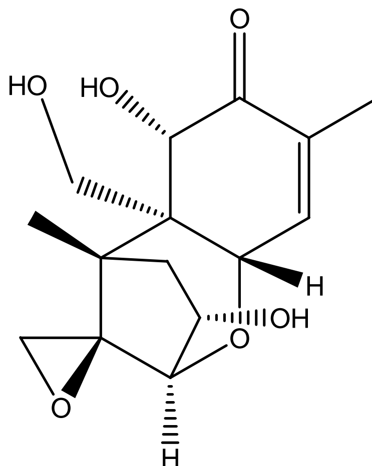


Figure 1.7 Structure of vomitoxin.

Polyketides are the most abundant fungal secondary metabolites and have an extremely broad range of biological activities and pharmacological properties. Polyketides are derived from the condensation of acetyl-SCoA with malonyl-ScoA units. For fungal polyketides, the enzymes responsible for their synthesis are type I polyketide synthases (Keller *et al.*, 2005). Examples of fungal polyketides include the mycotoxic aflatoxins and lovastatin, a cholesterol lowering agent.

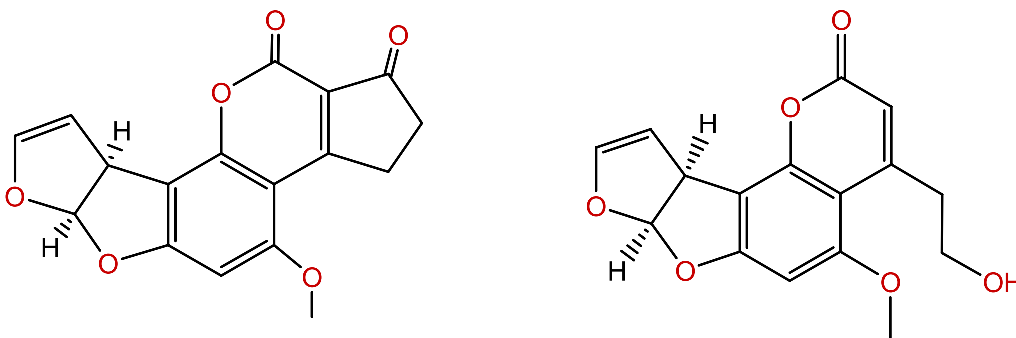


Figure 1.8 Structures of several types of aflatoxins, aflatoxin B1 (left) and aflatoxin B3 (right).

1.3 Fungal isolation

Fungi have proven and continue to be promising producers of novel bioactive compounds. They occupy many ecological niches and the majority of fungi are still awaiting discovery. Soil and less so plant litter has been the habitat of choice in the past for fungal isolation, however, soil and plant litter offer further potential since it has been shown that an abundance and diversity of microfungi exist in soil and litter which could be accessed using specialised techniques (Bills and Polishook, 1994).

Currently, other habitats such as plant tissues (endophytes) and the marine world are being explored in the search for rare and new fungi which hopefully will lead to the discovery of novel bioactive compounds. Habitats with extreme environments such as Antarctica (Arenz *et al.*, 2006) and hot springs are also promising sources for isolating rare fungi.

The type of fungal species that can be isolated from the environment is dependent on the isolation methods used. Conventional isolation methods which examine sporulating structures formed on plant material in moist chambers and dilution plating of soil have been routinely used in the past. These techniques tend to select for vigorous, fast growing fungi which are dominant and usually will overgrow the less vigorous fungi on artificial media. Many of these dominant fungi have been isolated and studied thoroughly and form the basis of present utilised fungal secondary metabolites. To attempt to isolate less readily culturable organisms, with the hope of finding new strains and consequent potential for novel bioactivity, more selective methods are required. Examples of selective methods are baiting, chemical pasteurization methods, environmental selection techniques and particle filtration.

Baiting is a technique in which the artificial medium is enriched nutritionally to favour organisms of interest. This way, the organism of interest will have better chance to grow. The baiting isolation technique has been widely used for isolation of cellulolytic fungi, keratinolytic fungi and entomopathogenic fungi (Klingen *et al.*, 2002). One example of a chemical pasteurization technique is ethanol and phenol pasteurization which is a

specialized technique usually used to selectively isolate ascomycetes from soil. In environmental selection techniques, the manipulation of several parameters such as temperature, pH, osmolarity can gives rise to different groups of fungi.

In this study, a particle filtration technique was used to maximize the number of fungal isolates obtained from soil and litter samples. This technique is one of a number of indirect methods for isolating fungi and a hybrid between the methods used in the isolation of soil fungi and leaf endophytic fungi. The Particle filtration technique enables the elimination of common and fast growing fungi which then enables the less culturable fungi to grow. With careful selection and the help of potent and non-lethal colony restriction agents as well as temperature, a high species level should be achieved with anticipated 'rarer' strains being isolated.

1.4 The aim of this study

It is hypothesised that the development and utilisation of selective isolation techniques for soil and litter fungi will result in the identification of new strains which have the potential to produce new bioactive metabolites. A contribution to New Zealand biodiversity will be made and hopefully new metabolite lead structures characterised with potential for pharmaceutical or agrochemical products.

Aims:

1. Isolation of litter fungi using a particle filtration technique.
2. Fungal identification by conventional and molecular techniques.
3. Screening for bioactivity in extracts of isolated fungi.
4. Characterisation of active metabolites.

Chapter 2

Methodology

2.1 Sampling

Soil samples from the South Island of New Zealand were collected from Klondyke Corner, Arthur's Pass. Leaf litter samples were collected from three sites. The first site was at Carew Falls (West Coast), the second at Mount French (West Coast) and the third in Kaituna Valley (Banks Peninsula). There were two types of leaf litter samples collected at the first two sites (Carew Falls and Mount French), Rimu leaf litter and mixed leaf litter of native trees. Only a mixed litter sample was collected at Kaituna Valley. All the samples were kept in sealed sterile bags and stored in a cold room prior to further processing.

2.2 Media

The medium used for initial fungal isolation from samples was potato dextrose agar (PDA) (BD) supplemented with 50 mg/L of chlortetracycline HCL (Sigma) and 250 mg/L of streptomycin sulphate (Sigma). For futher culturing and extraction, no antibiotic was included in the medium.

2.2.1 Preparation of Potato dextrose agar (PDA)

Potato dextrose agar was prepared according to the manufacturers recommendations. For culturing and extraction work, after autoclaving, the agar was poured into Petri dishes immediately, approximately 20 mL to 25 mL each plate. For isolation work, 6 mL of the antibiotic stock solution was added to cooling agar and poured into Petri dishes. All plates were stored at 4°C.

2.2.2 Preparation of stock antibiotic solution

100 mL of stock antibiotic solution was prepared. 500 mg of chlortetracycline HCL and 2500 mg of streptomycin sulphate were added to 50 mL distilled water. The mixture was stirred until fully dissolved and the solution poured into a 100 mL volumetric flask, made up to 100 mL with distilled water and mixed well. The antibiotic solution was then filtered using a 0.2 μm sterile filter into a sterile bottle and stored at -20°C.

2.3 Isolation of soil fungi and endophytes

2.3.1 Preparation of soil and leaf litter samples

Soil samples were taken from the cold room and air dried in a laminar flow unit, before proceeding with the isolation process. Leaf litter samples were surface sterilized before isolation.

The leaf litter samples were shredded by either hand or scissors, placed in a strainer and washed thoroughly under running tap water for at least 10 minutes. They were then drained and transferred into a beaker and further work conducted in a laminar flow.

First, the leaf pieces were immersed in 70% ethanol for 1 minute with a little agitation. Second, the leaf pieces were immersed in 50% NaOCl solution for 3 minutes with a little agitation and strained before immersing again in 70% ethanol for 30 seconds. Finally, the

leaf pieces were rinsed 3 times with sterile distilled water before spreading out onto sterile Whatman No. 1 filter papers and air dried under a stream of sterile air.

2.3.2 Particle filtration technique

A particle filtration technique was used to isolate fungi from the soil and leaf litter samples adapted from that of Bills and Polishook, (1994). The filtration system shown in **Figure 2.1** consisted of a micro-sieve of three interchangeable screen holding cylinders of molded PVC with inner diameter of 55 mm. The cylinders can be unscrewed easily for changing filters, cleaning and disinfecting.

All the apparatus involved in this technique was either sterilized by autoclaving or swabbing with 70% ethanol. The particle filtration device was disassembled and soaked in 70% ethanol. After that, the particle filtration was reassembled with 3 different size of polypropylene filters (Spectrum) incorporated into it. The 500 µm filter was put on top, 200 µm was put in the middle and the 105 µm filter was put at the bottom. Refer to **Figure 2.1** for detail of the device. The assembled column was held under a tap by a burette clamp mounted on a ring stand.

Both soil and leaf litter samples were fragmented separately using a Waring blender. 3 g of either soil or leaf litter sample were then put on the top of the filter column and the fragments were wet gradually by running a fine spray of tap water for 10 minutes over the sample.

After 10 minutes, the particle filtration column was disassembled and the 105 µm filter was taken out and placed into a sterile 50 mL centrifuge tube. 50 mL of sterile distilled water was added. Then, the tube was agitated to wash off the particles from the filter. The 105 µm filter was then taken out from the centrifuge tube and the particles allowed to settle. The particles were then washed twice with sterile distilled water before a final 1 mL of sterile distilled water was added to the centrifuge tube.

The 1 mL particle solution was then diluted 4 times with sterile distilled water before 100 µL was spread onto 4 PDA plates supplemented with antibiotics. All plates were sealed with either parafilm or plastic wrap and kept at 25°C in the dark.

2.3.3 Maintenance of fungal isolates

Isolation plates were observed daily for 30 days. Any visible colonies growing were subcultured immediately onto fresh PDA without antibiotics. All cultures were then incubated at 25°C.

2.3.4 Storage of fungal isolates

For working and short term storage, the fungal isolates were maintained on a PDA plate and kept at 25°C. For mid term storage, 7 to 8 mycelial discs of the fungal isolates were transferred into a cryovial tube filled will 1 mL of sterile distilled water and kept at 4°C.

For long term storage, 7 to 8 mycelial discs of each isolates were transferred into a cryovial tube filled with 1 mL of sterile 10% glycerol solution, kept at 4°C for 2 hours and then transferred into a freezer at -20°C overnight before placing in a -80°C freezer.

2.4 Identification of fungal isolates

Fungal isolates were identified morphologically using microscopy and genetically by sequencing the ITS region of the isolated fungal DNA.

2.4.1 Morphological identification of fungal isolates

Microscopic fungal structures that are crucial for fungal identification such as the fruiting structures and spores were examined using a compound microscope with magnification up to 400X. Stains used for slide preparation were either lactophenol cotton blue or Meltzer's solution or 0.03% lactofuschin. A small piece of fungal mycelium was scraped from the agar plate using either a needle or scalpel and placed into a drop of 70% ethanol

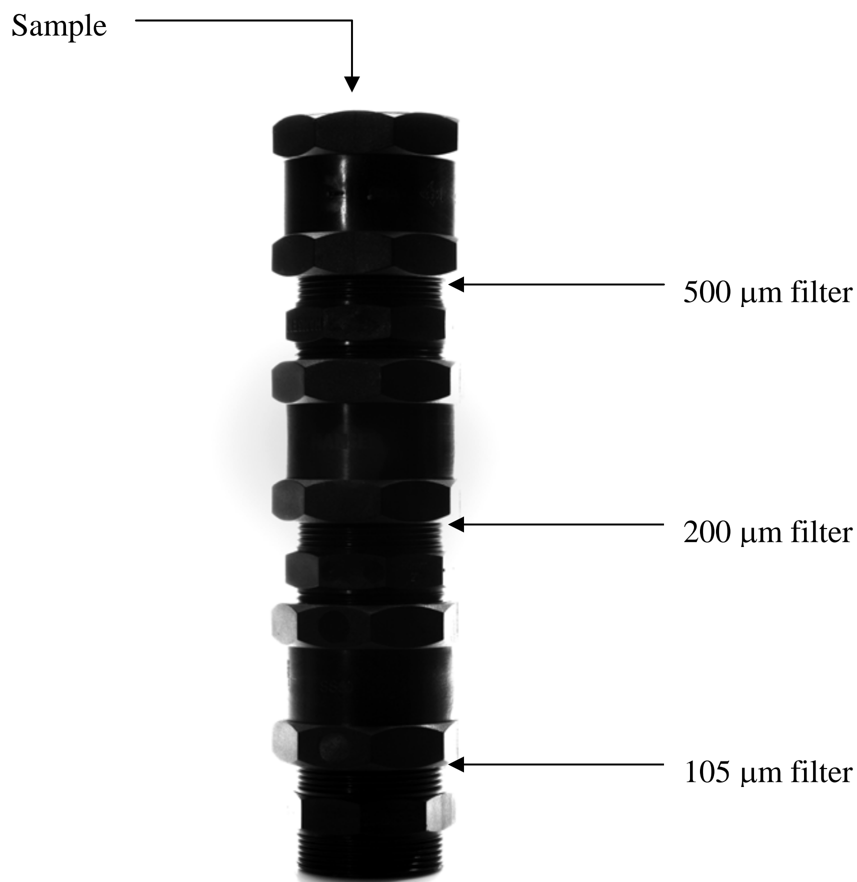


Figure 2.1 Particle filtration column. (Top) Filtration column showing positioning of the 3 filters. (Bottom) Interior of the filtration column showing filter supports within column.

on a microscope slide. After the ethanol evaporated, a drop of appropriate stain was applied to the fungal mycelium. After that, a cover slip was gently placed on top of the stain and pressed gently. The slide was now ready for examination under the compound microscope.

2.4.2 Sequencing of ITS region of fungal DNA

For DNA extraction, the fungal isolates were cultured onto plates of PDA overlaid with colourless cellophane and incubated for several days depending on the isolate at 25°C. Approximately 100mg of mycelium was harvested and frozen at -20°C. Mycelium was ground in liquid nitrogen, mixed with 500 μL of extraction buffer and incubated at 65°C for 40 minutes. The solution was then mixed with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and centrifuged at 10 000 rpm for 10 minutes. The supernatant was extracted again with one volume of chloroform-isoamyl alcohol (24:1). DNA was precipitated by addition of an equal volume of ice-cold isopropanol and centrifugation at 10 000 rpm for 10 minutes. Pellets were washed twice in 70% ethanol and air dried at 37°C. DNA was resuspended in 50 μL of MBG water and stored at -20°C prior to further analysis.

The entire ITS1-5.8s-ITS2 region of ribosomal DNA was amplified and sequenced using the primers ITS5 and ITS4 (White *et al.* 1990). PCR reactions with a total volume of 50 μL contained 0.4 μM of each primer (Invitrogen), 200 μM dNTPs (Innovative Sciences) 5 μL reaction buffer buffer , 2.5 mM $MgSO_4$, 1 μL BSA , 2 μL of template DNA and 0.7 U Expand HiFidelity Taq (Roche). The temperature profile for PCR was 30 cycles of 30 seconds at 94°C, 30 seconds at 56°C, 45 seconds at 72°C using an Eppendorf mastercycler. PCR products were visualised by gel electrophoresis and cleaned using PerfectPrep Gel cleanup kit (Eppendorf). PCR products were quantified using a spectrophotometer and sequenced in both forward and reverse directions (DNA Sequencing Facility, University of Canterbury, New Zealand). Forward and reverse sequences were assembled into consensus using ChromasPro Version 1.34

(Technelysium Pty Ltd). The sequence was then compared with the genebank database to determine the identity of the fungal isolates.

2.5 Extraction of isolates for bioassay and chemical studies

Fungal cultures prepared for extraction were grown on PDA plates at 25°C for 30 days. After 30 days, the agar was cut into pieces, transferred into a test tube and 25 mL of ethyl acetate added to the tube. The work was conducted in a fume cupboard. The agar was then homogenized using a homogenizer (Janke & Kunkle), test tube covered with aluminium foil and left in the fume cupboard overnight. The following day, the ethyl acetate layer was transferred into a 100 mL beaker using a 1 mL micropipette. Then, 20 mL of ethyl acetate was added to the test tube and left in the fume cupboard for at least 8 hours. The ethyl acetate layer was then transferred into a beaker. Another 20 mL of ethyl acetate was then added into the test tube and left in the fume cupboard for at least 6 hours and then the ethyl acetate layer transferred into a beaker.

The ethyl acetate extract was then filtered using a 0.45 μm PTFE membrane filter into a pre-weighed vial and dried using either nitrogen or a centrifugal evaporator and stored at 4°C. The dry weight of the concentrated extract was determined and dissolved in HPLC grade methanol to a concentration of 1 mg/mL.

2.6 Bioassays

Cytotoxicity and antimicrobial assays were conducted.

2.6.1 P388 quick screen assay

Murine cell line P388 (ATCC CCL 46, P388D1) used in this assay was grown in MEM (minimal essential media) supplemented with 10% fetal calf serum, 266 μg/mL of

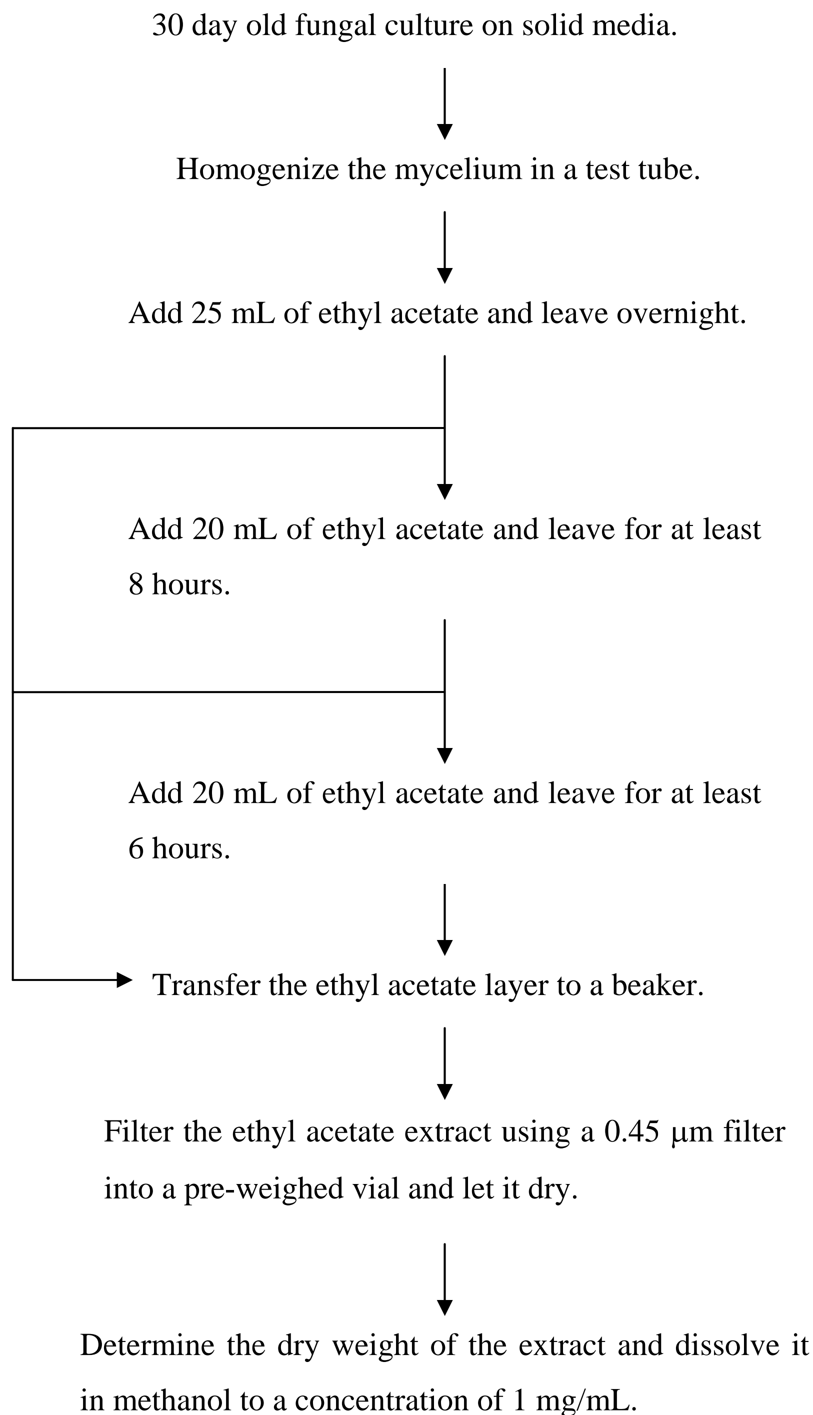


Figure 2.2 Summary of extract preparation process.

penicillin, 132 μg/mL of streptomycin, 2 mM L-glutamine, 2.2 g/L of $NaHCO_3$ and 7.4 mM HEPES. The negative control used was a cell free medium while the positive control used was analyte free cell.

5 μL of crude extracts (1 mg/mL) were pipetted into a 96 well microtitre plate which was then left to dry in a fume cupboard. After that, 150μL of the P388 cells were added to each well and incubated at 36°C for 3 days. 20 μL of MTT tetrazolium, a yellow dye, were added into each well and incubated for 4 hours at 36°C. Non-viable cells cannot metabolise this dye, whereas viable cells reduce this dye to MTT formazan resulting in an intense purple colour. Then, 170 μL of 0.08 HCl in isopropanol was added to each well. Finally, the cell viability can be calculated by measuring the absorption of each well at 540 nm, subtracting the absorption at 690 nm and using the negative and positive control as 0% and 100% growth reference respectively. Extracts that inhibit at least 50% of the cell growth were then subjected for IC_{50} assay.

2.6.2 P388 IC_{50} assay

P388 IC_{50} assay consists of a two dilution series of the sample of interest followed by incubation with P388 cells in microtitre plates as described in **2.6.1**.

The concentration of sample required to reduce cell growth by 50% when compared to controls, is expressed as an IC_{50} (Inhibitory Concentration) in ng/mL. The concentration is determined by using the absorbance values obtained at 540 nm after the yellow dye MTT tetrazolium is reduced to purple MTT formazan. Result that was expressed as $< IC_{50}$ or $> IC_{50}$ value indicates that the result is off scale and the sample needs to be retested at lower or higher concentrations respectively.

2.6.3 Antimicrobial quick screen assay

The Gram-positive bacterium, *Bacillus subtilis*, Gram-negative bacterium, *Pseudomonas aeruginosa* and the fungus, *Candida albicans*, were used for the purpose of this assay. The positive and negative controls used were the same as described in **2.6.1**. For activity

against *C. albicans*, 20 μL of crude extracts (1 mg/mL) were pipetted into a 96 well microtitre plate, while for the activity against bacteria, 10 μL of crude extracts (1 mg/mL) were pipetted into a 96 well microtitre plate. 200 μL of appropriate microorganisms were then added into each well and left for 24 hours at 30°C. After that, 30 μL of resazurin, a blue dye, was added into each well and left for another 24 hours (plates containing *Candida albicans* and *Pseudomonas aeruginosa*) or half to an hour (plates containing *Bacillus subtilis*) at 30°C. Resazurin is a growth indicator and the presence of bacteria or fungi is indicated by oxygen emission which reacts with resazurin, generating a pink color. The cell viability can then be calculated the same way as in **2.6.1**, but measuring the absorption at 600 nm and subtracting the absorption at 690 nm.

2.7 High Pressure Liquid Chromatography (HPLC)

Analytical HPLC was carried out using Dionex liquid chromatograph equipped with a UVD 340U diode array detector and connected to an Alltech ELSD 800. The column used was Phenomenex Luna C18 (10 × 250 mm, 5 μm). The standard gradient program was: 2 minutes of 10% ACN/H_2O; a linear gradient to 75% ACN/H_2O for 12 minutes; isocratic at 75% for another 10 minutes; a linear gradient for 2 minutes to 100% ACN/H_2O followed by isocratic at 100% ACN for 4 minutes then returned to 10% ACN/H_2O in 2 minutes and re-equilibrated for 8 minutes with a flow rate of 1 mL/min at 40°C.

2.7.1 HPLC screening

30 μg of samples were injected into the HPLC. The samples were analysed based on reverse phase chromatography with the standard gradient as in **2.7**. For the purpose of this screening the mili-Q H_2O used was acidified with 0.05% trifluoroacetic acid (TFA). The presence of metabolites were then indicated by both UV and ELSD traces, which were compared with database spectra to identify metabolites.

2.7.2 P388 HPLC assay

250 µg of selected crude extracts were injected into the HPLC. Samples were analysed by reverse phase chromatography using the standard gradient given in **2.7**. The eluent was collected in a 96 well polystyrene mictotitre plate. From this master plate, based on the IC_{50} results, 5 µL, 50 µL or 100 µL were transferred into a daughter plate. The daughter plate was then dried using a centrifugal evaporator and assayed against P388 cells as described in **2.6.1**.

2.7.3 Antimicrobial HPLC assay

250 µg of selected crude extracts were injected into the HPLC. The samples were analysed based on reverse phase chromatography using the standard gradient as given in **2.7**. The eluent was collected in a 96 wells polystyrene mictotitre plate and dried using the centrifugal evaporator before being assayed against appropriate microorganisms as described in **2.6.3**.

2.8 Preparation of samples for CapNMR

750 µg of crude extracts were injected into the HPLC. Samples were analysed based on reverse phase chromatography with the standard gradient as described in **2.7**. The eluent was collected in a 96 well polypropylene microtitre plate and dried using the centrifugal evaporator. The amount of samples were then determined based on the ELSD trace. The sample was only subjected to CapNMR if the ELSD trace was at least 500% of the ELSD base peak.

2.9 Capillary-probe Nuclear Magnetic Resonance (CapNMR)

^{1}H, COSY, HSQC and HMBC experiments were all conducted on a Varian INOVA 500 spectrometer at 23°C and 500 MHz. The 500 MHz Protasis Capillary NMR probe was

attached to the NMR. Coupling constants (J) were expressed in Hz while chemical shifts were expressed in parts per million (ppm) on the δ scale.

From **2.8**, the well of interest containing pure or partially pure compound was selected and dissolved in 6 μL of deuterated methanol, and injected into an inlet capillary using a syringe. A further 11 μL of deuterated methanol was injected to push the sample in the probe. After the reading was recorded, the sample was collected back into silated HPLC vials or inserts and kept at 4°C.

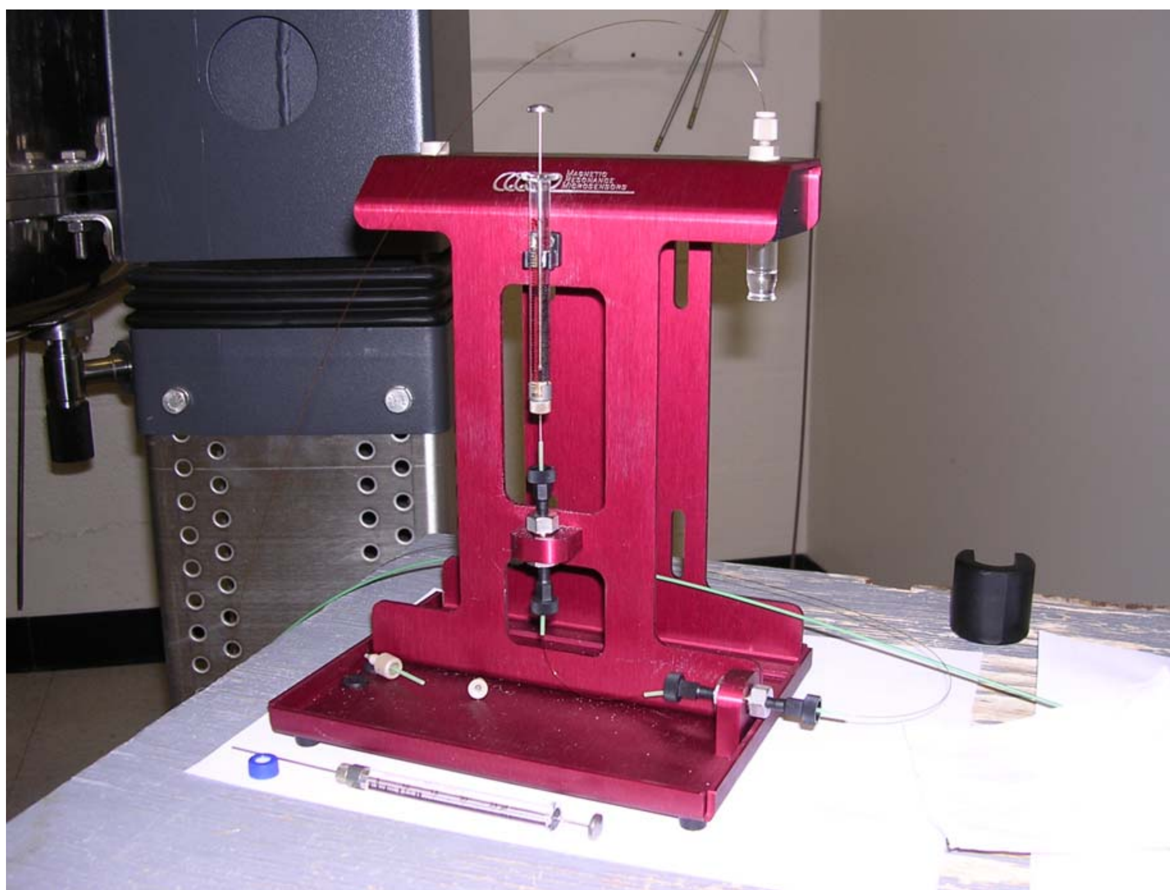


Figure 2.3 CapNMR

2.10 Electrospray ionization mass spectrometry (ESIMS)

Electrospray ionization mass spectra were recorded in positive and negative modes on a Micromass (Machester, UK) quadrupole time of flight (QTOF-2) mass spectrometer. The measurements were performed by Dr. Marie Squire and Mr. Robert Stainthorpe (Department of Chemistry, University of Canterbury).

2.11 Glassware deactivation (silanization) for CapNMR

All items were immersed into 5% dimethyldichlorosilane (DMDCS) solution for 5 to 30 minutes. After that, the 5% DMDCS solution was poured off and the items were rinsed with methylene chloride immediately. Then, the items were immersed in methanol for 15 to 30 minutes. Finally, the methanol was drained off and items kept in 100°C oven to dry completely.

Chapter 3

Preliminary screening

3.1 Fungal isolates from soil and leaf litter

A total of 232 fungal isolates were obtained from the soil (60 isolates) and leaf litter samples (172 isolates) (**Table 3.1**). A preliminary identification of the fungal isolates was made based on morphological and cultural characteristics. At this stage, many could only be identified to the genus level, however, most of the fungal isolates obtained could not be identified morphologically because of the absence of spores. For those non-sporing active isolates, DNA sequencing was used in an attempt to clarify their identity.

The total number of fungal cultures isolated here, is much less than that reported by Bills and Polishook (1994) who isolated a total of 1709 cultures from leaf litter collected from four different sites. Paulus *et al.* (2003) isolated 1365 cultures from leaf litter collected from eight different samples. In their studies however, greater replication and several media types for primary isolation were used.

Most of the fungal cultures isolated in this study were sterile and a number of slow growing fungi have also been isolated. This is consistent with the results of Bills and Polishook (1994) and Paulus *et al.* (2003) who noted isolation of sterile fungi with this technique.

Table 3.1 Fungal isolates obtained from soil and leaf litter samples.

Sampling sites	Samples	Number of isolates
Klondyke Corner	Soil	60
Carew Falls	Rimu leaf litter	42
	Mixed leaf litter	30
Mount French	Rimu leaf litter	18
	Mixed leaf litter	63
Kaituna Valley	Mixed leaf litter	19
	Total	**232**

3.2 Selection of fungal isolates for bioactivity screening

From a total of 232 fungal isolates, only 108 of the isolates were selected for bioactivity screening. Of the 108 fungal isolates, 85 isolates were selected from the leaf litter samples while 23 isolates were selected from the soil sample. The fungal isolates were selected randomly based on their cultural characteristics especially colour which often represents compounds of interest. Some isolates of common species such as *Aspergillus sp.* and *Penicillium sp.* were also subjected for bioassay since they are members of very productive genera. **Figure 3.1** shows those fungal isolates selected for bioactivity screening.

All cultures were extracted with ethyl acetate and the extracts subjected to the quick screen assay against P388 murine leukemia cell line, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Candida albicans*. The results of the assays are shown in **Table 3.2**.

Figure 3.1 Examples of fungal isolates selected for bioactivity screening.

Penicillium sp. (KV2)

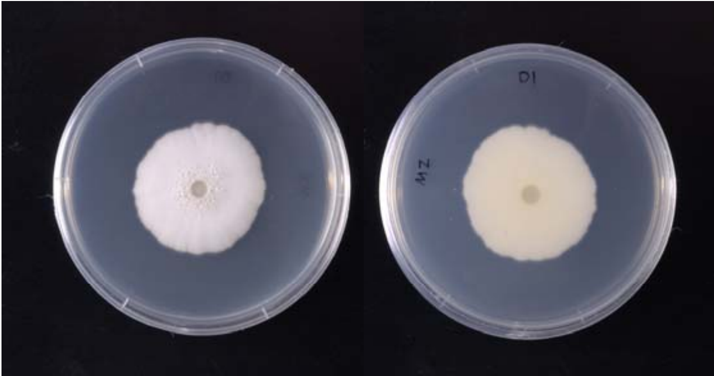
Penicillium sp. (RF14)

Aspergillus sp. (C5)

Figure 3.1 Continued

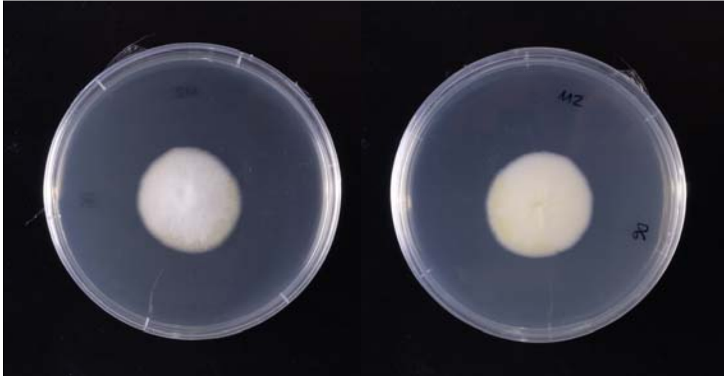
Beauveria malawiensis

Beauveria sp. (MC3)



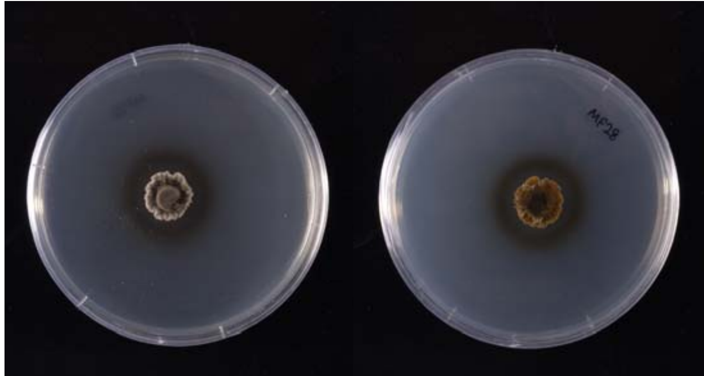
Verticillium sp. (D1)

Figure 3.1 Continued



Tolypocladium inflatum

Unidentified fungal culture (MF31)



Unidentified fungal culture (MF28)

Table 3.2 Bioactivity of fungal isolates from soil and leaf litter samples.

Isolate	Extract	Quick screen (% of cell growth)			
		P388	***B. subtilis***	***P. aeruginosa***	***C. albicans***
MC3	F8051	36.3	66.2	104.2	104.7
MC4	F8052	6.8	68.0	100.5	100.1
MC13	F8053	77.5	100.0	102.7	102.5
MF2	F8054	79.0	101.4	99.9	100.7
MF7	F8055	22.8	92.3	102.6	100.7
MF17	F8056	98.7	101.3	101.9	100.8
MF18	F8057	41.3	84.0	102.9	103.7
MF19	F8058	98.5	100.1	103.7	103.1
RC2	F8059	64.6	86.2	100.6	102.7
RC3	F8060	66.3	90.0	102.0	101.9
RC7	F8061	87.1	93.0	101.9	102.3
RF2	F8062	43.5	92.6	100.9	100.9
MC5	F8215	79.9	92.8	101.1	104.6
MC7	F8216	37.9	83.9	101.6	85.2
MC11	F8217	18.2	87.3	101.6	92.0
RF3	F8218	66.1	96.6	107.5	105.4
RF6	F8219	31.3	94.8	99.4	106.4
MF14	F8220	49.9	97.9	100.9	112.8
MF17	F8221	2.4	98.1	103.3	84.1
RC1	F8222	52.4	72.8	101.2	94.9
RC9	F8223	7.5	93.2	107.4	88.9
MC10	F8224	102.0	84.1	107.5	97.3
MC15	F8225	68.3	85.1	100.5	99.6
MF3	F8226	82.2	94.2	101.8	95.0
MF9	F8227	59.3	93.7	101.2	78.1
RC11	F8228	40.6	92.9	105.0	81.4

Table 3.2 Continued

Isolate	**Extract**	**Quick screen (% of cell growth)**			
		P388	***B. subtilis***	***P. aeruginosa***	***C. albicans***
RF9	F8229	73.7	77.9	105.7	101.5
RF7	F8230	38.7	90.8	74.3	106.2
MC1	F8231	31.2	83.5	101.2	101.4
MF1	F8232	92.6	92.4	100.4	85.1
MF4	F8233	37.3	102.4	100.4	96.8
MF5	F8234	94.8	99.0	104.4	105.7
MF11	F8235	90.5	101.3	105.0	105.8
MF20	F8236	65.9	97.5	99.8	109.9
MF31	F8237	90.3	91.9	100.3	97.9
MC6	F8239	14.1	95.8	99.1	106.0
MC8	F8240	72.3	90.7	105.5	99.4
MC16	F8241	115.8	96.1	105.4	98.9
MC20	F8242	108.2	91.2	110.1	111.2
MF8	F8243	38.8	88.3	100.2	109.8
MF15	F8244	74.5	96.8	97.3	106.6
MF24	F8245	69.2	98.4	99.7	96.4
RF5	F8246	76.5	91.3	104.8	102.9
RF8	F8247	66.4	89.4	105.1	111.5
RF10	F8248	52.8	99.2	104.2	101.0
RF11	F8249	79.9	101.8	99.2	92.5
RC5	F8250	30.3	94.3	104.2	104.9
RC10	F8251	57.2	98.8	105.6	105.5
MC2	F8252	82.7	97.2	105.6	100.2
MC12	F8253	35.4	90.3	104.9	109.9
MC14	F8254	69.2	91.2	104.8	106.2
MC17	F8255	92.9	90.6	100.2	108.0

Table 3.2 Continued

Isolate	Extract	Quick screen (% of cell growth)			
		P388	*B. subtilis*	*P. aeruginosa*	*C. albicans*
MF6	F8256	3.5	88.4	99.9	103.3
MF12	F8257	47.5	91.7	99.1	103.9
MF26	F8258	39.6	101.5	90.3	107.3
MF25	F8259	79.8	93.6	99.7	111.6
KV1	F8260	23.5	91.5	99.7	106.1
KV2	F8261	63.3	77.7	104.9	104.1
KV7	F8262	6.1	19.0	24.3	30.7
KV9	F8263	5.5	39.2	104.5	103.2
KV14	F8264	32.3	72.9	95.6	110.8
KV15	F8265	58.3	70.8	99.9	86.4
KV11	F8266	20.6	101.7	105.6	97.7
KV12	F8267	13.8	82.6	99.9	97.1
MC18	F8417	56.0	99.9	-	87.4
RF4	F8418	41.4	99.7	103.8	89.0
A3	F8419	17.8	100.9	103.9	88.2
B3	F8420	24.3	98.2	103.1	69.6
C5	F8421	42.7	102.9	104.2	95.1
D1	F8422	66.2	103.3	112.4	97.7
D3	F8423	53.8	104.1	104.7	96.8
D4	F8424	60.2	102.0	104.2	91.7
D5	F8425	56.9	103.3	107.1	103.0
J1	F8426	94.3	101.6	103.6	73.6
J2	F8427	85.8	102.1	111.1	94.5
KV4	F8428	73.0	101.0	104.1	107.9
KV5	F8429	63.6	101.4	105.7	93.1
KV6	F8430	72.9	92.8	110.8	91.0

Table 3.2 Continued

Isolate	Extract	Quick screen (% of cell growth)			
		P388	***B. subtilis***	***P. aeruginosa***	***C. albicans***
KV8	F8431	19.6	98.7	106.6	103.5
KV10	F8432	81.2	97.0	113.0	101.3
KV13	F8433	8.6	100.2	106.3	102.6
MC9	F8434	76.7	97.8	104.0	92.6
MC22	F8435	20.5	103.1	105.2	70.1
MF10	F8436	63.1	102.6	102.2	95.5
MF23	F8437	86.3	104.2	103.3	94.2
RC12	F8438	37.5	103.2	102.4	105.6
RC13	F8439	1.9	96.4	101.6	100.2
D6	F8440	36.3	100.3	101.8	98.3
L	F8441	-0.3	102.8	102.0	98.5
MC21	F8442	74.0	103.5	102.1	86.2
MF27	F8443	8.0	102.0	103.0	87.0
RF14	F8444	75.6	102.3	102.9	92.7
G1	F8445	33.1	104.3	111.3	93.6
G2	F8446	-0.3	102.8	110.9	95.7
G3	F8447	50.6	103.9	103.5	85.2
G4	F8448	51.8	103.0	105.6	88.1
H1	F8449	73.4	102.1	101.4	76.0
H2	F8450	44.8	103.9	103.3	92.4
H4	F8451	35.7	103.0	111.3	84.1
H5	F8452	45.9	101.3	111.0	90.9
H6	F8453	62.9	103.3	111.9	94.6
H7	F8454	76.0	100.8	101.2	75.5
H8	F8455	60.2	103.0	103.9	89.4
H9	F8456	68.7	100.3	103.8	75.8

Table 3.2 Continued

Isolate	Extract	Quick screen (% of cell growth)			
		P388	***B. subtilis***	***P. aeruginosa***	***C. albicans***
KV16	F8457	26.7	103.4	110.9	37.4
KV17	F8458	19.0	102.7	110.4	95.1
KV18	F8459	23.3	103.6	103.8	35.3
KV19	F8460	6.4	103.7	103.7	97.6

The results above show the percentage of cell growth and the lower the percentage, the more active the extract is. Of the 108 extracts assayed, 47 (43.5%) were active only against P388 cells, one (0.9%) against both P388 cells and *Bacillus subtilis* and one (0.9%) against P388 and all three microorganisms.

Extracts that inhibited at least 50% of the growth of P388 cells and gave a good HPLC trace were then subjected to IC_{50} assay to determine the concentration required to inhibit the growth of the cells before being selected for further investigations. The results for the IC_{50} assay are shown in **Table 3.3**. While extracts that inhibited at least 50% of the growth of microbial cells and gave a good HPLC trace were selected for further investigations.

Table 3.3 P388 IC_{50} assay.

Isolate	Extract	IC_{50} (ng/mL)
MF7	F8055	14 410
MF18	F8057	4 157
MC6	F8239	13 186
RC5	F8250	25 287
MC12	F8253	21 172
MF6	F8256	3 585

Table 3.3 Continued

Isolate	Extract	IC_{50} (ng/mL)
KV1	F8260	< 975
KV7	F8262	3 002
KV9	F8263	1 950
KV14	F8264	41 705
KV11	F8266	< 975
A3	F8419	4 776
KV13	F8433	> 125 000
RC13	F8439	4 134
L	F8441	3 377
MF27	F8443	55 675
G2	F8446	2 604
H2	F8450	48 212
H4	F8451	32 736
H5	F8452	44 092
KV18	F8459	5 484

Following bioassay, the active extracts were subjected to HPLC assay to determine which peak(s) is/are responsible for the bioactivity before NMR studies. Not all of the active extracts were able to be investigated because of time constraints and quantity of extract available. In addition there were a few interesting non-active compounds from both active and non-active extracts investigated further by NMR. In summary, 6 extracts were selected for further investigation, 5 active extracts and one non-active extract. 9 compounds were isolated from these 6 extracts. Of these 9 compounds, 5 were active against P388 cells only, one was active against both P388 cells and *Bacillus subtilis* and 3 were not active.

Chapter 4

Secondary metabolites from *Aspergillus sp.* (MF7)

4.1 Introduction

The genus *Aspergillus* was first recorded by Pietro Antonio Micheli, an Italian priest and biologist, in 1727 (Samson, 1994) and *Aspergillus* species were the first fungal organisms cultivated on artificial media and studied for their biochemical properties. They are of worldwide occurrence and some demonstrate oligotrophy where they are capable of growing in nutrient depleted environments such as damp walls. *Aspergillus* is a hyphomyceteous genus, however, the perfect state of some Aspergilli has been reported and placed in ascomyceteous genera such as *Eurotium, Neosartorya*, *Emericella* and *Chaetosartorya*.

Aspergillus is characterized by the presence of a conidiogenous structure, the aspergillum, consisting of a stipe with a bent or T-shaped base (foot cell) and an inflated apex (vesicle) bearing simultaneously produced, conidium-bearing cells (phialides). Phialides may be borne directly on the vesicle (uniseriate aspergillum) or with a layer of cells (metulae) between the vesicle and phialides (biseriate aspergillum). Raper and Fennel (1965) divided the genus into 18 groups and accepted 132 species with 18 varieties. Since then, many new taxa have been described and the validity of these species has been reviewed and compiled by Samson (1979; 1992).

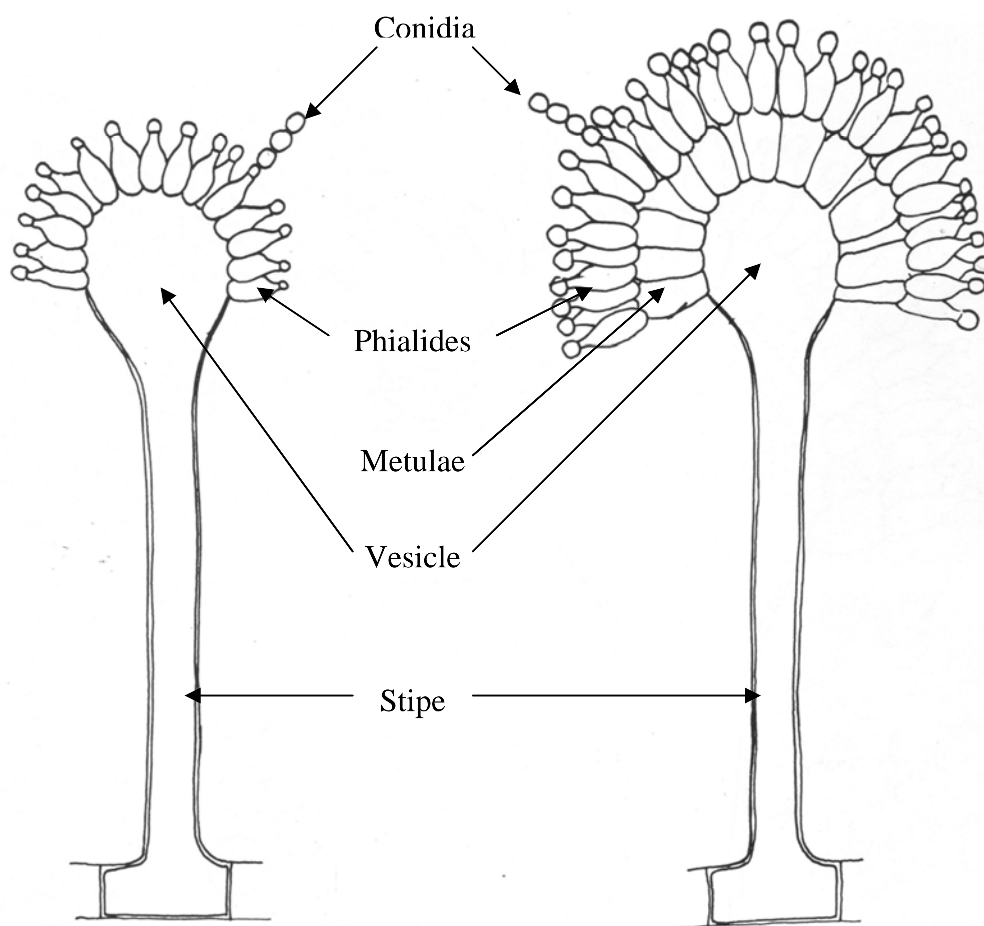


Figure 4.1 Characteristics of the conidiophores of *Aspergillus spp.*

Aspergilli are of importance in many respects. Some *Aspergillus spp.* possess a broad range of biological activities and have been used in the search for novel active metabolites. For example, *Aspergillus fumigatus* has been found to produce various types of antimicrobial compounds such as fumifungin (Mukhopadhyay *et al.*, 1987), synerazol (Ando *et al.*, 1991) and fumagillin (Han *et al.*, 2000). The genus *Aspergillus* is also a versatile producer of enzymes and citric acid and is used in the fermentation industry for large scale production of these products. The range of enzymes produced by species of *Aspergillus* is being steadily extended by the use of recombinant DNA technology (Harvey and McNeil, 1993). In addition, Aspergillus species have been used over the

centuries by humans to make food and beverages (Kendrick, 1992), especially in Asian countries such as Japan, again through fermentation processes.

Aspergillus species are also known for their ability to produce toxic metabolites, mycotoxins, which can cause serious effects in humans, animals and plants. For example, aflatoxin produced by *Aspergillus flavus* is both toxic and carcinogenic for humans and animals. *Aspergillus spp.* are of increasing importance as human pathogens. With the increase in immunocompromised patients, the incidence of *Aspergillus* is showing a consequent increase. *Aspergillus fumigatus* is the most notable of these pathogens and causes range of conditions from allergic bronchopulmonary aspergillosis to pulmonary aspergilloma to invasive aspergillosis. *Aspergillus* is also recorded as a plant pathogen, particularly of fruit such as grapes and citrus (Logrieco *et. al.*, 2007).

4.2 *Aspergillus sp.* (MF7)

Aspergillus sp. (MF7) was isolated from leaf litter samples collected from Mount French (West Coast). Based on its ITS sequence, it was identified as *Aspergillus versicolor*. **Figure 4.2** shows colonies of MF7 on PDA. The colony is white at first, turning to yellow, orange-yellow to yellow green, often intermixed with flesh to pink colours. Conidiophores are hyaline or slightly pigmented with smooth walls. Vesicles are subglobose to ellipsoidal and phialides borne on metulae. Conidia globose, brownish and conspicuously echinulate. These characteristics are consistent with its designation as *Aspergillus versicolor*.

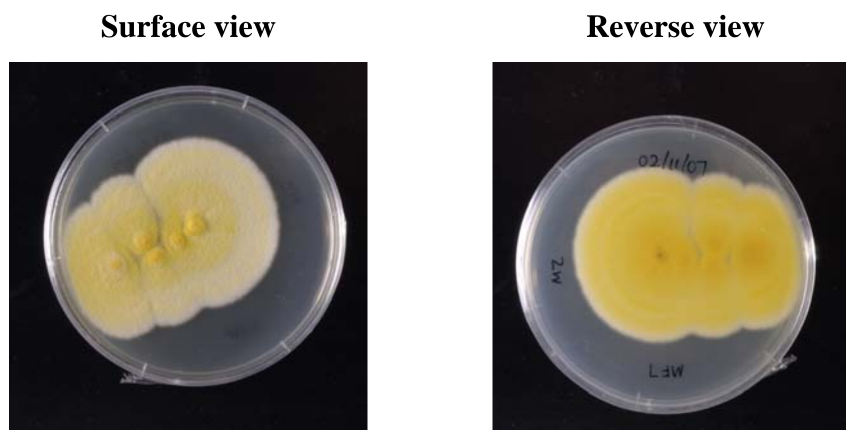


Figure 4.2 14 day old colonies of *Aspergillus versicolor* (MF7) on PDA.

4.2.1 Compound isolated from *Aspergillus versicolor* (MF7)

The crude extract from *Aspergillus versicolor* (MF7) was yellow in colour and cytotoxic in the P388 assay (IC_{50} value of 14 410 ng/mL). From the HPLC trace, one main peak could be detected at 17.3 minutes. The UV profile of the peak had a maximum absorbance at 205, 245 and 324 nm. Bioactive profiling confirmed that the one main peak (17.3 minutes) contained the cytotoxic compound.

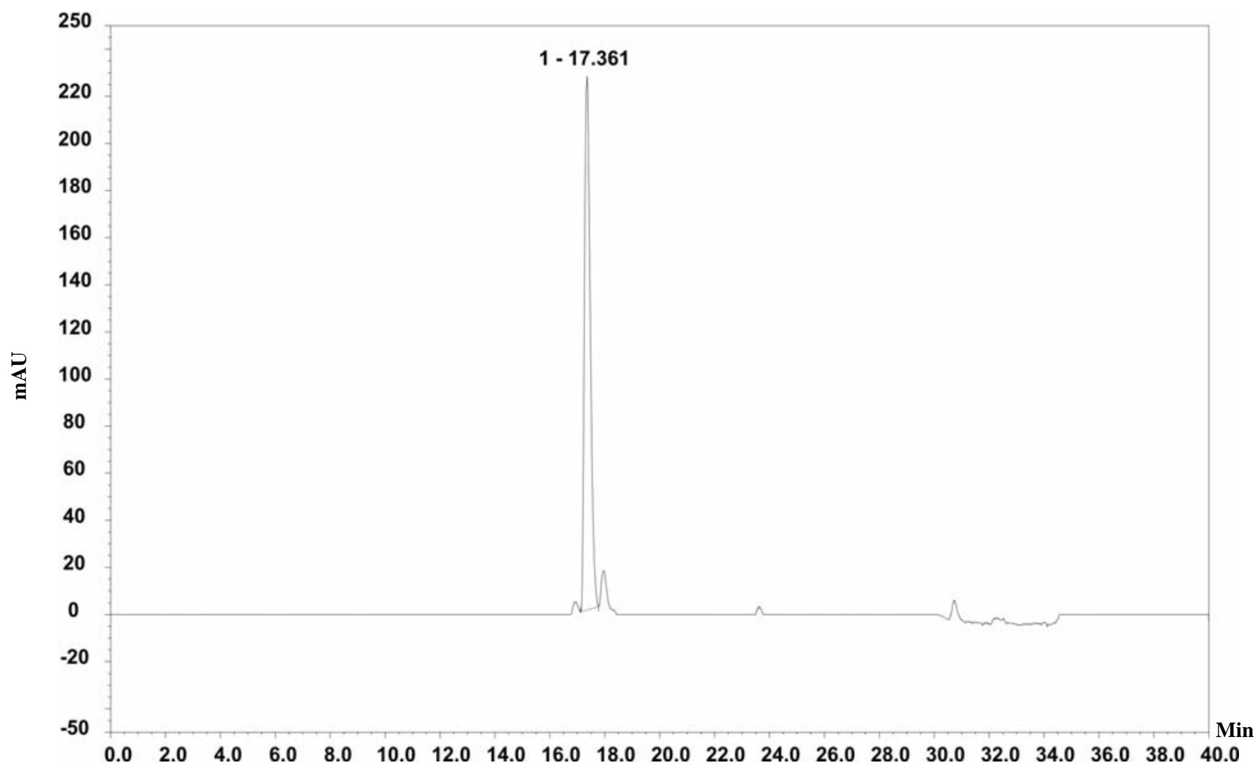


Figure 4.3 ELSD chromatogram of extract from *Aspergillus versicolor* (MF7).

The UV profile of this compound was compared with the UV library which revealed that the peak corresponded to that of sterigmatocystin. Both the UV profile and the retention time were almost perfectly matched. For further confirmation of the identity of the compound, its molecular weight was also determined by mass spectrometry and found to be 324 Da (major ions at 325.3 m/z $[M+H]^+$ and 671.5 m/z $[2M+Na]^+$), which is identical to that of sterigmatocystin.

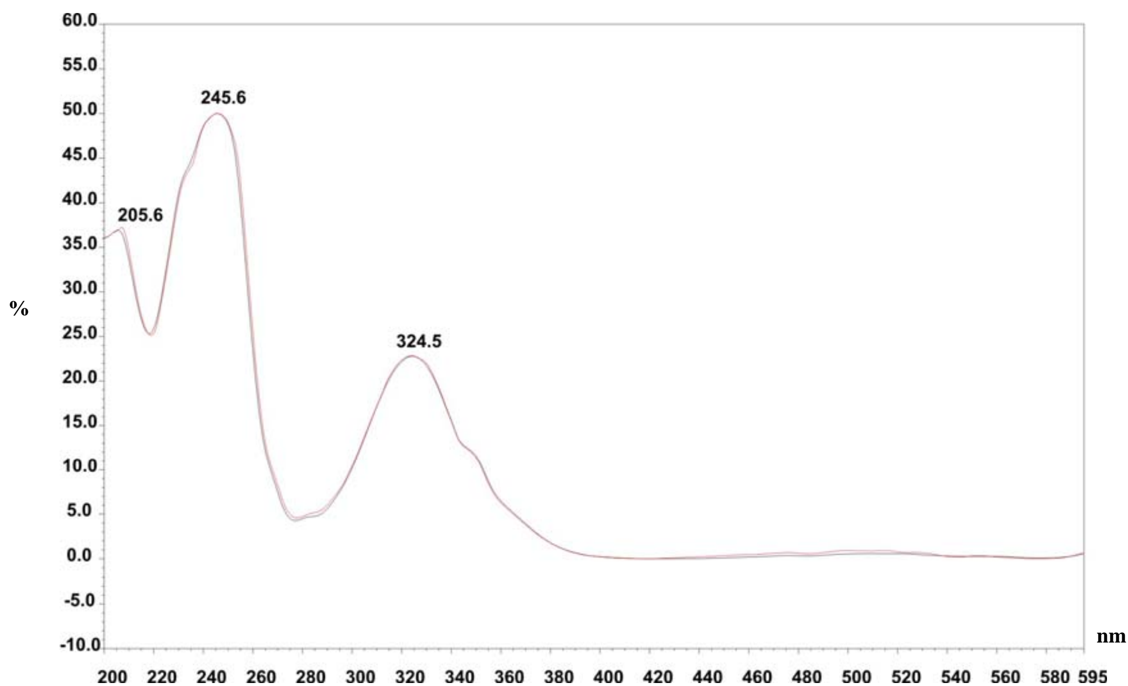


Figure 4.4 UV profile of the compound (black) eluted at 17.3 minutes matched with UV profile of sterigmatocystin (red) in the UV library database.

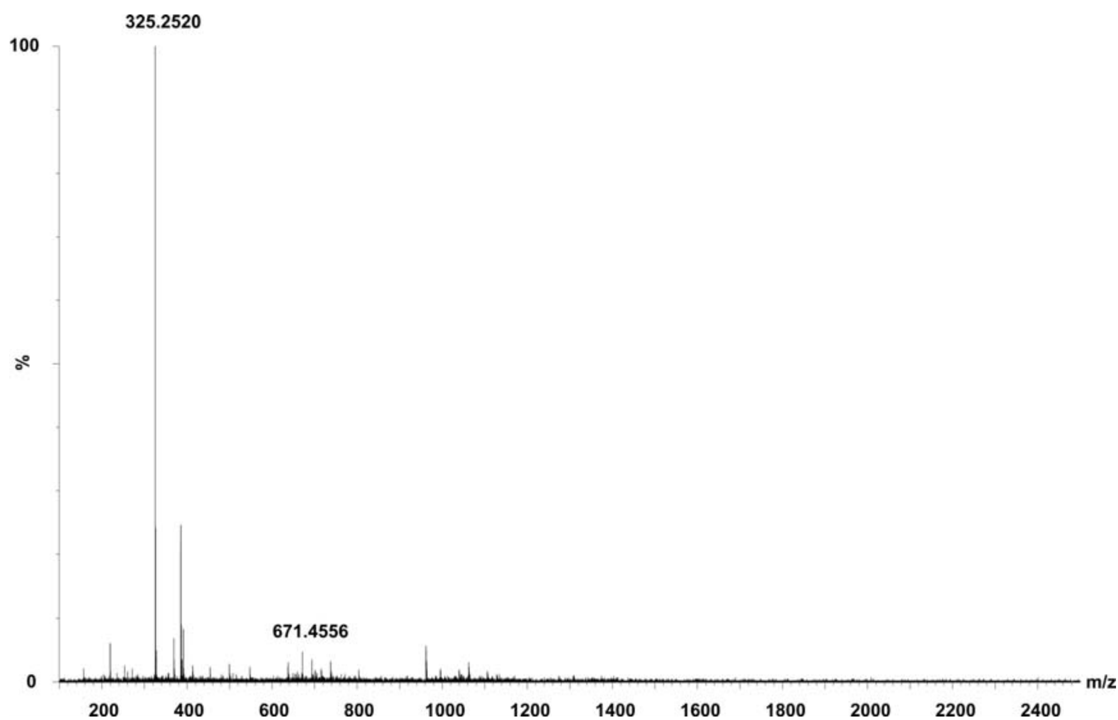


Figure 4.5 ESI-MS spectrum for the active compound eluted at 17.3 minutes.

4.3 Sterigmatocystin

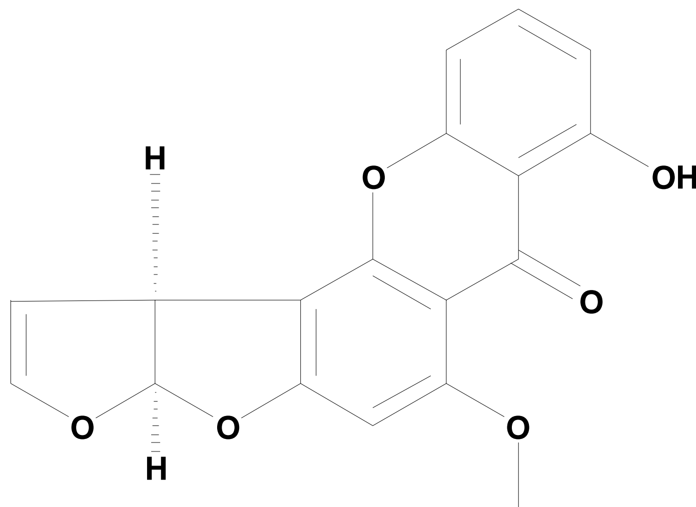


Figure 4.6 Structure of sterigmatocystin.

Sterigmatocystin ($C_{18}H_{12}O_6$) is a toxic metabolite structurally closely related to the aflatoxins and consists of a xanthone nucleus attached to a bifuran structure. Sterigmatocystin has been reported to be produced by various species of *Aspergillus* including *Aspergillus versicolor* and *Aspergillus nidulans* (Cole and Schweikert, 2003) and also by *Bipolaris sorokiniana* (Rabie and Steyn, 1976). It crystallizes as pale yellow needles and is readily soluble in methanol, ethanol, acetonitrile, benzene and chloroform.

The toxic effects of sterigmatocystin are much the same as those of aflatoxin B1. It is thus considered as a potent carcinogen, mutagen and teratogen. Toxic effects of sterigmatocystin-fed laboratory animals have included kidney and liver damage and diarrhoea. Skin and hepatic tumours are induced in rats by dermal application. Cattle exhibiting bloody diarrhoea and loss of milk production. Aside from being toxic itself, it also acts as a biogenetic precursor in the biosynthesis of aflatoxins by *Aspergillus parasiticus* and *Aspergillus flavus* (Rabie and Steyn, 1976).

The International Agency for Research on Cancer has classified sterigmatocystin as a group 2B substance indicating its potential carcinogenicity. In practice, the risk is relatively low because this substance usually appears in moldy or poor quality products such as wheat, maize, animal feed, hard cheese, pecan nuts and green coffee beans where the chance of daily exposure for most people is very low.

Chapter 5

Secondary metabolites from *Penicillium sp.* (KV1 and KV11)

5.1 Introduction

Penicillium is a genus that is universal in occurrence. The name *Penicillium* comes from the word “brush”, which refers to the appearance of the spore bearing structures which resembles a brush or candelabra in *Penicillium*. Penicillia are versatile and opportunistic. *Penicillium* is a hyphomyceteous genus but the teleomorphs for many Penicillia are now known and placed in the ascomyceteous genera *Eupenicillium* and *Talaromyces*.

Penicillium colonies usually growing rapidly in shades of green but can also be white, grey, blue or yellow, mostly consisting of a dense felt of conidiophores. Conidiophores are single (mononematous) or bundled (synnematous), consisting of a single stipe terminating in either a whorl of phialides (simple, monoverticillate) or in a penicillus. The penicillus containing branches and metulae (penultimate branches which bear the whorl of phialides). All cells between metulae and stipe are referred to as branches. The branching pattern can either be one-stage branched (biverticillate-symmetrical), two-stage branched (biverticillate- asymmetrical) or three or more stage branched. The conidiophores arise from the substrate (velvety), from aerial hyphae (lanose), from prostrate bundled hyphae (funiculose) or from erect loosely or compactly bundled hyphae

(fasciculate or synnematous). Conidiophores are hyaline, smooth or rough walled. Phialides are usually flask-shaped consisting of a cylindrical basal part and a distinct neck or lanceolate. Conidia usually in long dry chains, divergent or in columns, globose, ellipsoidal, cylindrical or fusiform, hyaline or greenish, smooth or roughed walled (Samson and Hoekstra, 1984).

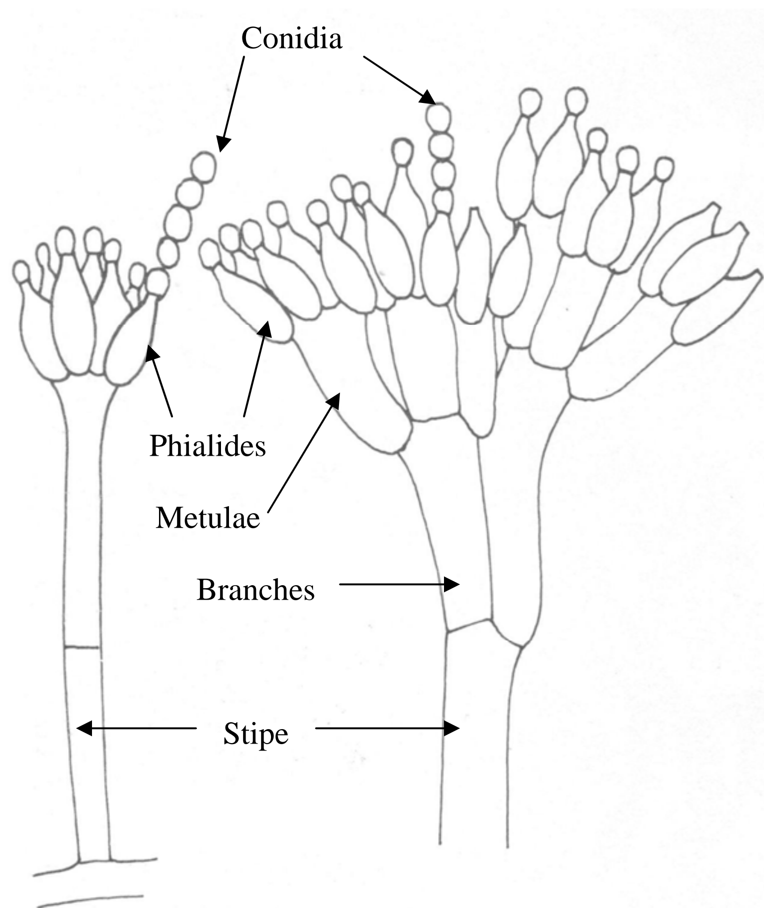


Figure 5.1 Structure of the penicillus.

Penicillium spp. are common causes of fungal spoilage in fruits and vegetables. *Penicillium italicum* and *Penicillium digitatum* are known to infect citrus fruits, while *Penicillium expansum* is known to cause apple fruit decay. *Penicillium expansum* not only causes fruit decay but also produces the carcinogenic mycotoxin, patulin. This toxin may rise to unacceptable levels in fruit destined for processing and may also result in off

flavours. *Penicillium* may also cause disease in humans with *Penicillium marneffii* as a prime example causing Penicillinosis.

The importance of *Penicillium* can be seen in many ways. Like *Aspergillus spp.*, *Penicillium spp.* possess a broad range of biological activities. Kurobane *et al.*, (1981) reported the production of fulvic acid by *Penicillium brefeldianum* which possessed antiviral, antifungal, antioxidant and antibiotic activities. Nam *et al.*, (2000) has reported the production of 8-O-methylsclererotiorinamine from *Penicillium multicolor* which showed antimicrobial activity. However, the greatest discovery related to *Penicillium* was made by Alexander Fleming in 1929 who discovered penicillin from *Penicillium notatum*. Penicillin is considered as the drug of the age and precursor to many developments in biotechnology especially the pharmaceutical industry. *Penicillium spp.* such as *Penicillium roquefortii* and *Penicillium camambertii* have also been used in the food industry especially in cheese making to produce cheese such as roquefort and camembert.

5.2 *Penicillium sp.* (KV1) and (KV11)

Two species of *Penicillium* were selected for further investigations designated KV1 and KV11. Both species were of different colony and morphology, but identification to species level was not attempted.

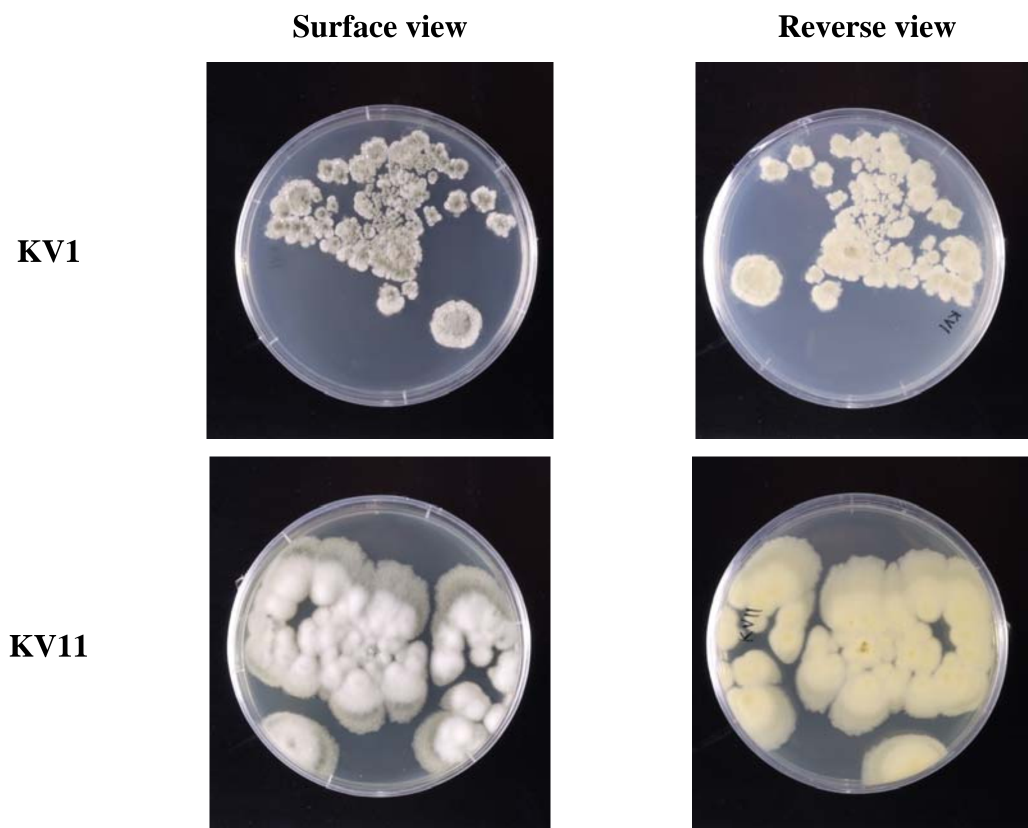


Figure 5.2 14 day old colonies of KV1 and KV11 on PDA.

5.3 Compounds isolated from *Penicillium sp.* (KV1)

Based on the quick screen assays, it was shown that the KV1 extract was active against P388 cells with an IC_{50} value of < 975 ng/mL. From the HPLC trace two main peaks could be detected. The first peak eluted at 8.6 min and the second peak at 15.0 min.

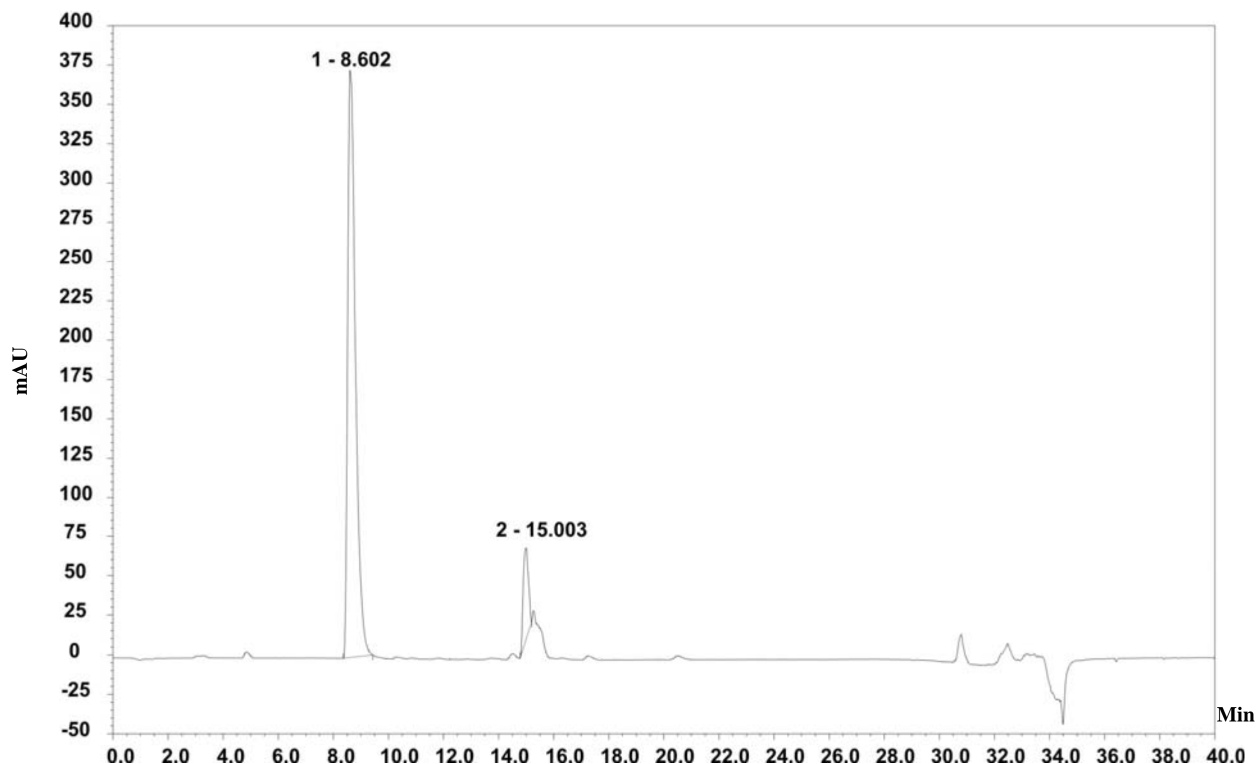


Figure 5.3 ELSD chromatogram of extract from *Penicillium sp.* (KV1).

5.3.1 KV1A: Compound eluted at 8.6 minutes

The UV profile of the first peak showed maximum absorbance at 212, 267 and 301 nm. HPLC bioactivity profiling revealed the first peak was not the one responsible for the cytotoxic activity, however, the compound was still pursued because of the lack of matching profiles within the UV library database. An aliquot of this compound was submitted for mass spectrometry and NMR spectroscopy for further investigation.

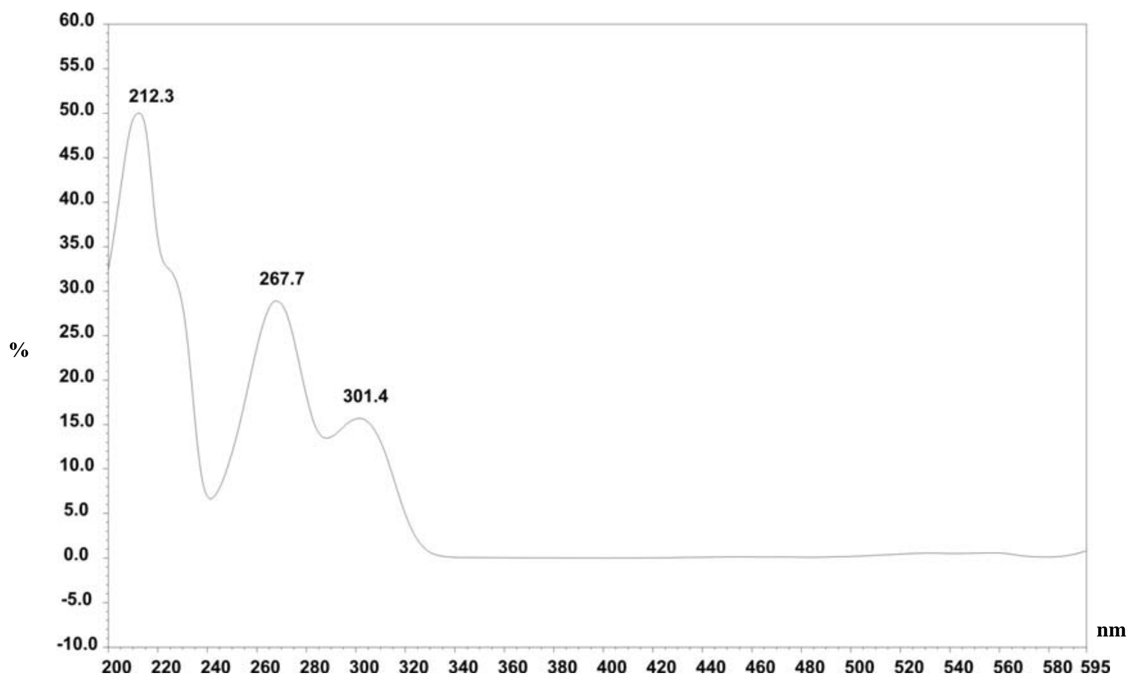


Figure 5.4 UV profile of KV1A (8.6 min).

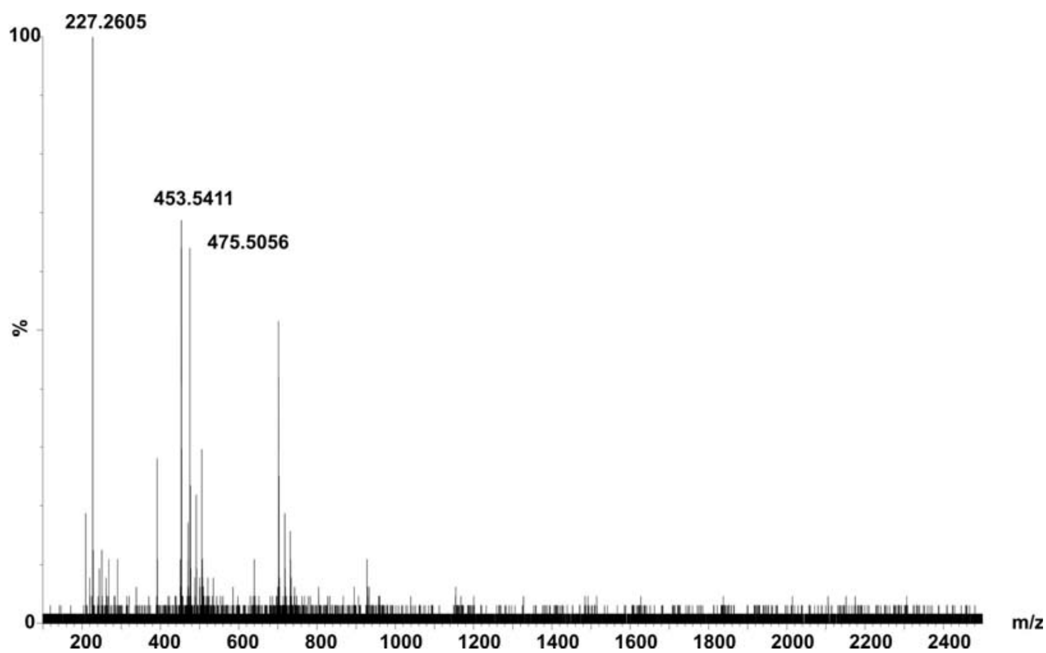


Figure 5.5 ESI-MS spectrum for KV1A (8.6 min).

As indicated in **Figure 5.5**, the ESI-MS showed major ions at 227.3 m/z $[M+H]^+$, 453.5 m/z $[2M+H]^+$ and 475.5 m/z $[2M+Na]^+$. This suggested that the molecular weight of the compound was 226 Da.

The proton NMR spectrum of KV1A is shown in **Figure 5.6**. From the spectrum, it was assumed that the peak was actually a mixture of two isomeric compounds. A search was then conducted using the AntiMarin database. The data searched on was one singlet methyl signal (1.63 ppm) and one sp^3 methine signal (4.37 ppm). The molecular weight entered was 225-227 Da. The search gave 3 hits, trihydroxymellein, 3,4,6-trihydroxymellein and 2,4-dihydroxy-6-(1-hydroxyacetonyl)benzoic acid. Trihydroxymellein and 3,4,6-trihydroxymellein are an inseparable part of diastereoisomers known as 3,4,6,8-tetrahydroxy-3-methyl-3,4-dihydroisocoumarin. The chemical shift of the methyl group of this compound should be about 1.5 ppm which is about the same as shown in the proton NMR spectrum (1.63 ppm). Based on these evidences, KV1A was identified as 3,4,6,8-tetrahydroxy-3-methyl-3,4-dihydroisocoumarin. There are two possible diastereoisomers of this compound. Since there were inseparable, it was impossible to determine which peaks in the proton NMR spectrum arose from which isomer. The structures of the isomeric compounds are as shown in **Figure 5.7**, or their enantiomers. This inseparable mixture has previously been reported by Ayer *et al.*, (1987).

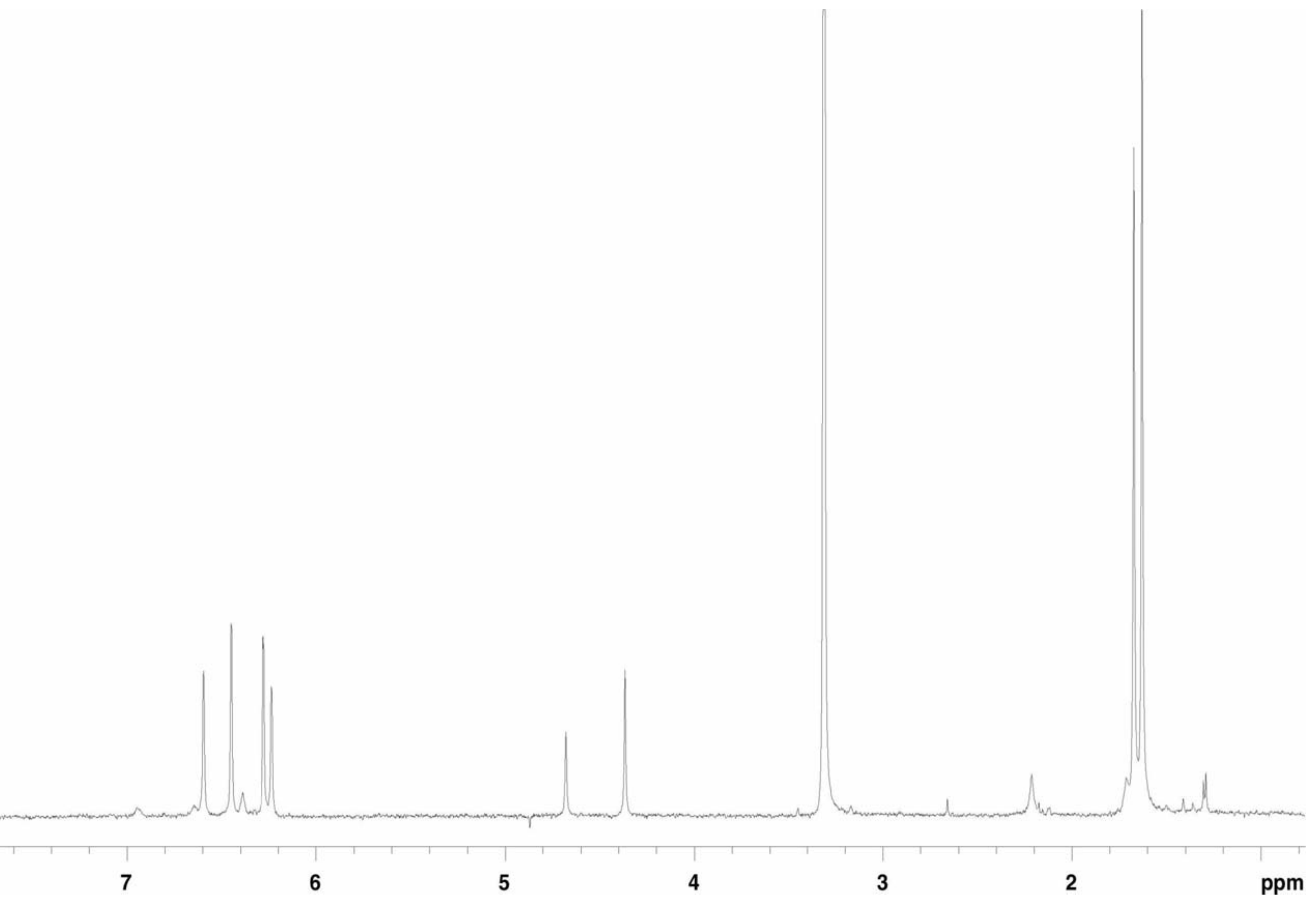


Figure 5.6 Proton NMR spectrum of KV1A.

5.3.1.1 3,4,6,8-tetrahydroxy-3-methyl-3,4-dihydroisocoumarin

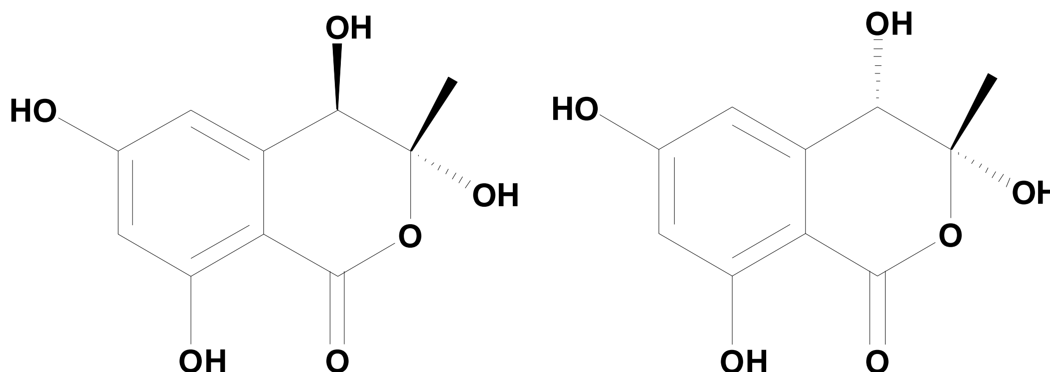


Figure 5.7 Diastereoisomeric structures of 3,4,6,8-tetrahydroxy-3-methyl-3,4-dihydroisocoumarin.

3, 4, 6, 8- tetrahydroxy- 3 -methyl-3, 4-dihydroisocoumarin ($C_{10}H_{10}O_6$) is non-active compound and was previously isolated by Ayer *et al.* (1987) as a metabolite of *Ceratocystis* minor, a fungus associated with the blue stain disease of Canadian pine trees. From literature review, little information could be found on these compounds. It has been reported, however, that this compound was used in the synthesis of acetophthalidin, a metabolite which inhibits the progression of the mammalian cell cycle by Nomoto and Mori, (1997).

5.3.2 KV1B: Compound eluted at 15 minutes

The UV profile of the second peak had a maximum absorbance at 215, 250 and 304 nm. Bioactivity profiling established that this peak was the one responsible for the cytotoxic activity of the crude extract.

The UV profile of the compound when searched in the UV library revealed that the peak corresponded to that of mycophenolic acid. Both the UV profile and the retention time fitted almost perfectly. For further confirmation of the identity of the compound, the molecular mass of the compound was measured. From the ESI-MS, the molecular weight of the compound was 320 Da (major ions at 321.4 m/z $[M+H]^+$, 343.4 m/z $[M+Na]^+$,

641.8 m/z $[2M+H]^+$ and 663.8 m/z $[2M+Na]^+$), which corresponding exactly of mycophenolic acid.

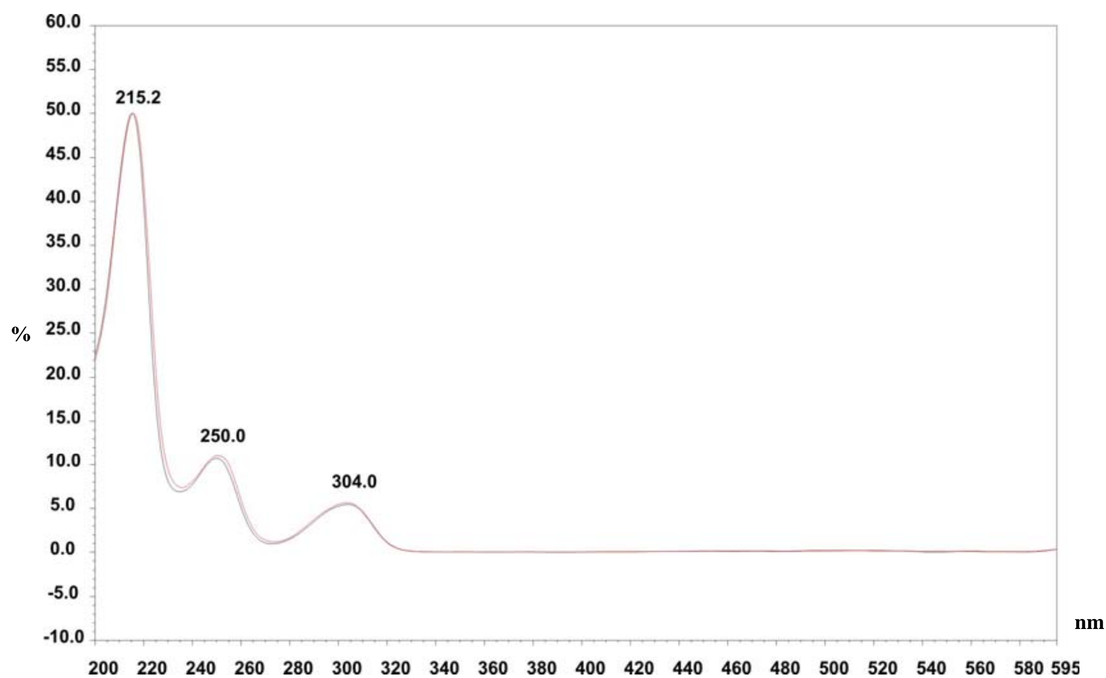


Figure 5.8 UV profile of KV1B (black) (15.0 min) matched with the UV profile of mycophenolic acid (red) in the UV library database.

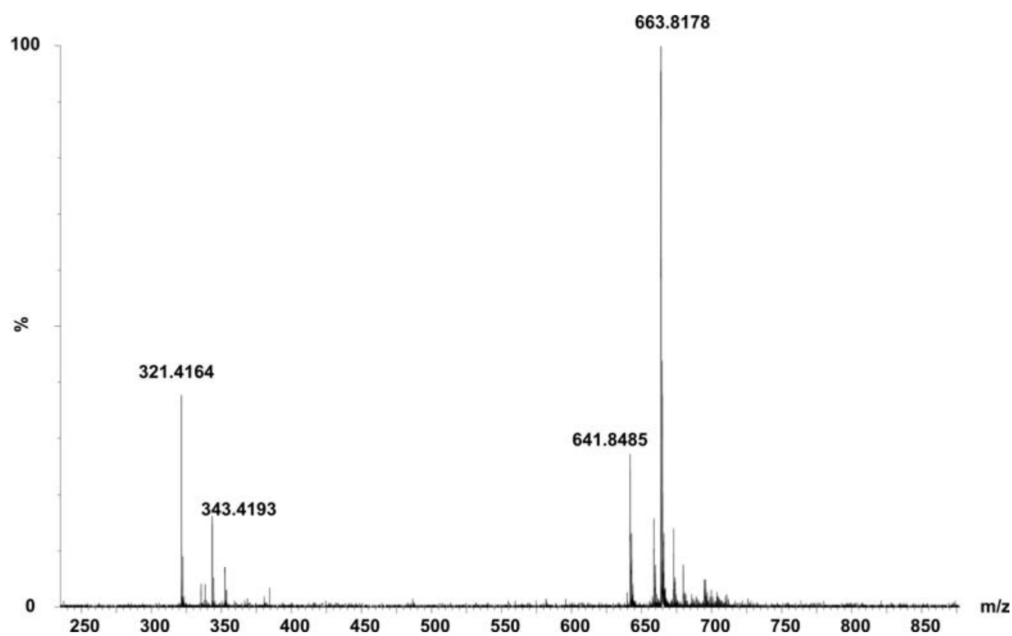


Figure 5.9 ESI-MS spectrum for KV1B (15 min).

5.3.2.1 Mycophenolic acid

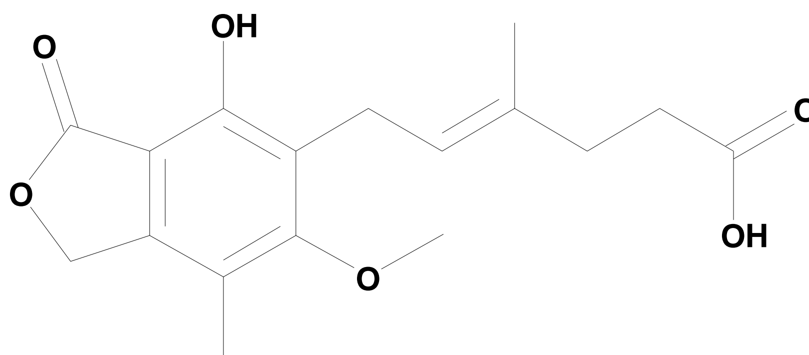


Figure 5.10 Structure of mycophenolic acid.

Mycophenolic acid ($C_{17}H_{20}O_6$) was first discovered by an Italian physician, Bartolomeo Gosio, in 1893 (Bentley, 2000). Mycophenolic acid or mycophenolate is an immunosuppressant drug used to prevent rejection in organ transplantation (Bentley, 2000). It is usually combined with cyclosporine and a steroid medication. It is commercially available as a prodrug mycophenolate mofetil. Mycophenolate mofetil is metabolized in the liver to the active moiety mycophenolic acid. Recently, the salt, mycophenolate sodium, has also been introduced.

Besides being used as an immunosuppressant, mycophenolic acid has also been used to treat autoimmune skin disorders such as psoriasis and sarcoidosis. Psoriasis is a disease which affects skin and joints. It commonly causes red scaly patches to appear on the skin. Sarcoidosis also known as Besnier-Boeck disease is an immune system disorder characterized by non-caseating granulomas. Any organs can be affected, but granulomas usually appear in the lungs or the lymph nodes.

The used of mycophenolic acid to treat sickness also causes several side effects. Common adverse drug reactions are allergic reaction, unusual tiredness, painful urination and severe nausea. Mycophenolate sodium is commonly associated with fatigue, headache and cough. Intravenous (IV) administration of mycophenolate mofetil is commonly

associated with thrombophlebitis and thrombosis. Infrequent adverse effects include insomnia, skin rash and dizziness.

5.4 Compounds isolated from *Penicillium sp.* (KV11)

Based on the quick screen assays, it was shown that the KV11 extract was active against P388 cells with an IC_{50} value of < 975 ng/mL. From the HPLC trace three main peaks could be detected. The first peak eluted at 15 min, the second peak at 16.7 min and the third peak at 17.6 min.

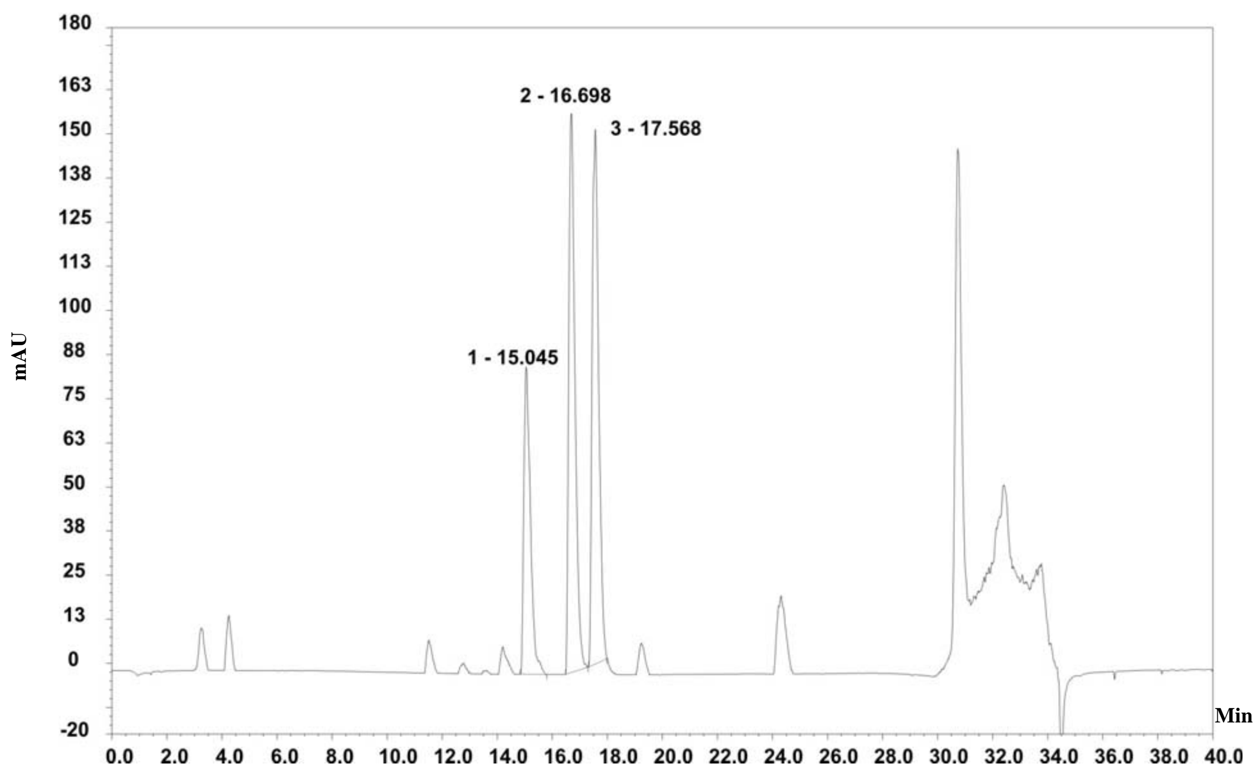


Figure 5.11 ELSD chromatogram of extract from *Penicillium sp.* (KV11).

5.4.1 KV11A: Compound eluted at 15 minutes

The UV profile of the first peak had a maximum absorbance at 212, 235 and 291 nm. P388 HPLC assay showed that the first peak was not the one responsible for the cytotoxicity. The UV profile of the compound when searched in the UV library revealed that the peak corresponded to that of griseofulvin. Both the UV profile and the retention time fitted almost perfectly.

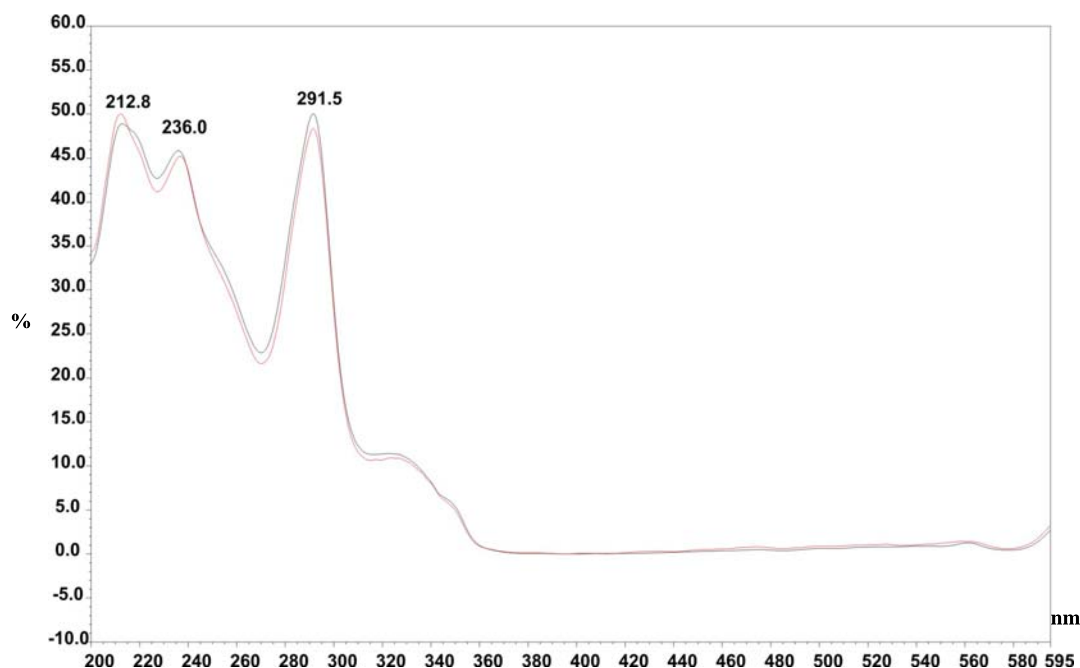


Figure 5.12 UV profile of KV11A (black) eluted at 15 min matched with the UV profile of griseofulvin (red) in the UV library database.

To further confirm the identity, the molecular weight of KV11A was determined. From the mass spectrum, it was concluded that the molecular weight was 352 Da (major ions at 353.1 m/z $[M+H]^+$ and 705.1 m/z $[2M+H]^+$), exactly the same as griseofulvin.

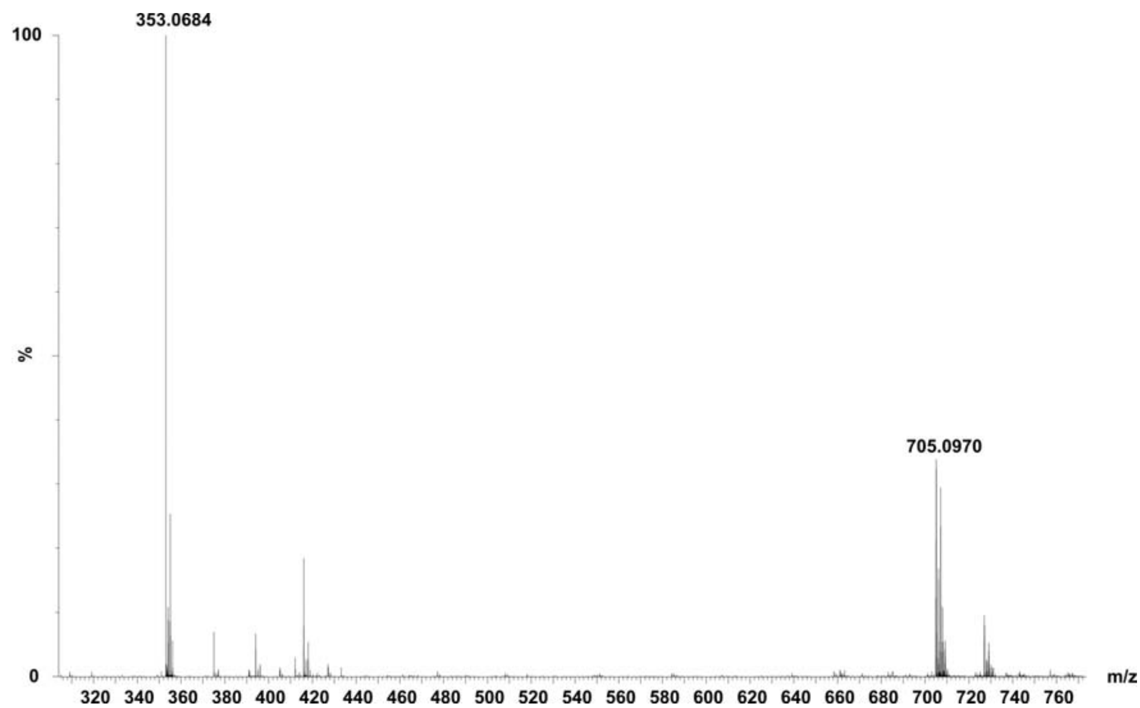


Figure 5.13 ESI-MS spectrum for KV11A eluted at 15 minutes.

5.4.1.1 Griseofulvin

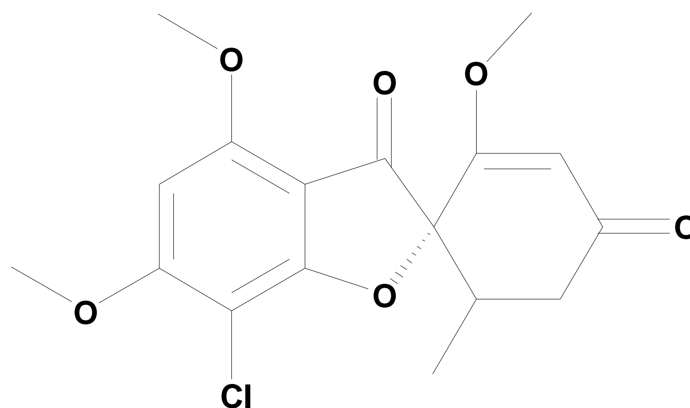


Figure 5.14 Structure of griseofulvin.

Griseofulvin ($C_{17}H_{17}ClO_6$) has been used in the treatment of dermatophyte infections for the past 40 years. It was first isolated from *Penicillium griseofulvum* by Oxford *et al.* in 1939. The useful biological activity against dermatophytes was not noticed at that time. The studies of Blank and Roth later confirmed the role of griseofulvin in dermatological therapeutics (Finkelstein *et al.*, 1996).

It has been used for the treatment of infections caused by *Epidermophyton*, *Trichophyton* and *Microsporum spp.* which cannot be cured by topical antifungal therapy. Griseofulvin has also been used for treatment of various other diseases including lichen planus, scleroderma, gout, herpes zoster and monilethrix, however, it is not considered to be the first line treatment for these diseases (Erkel, 1997).

Studies of the mode of action of griseofulvin revealed that it binds to tubulin, interfering with microtubule function, hence inhibiting mitosis. Side effects caused by using griseofulvin are hives, confusion, fatigue, itching, swelling and inability to fall or stay asleep.

5.4.2 KV11B: Compound eluted at 16.7 minutes

The UV profile of the second peak had a maximum absorbance at 216, 256 and 306 nm. P388 HPLC assay showed that this peak was responsible for the cytotoxic activity. The UV profile of the compound was searched against the UV library database which revealed that the peak corresponded to that of cycloaspeptide. Both the UV profile and the retention time were almost perfectly matched. However, there are several types of cycloaspeptide. In order to confirm which cycloaspeptide was isolated from this extract, an aliquot of the compound was submitted for mass spectrometry and NMR spectroscopy for further investigations.

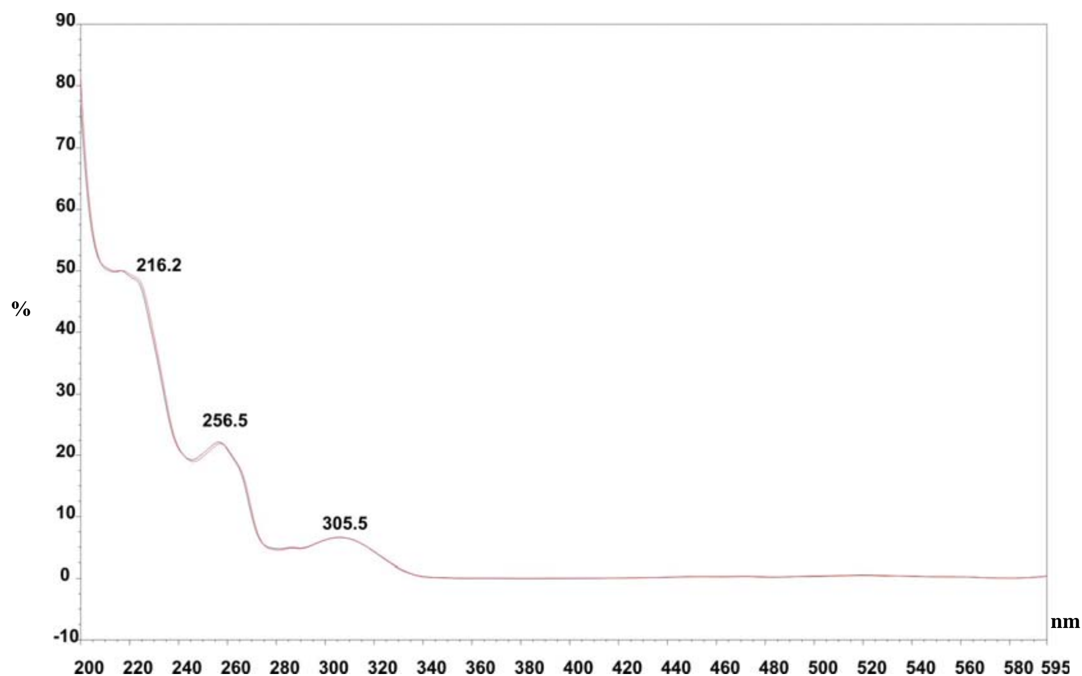


Figure 5.15 UV profile of KV11B (black) eluted at 16.7 min matched with the UV profile of cycloaspeptide (red) in the UV library database.

As indicated in **Figure 5.16**, the ESI-MS showed major ions at 642.3 m/z $[M+H]^+$ and 1283.5 m/z $[2M+H]^+$. This suggested that the molecular weight of the compound was 641 Da.

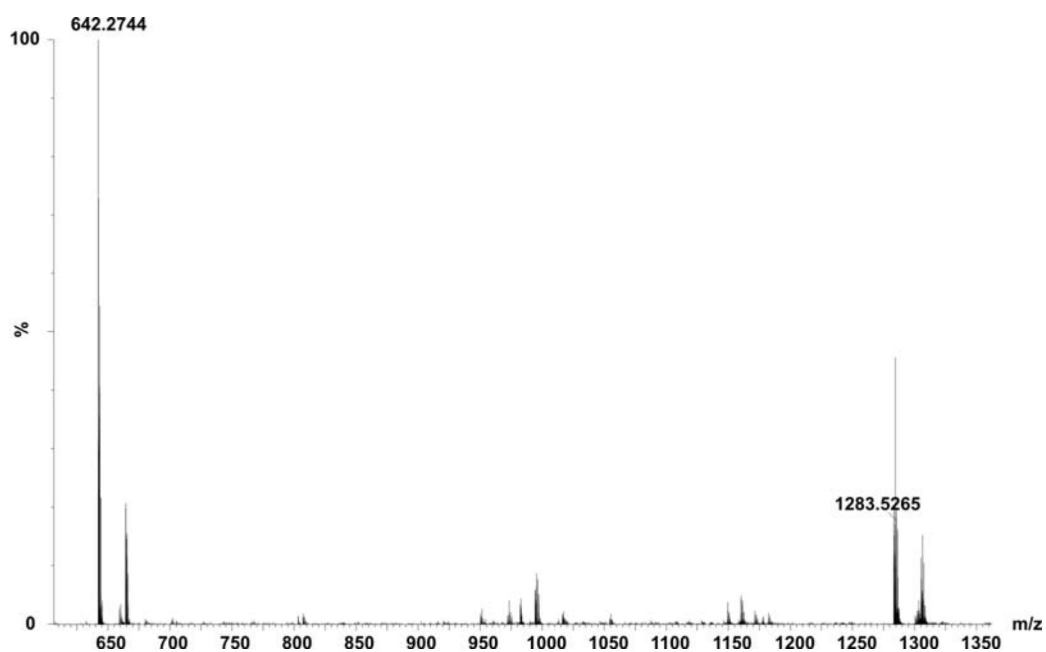


Figure 5.16 ESI-MS spectrum of KV11B eluted at 16.7 min.

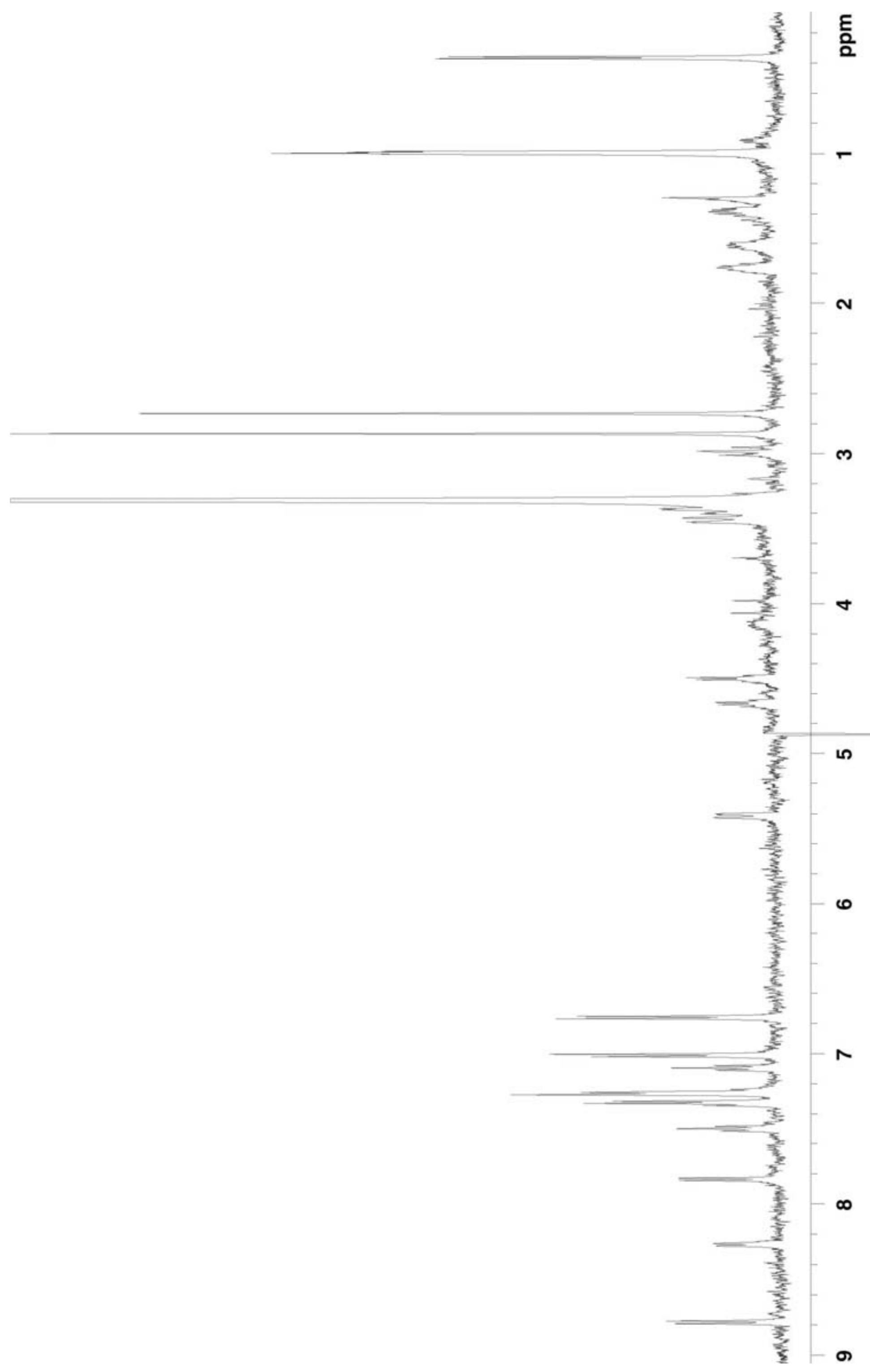


Figure 5.17 Proton NMR spectrum of KV11B.

In the ^{1}HNMR spectrum of KV11B (**Figure 5.17)**, five methyl signals of which two were doublet methyl groups (0.36 and 0.99 ppm), one triplet methyl group (1.00 ppm) and 2 N-methyl (2.73 and 2.87 ppm) were recognized. When this data and the molecular weight (640-642 Da) was searched against AntiMarin, 2 hits, cytorhodin L and cycloaspeptide A were identified. Since the initial identity of the isolated compound was cyclosapetide as shown in the UV library, it was concluded that KV11B was cycloaspeptide A.

5.4.2.1 Cycloaspeptide A

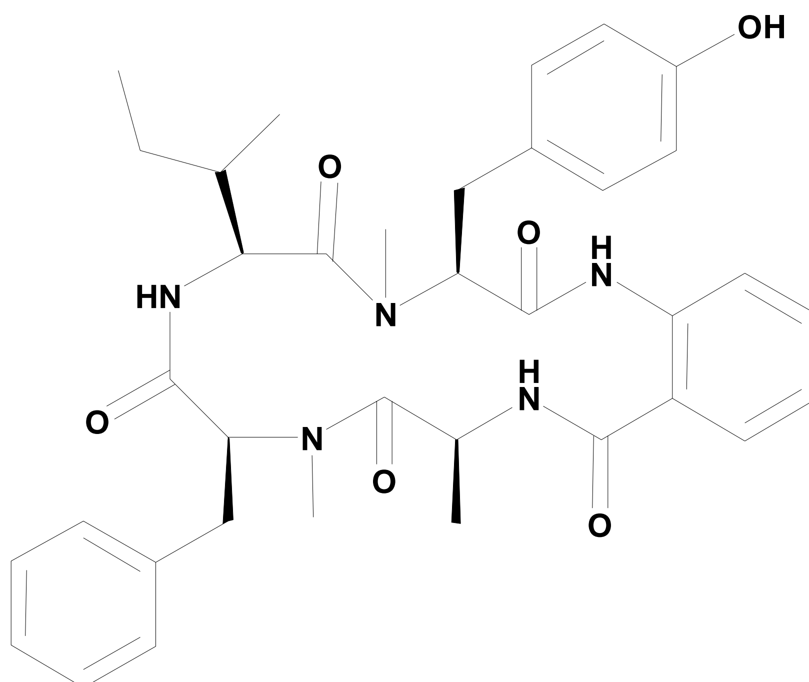


Figure 5.18 Structure of cycloaspeptide A.

Little has been reported on the activity of cycloaspeptide A ($C_{36}H_{43}N_5O_6$), however moderate activity against *Plasmodium falciparum* was shown by Dalsgaard *et al.*, (2005) who also indicated that cycloaspeptide A is inactive against P388 cells. This is inconsistent with the results obtained here where moderate activity was seen.

5.4.3 KV11C: Compound eluted at 17.6 minutes

The UV profile of the third peak had a maximum absorbance at 230, 237 and 245 nm. P388 HPLC assay showed that this peak was also responsible for the cytotoxic activity. The UV profile of the compound was searched against the UV library database. No match was found. An aliquot of this compound was submitted for mass spectrometry and NMR spectrometry for further investigations.

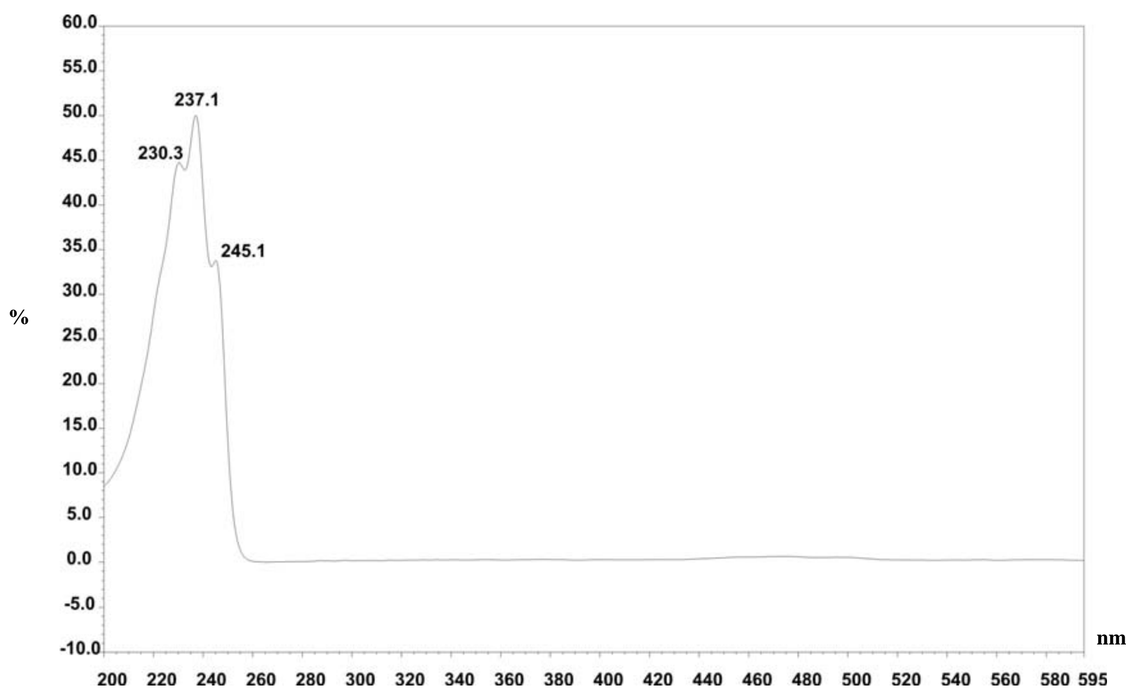


Figure 5.19 UV profile of KV11C (17.6 min).

As indicated in **Figure 5.20**, the ESI-MS shows major ions at 391.3 m/z $[M+H]^+$, 781.5 m/z $[2M+H]^+$ and 803.4 m/z $[2M+Na]^+$. This indicated that the molecular weight of the compound was 390 Da.

Figure 5.20 ESI-MS spectrum for KV11C (17.6 min).

From the ^{1}HNMR spectrum of KV11C **(Figure 5.21)**, three methyl signals of which two were doublet methyl groups (0.92 and 1.13 ppm) and one a triplet methyl group (1.00 ppm) could be identified. The molecular weight was also entered (389-391 Da). The result of the search gave 2 hits, mevastatin and mevinic acid. Comparison of the 1D proton NMR data with published data (Keck and Kachensky, 1986) of synthetic mevastatin combined with the analysis of 2D proton NMR data established that KV11C was mevastatin.

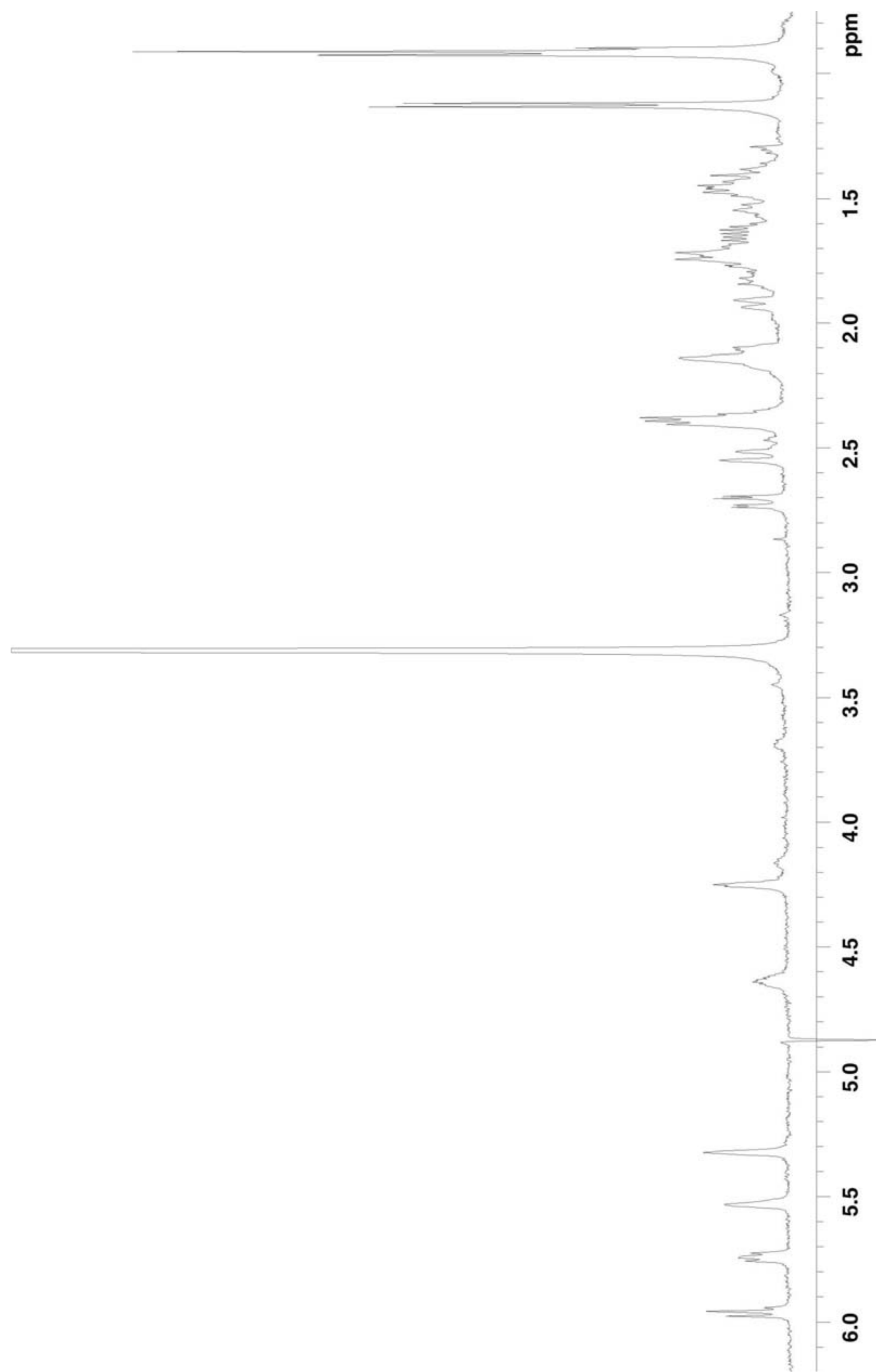


Figure 5.21 Proton NMR spectrum of KV11C.

Assigments	^{1}H NMR (ppm)(mult) reported here (CD_3OD)	^{1}H NMR (ppm)(mult) reported in the literature ($CDCl_3$)
18	0.91 (t)	-
23	0.92 (d)	0.90 (d)
22	1.13 (d)	1.12 (d)
19	1.50, 1.70 (m)	-
14	1.75, 1.92 (m)	1.92 (m)
	2.14 (m)	2.14 (m)
1, 20	2.39 (m)	2.36 (m)
16	2.53 (m)	-
	2.72 (m)	2.70 (m)
15	4.25 (m)	4.35 (m)
13	4.64 (m)	4.46 (m)
8	5.33 (m)	5.34 (m)
5	5.54 (m)	5.56 (m)
2	5.74 (m)	5.73 (dd)
3	5.96 (m)	5.98 (d)

Table 5.1 Comparison of the ^{1}H data of mevastatin with published literature (Keck and Kachensky, 1986).

5.4.3.1 Mevastatin

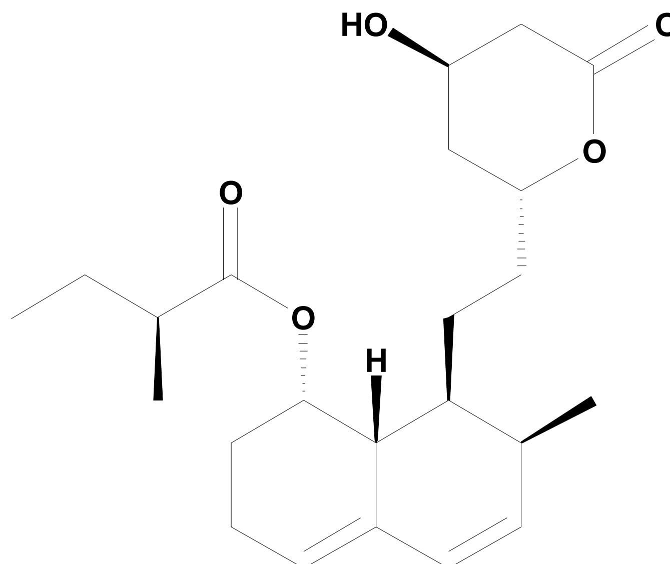


Figure 5.22 Structure of mevastatin.

Mevastatin ($C_{23}H_{34}O_5$), also known as compactin or ML-236 B, is a hypolipidemic agent that belongs to the statin class of compounds. It was first isolated in the 1970s from *Penicillium citrinum*. Mevastatin works as a competitive inhibitor of 3-hydroxy-3-methyl-glutaryl (HMG)-CoA reductase, which is a regulatory enzyme for cholesterol biosynthesis (Chakravarti and Sahai, 2004).

Cholesterol is an essential component of cell membranes and a precursor of various hormones, bile salts and vitamins. It is synthesized from acetyl-CoA via a series of more than 20 enzymatic reactions. This synthetic pathway is mainly regulated by the activity of the enzyme HMG-CoA reductase, which catalyzes the reduction of HMG-CoA to mevalonate (Endo, 1985).

Inhibition of the conversion of HMG-CoA to mevalonate by mevastatin will result in accumulation of HMG-CoA that are metabolized to simpler compounds, thereby making it an ideal compound for treatment of atherosclerosis. Aside from being used as an inhibitor in cholesterol biosynthesis, mevastatin also acts as a precursor of other statins such as pravastatin.

Chapter 6

Secondary metabolites from *Phoma sp.* (MF18 and KV9)

6.1 Introduction

Phoma is the largest and most widely distributed genus of the Sphaeropsidales, with species reported from soil, as saprophytes on various plants and on dead plant material. It has a complicated history since it was initially introduced more than 180 years ago. During this period, thousands of non-stromatic species of pycnidial fungi which produce hyaline conidia without septa were described according to Saccardo's concept. This system placed emphasis on host specificity and presence or absence of a septum. The first modern studies on *Phoma* were carried out by Dennis (1946), introducing in vitro characters. This formed the basis for a more sophisticated and natural circumscription of the Saccardoan form-genera by Kovics *et al.*, 2005.

Phoma colonies can be characterized by having pigmented separate pycnidia (pycnidial conidiomata), usually each with single ostioles, sometimes multiostiolate. Conidiogenous cells with a narrow phialidic opening, more or less the same structure as inner-cells of pycnidia, producing abundant slimy conidia. Conidia are usually one-celled, ellipsoidal to cylindrical, hyaline or coloured. Chlamydospores in some species formed in single chains or aggregated in dicytochlamydospores (Samson and Hoekstra, 1984).

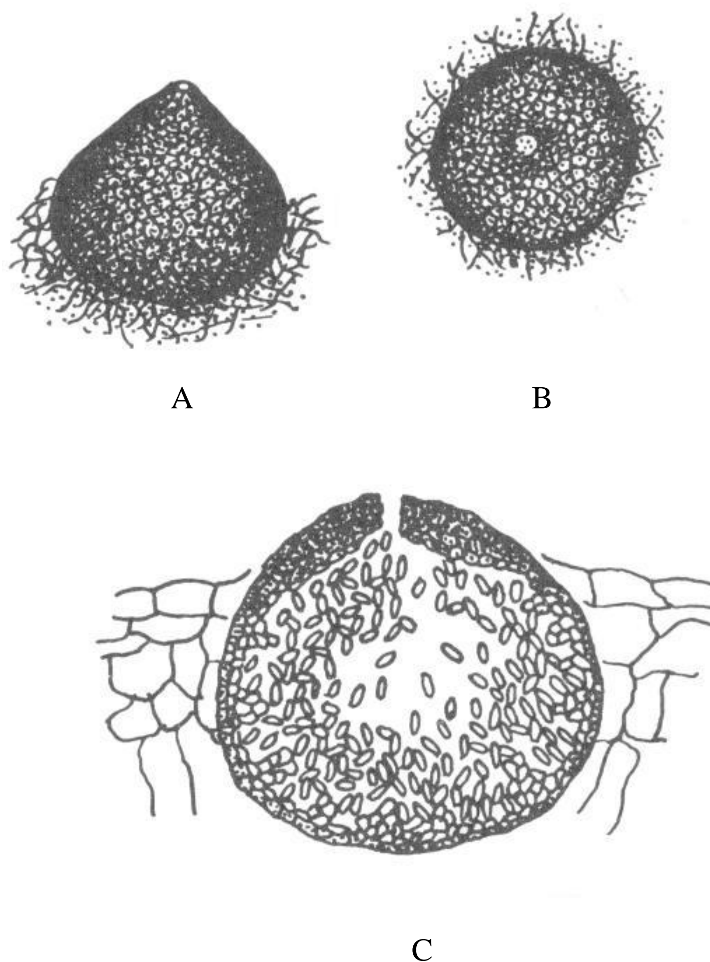


Figure 6.1 Pycnidium of *Phoma sp.* (A) Side view; (B) top view; (C) Conidia within pycnidial cavity.

Phoma has been reported to produce some interesting active metabolites with some of importance to the pharmaceutical industry. Alvi *et al.* (1997) a reported the production of phomacins, an antitumor agent, from *Phoma sp.* isolated from a soil sample and Singh *et al.* (1997) also reported the production of an antitumor agent, fusidienol A, from a *Phoma sp.* isolated from a vegetation sample.

6.2 *Phoma sp.* (MF18) and (KV9)

Both cultures, MF18 and KV9, were isolated from leaf litter samples. The sample for MF18 was collected from Mount French (West Coast), while the sample for KV9 was collected from the Kaituna Valley, Banks Peninsula. Red pigmentation was produced by KV9 mycelial and also into the agar while no pigmentation was visible in MF18 cultures. Neither culture produced any fruiting structures so identification was made by ITS sequencing. However, identification to the species level was not possible and different gene sequences would be required to give a more definitive identification.

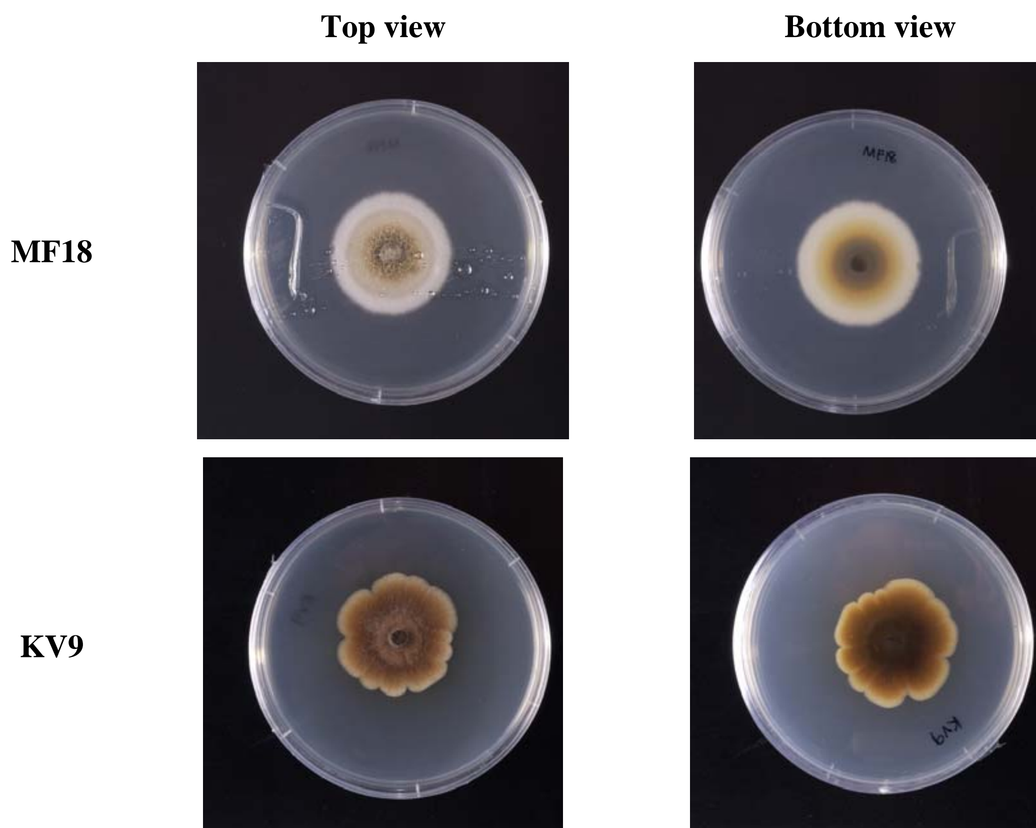


Figure 6.2 14 day old colonies of MF18 and KV9 on PDA.

6.3 Compound isolated from *Phoma sp.* (MF18)

Quick screen assays established that the MF18 extract was active against P388 cells (IC_{50} 4 157 ng/mL). From the HPLC trace one main peak could be detected at 10 min. The UV profile of the peak had a maximum absorbance at 249.1 nm. Further tests with P388 HPLC assay confirmed that the one main peak, MF18A, was responsible for the cytotoxic effect.

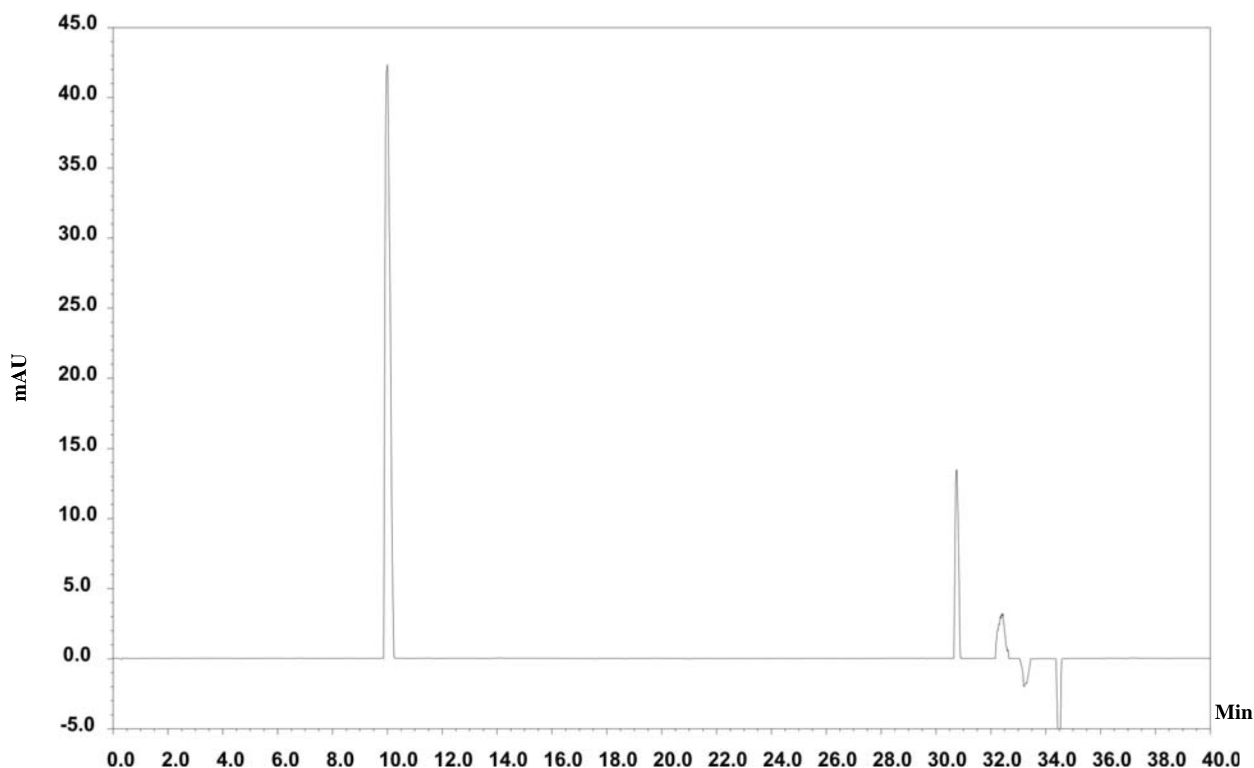


Figure 6.3 ELSD chromatogram of extract from *Phoma sp.* (MF18).

The UV profile of MF18A was then compared against the UV library database but no match was found. An aliquot of MF18A was submitted for mass spectrometry and NMR spectroscopy for further investigations.

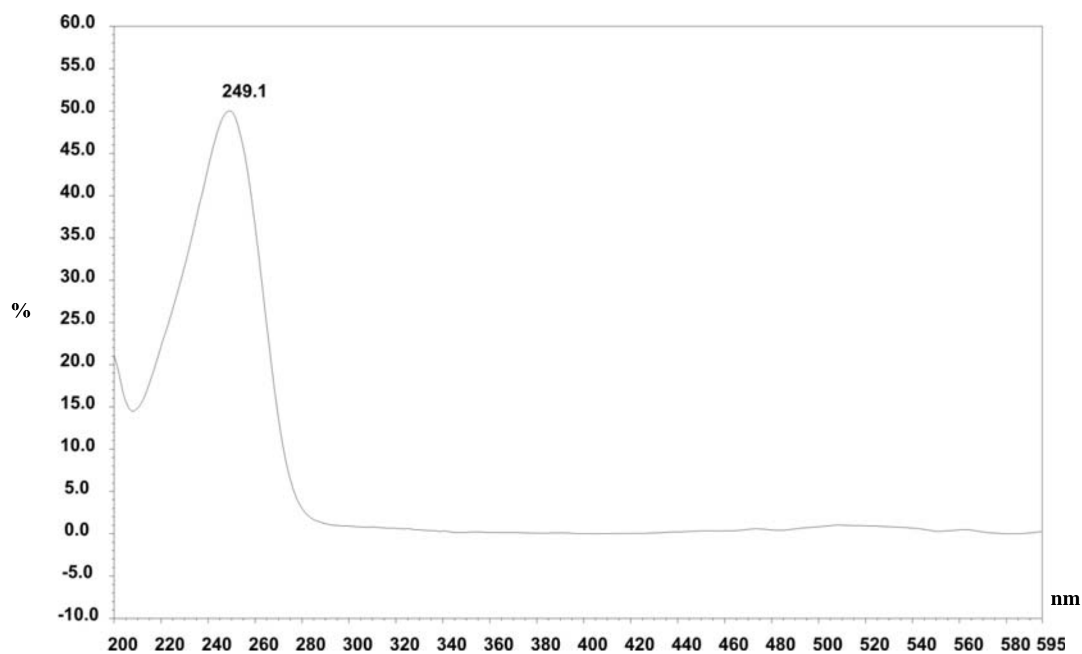


Figure 6.4 UV profile of MF18A.

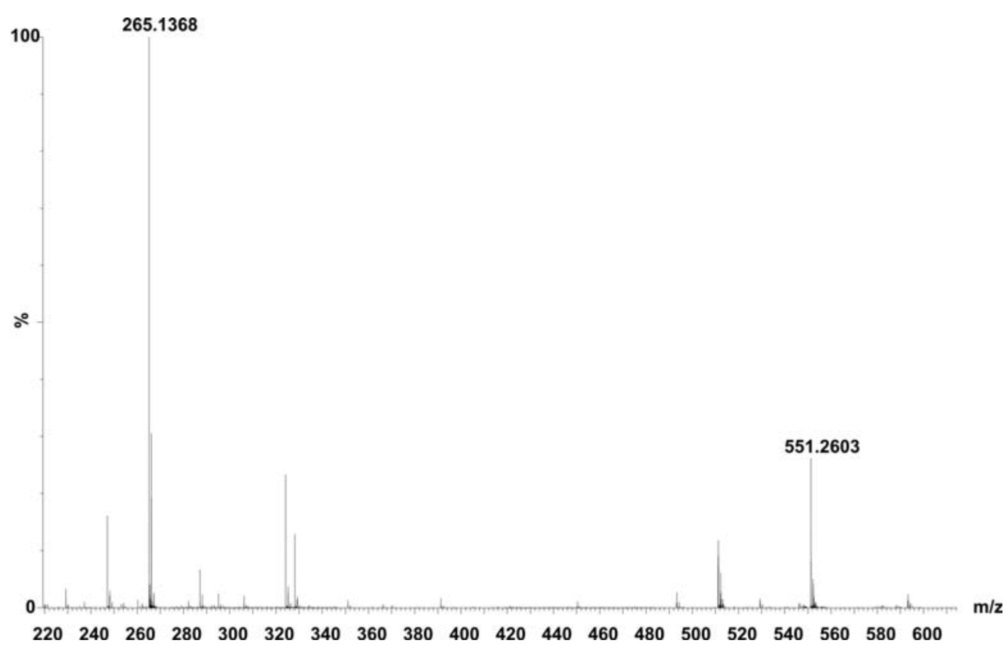


Figure 6.5 ESI-MS spectrum for MF18A.

As indicated in **Figure 6.5**, the ESI-MS showed major ions at 265.1 m/z $[M+H]^+$ and 551.3 m/z $[2M+Na]^+$. This suggested that the molecular weight of MF18A was 264 Da.

Figure 6.6 shows the proton NMR spectrum of MF18A. A search was made using the AntiMarin database with input of two methyl signals of which one was singlet methyl group (1.26 ppm) and the other was a doublet methyl group (1.24 ppm). The molecular weight entered was 640-642 Da. The search resulted in five hits, 9α-hydroxyparthenolide, dechloromikrolin, phomenone, phomadecalin D and PF-E99376-1. Comparison of the ^{1}HNMR data against published data (Kitahara *et al.*, 1991) confirmed that MF18A was phomenone.

Assignment	**^{1}H NMR (ppm)(mult) reported here** (CD_3OD)	**^{1}H NMR (ppm)(mult) reported in the literature** ($CDCl_3$)
15	1.24 (d)	1.27 (d)
14	1.26 (s)	1.24 (s)
2	1.36 (dddd)	1.45 (dddd)
4	1.72 (dq)	1.82 (dq)
2’	2.12 (dddd)	2.17 (dddd)
1’	2.35 (ddd)	2.36 (ddd)
1	2.61 (ddt)	2.54 (ddt)
6	3.40 (s)	3.38 (s)
3	3.59 (ddd)	3.64 (ddd)
12’	4.18 (d)	4.20 (d)
12	4.29 (d)	4.33 (d)
13’	5.21 (s)	5.33 (s)
13	5.26 (s)	5.40 (s)
9	5.72 (d)	5.79 (d)

Table 6.1 Comparison of ^{1}HNMR data of phomenone with published literature (Kitahara *et al.*, 1991).

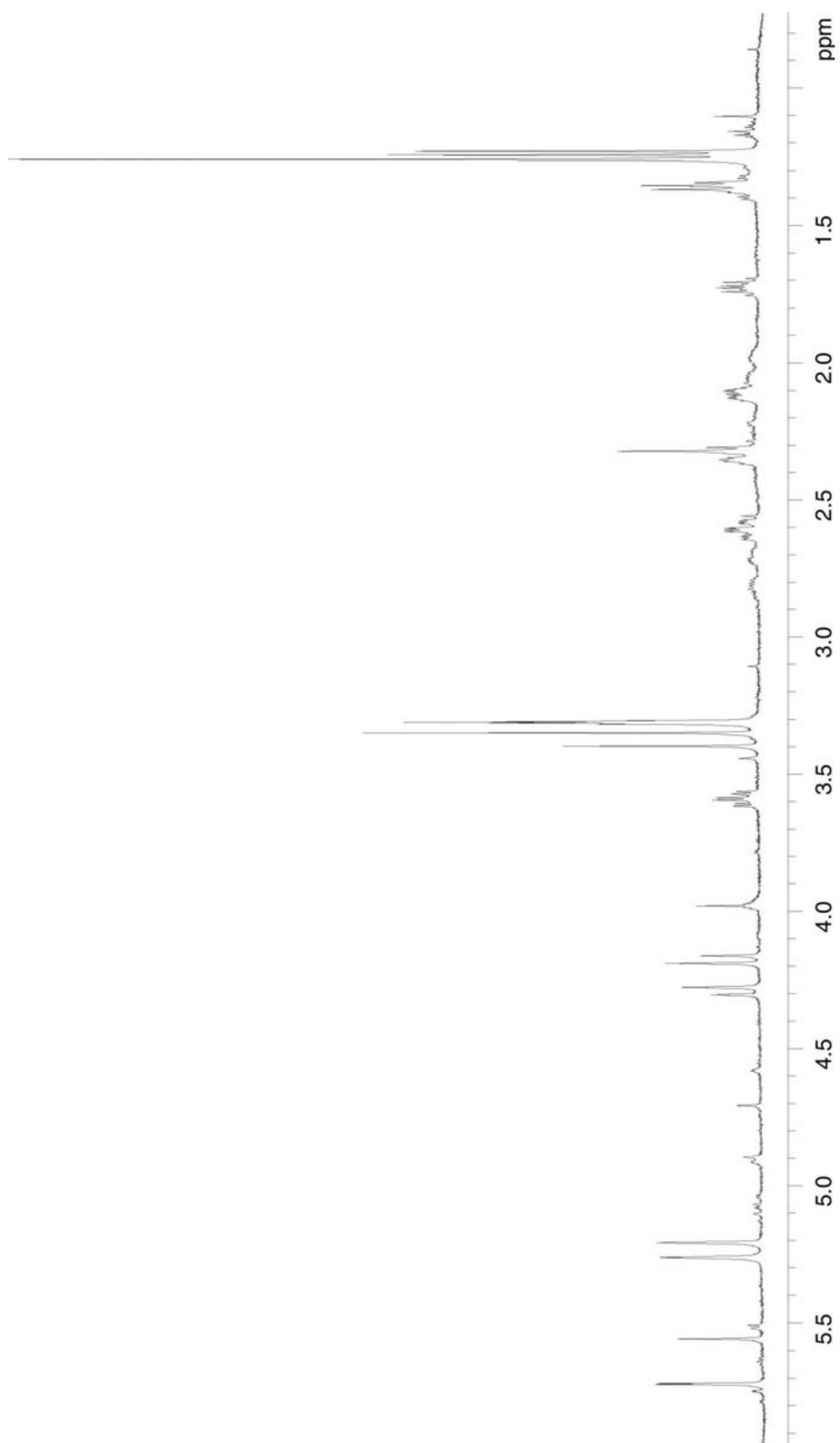


Figure 6.6 Proton NMR spectrum of MF18A.

6.3.1 Phomenone

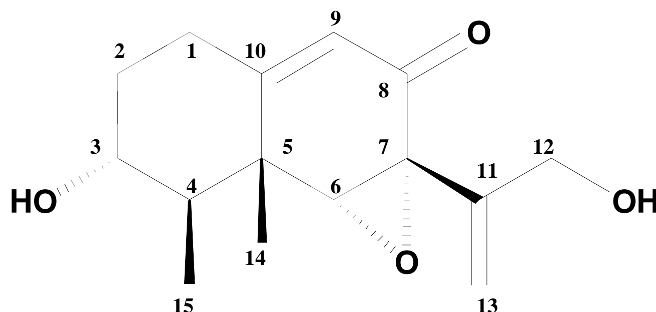


Figure 6.7 Structure of phomenone.

Phomenone is a known phytotoxic and mycotoxic sesquiterpene which has the eremofilane ring structure similar to PR toxin. It was first identified from a pathogenic fungus, *Phoma exigua* var. *inoxydabilis* (Kitahara *et al.*, 1991).

In the 1970s and 80s, phomenone was shown to act as a growth inhibitor of tomato (Capasso *et al.*, 1986). Twenty years later, people started to become aware of the cytotoxic activity of phomenone. Isaka *et al.*, (2000) and Seephonkai *et al.*, (2002) reported that phomenone exhibits strong cytotoxicity to KB and BC-1 cancer cells. We also demonstrated that it was active against P388 cells. However, phomenone also shows toxicity against normal cells which led to its limitation for further clinical study and biopharmaceutical uses. Currently, work is being undertaken to modify its structure in an attempt to reduce toxicity (Weerapreeyakul *et al.*, 2007).

6.4 Compound isolated from *Phoma sp.* (KV9)

Based on the quick screen assays, it was shown that the KV9 extract was active against *Bacillus subtilis* and P388 cells (IC_{50} 1 950 ng/mL). From the HPLC trace one main peak, KV9A, could be detected at 18.7 min. The UV profile of KV9A had a maximum absorbance at 241.5, 305.5 and 484.4 nm. Further bioactivity profiling (P388 and

antimicrobial) confirmed that the single main peak was responsible for both the cytotoxic and antimicrobial effects.

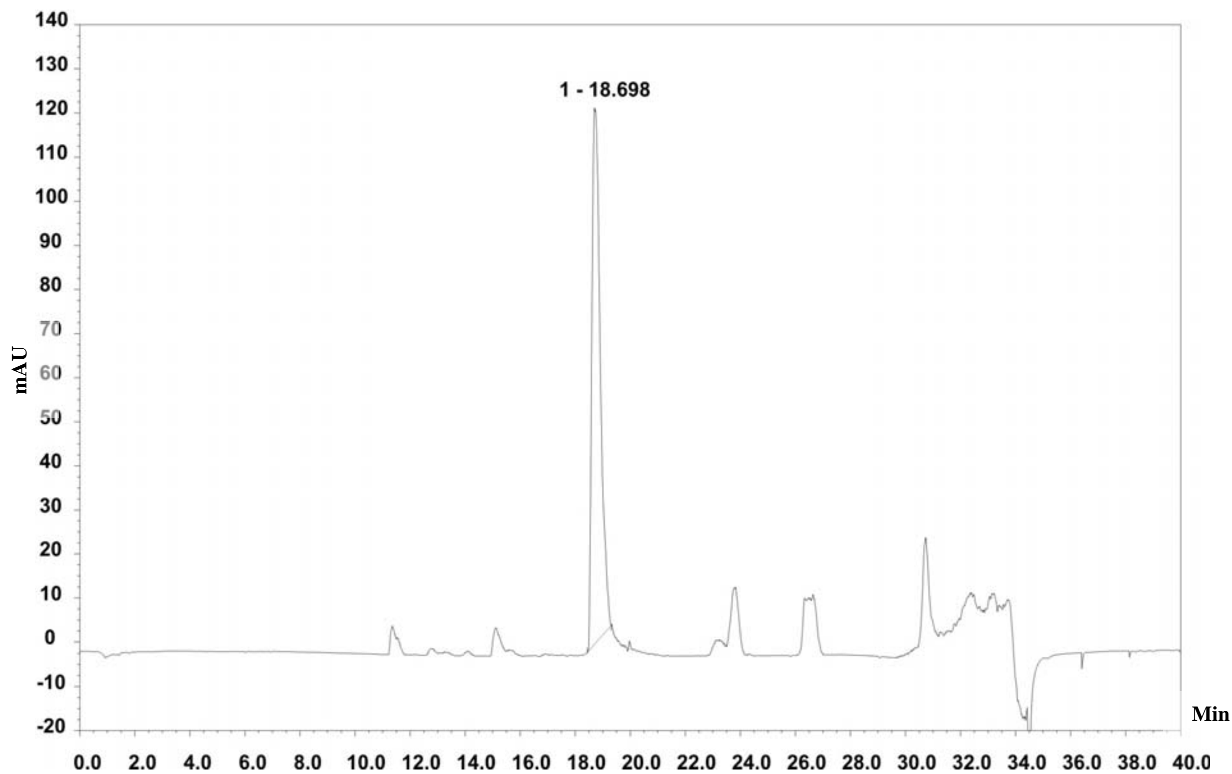


Figure 6.8 ELSD chromatogram of extract from *Phoma sp.* (KV9).

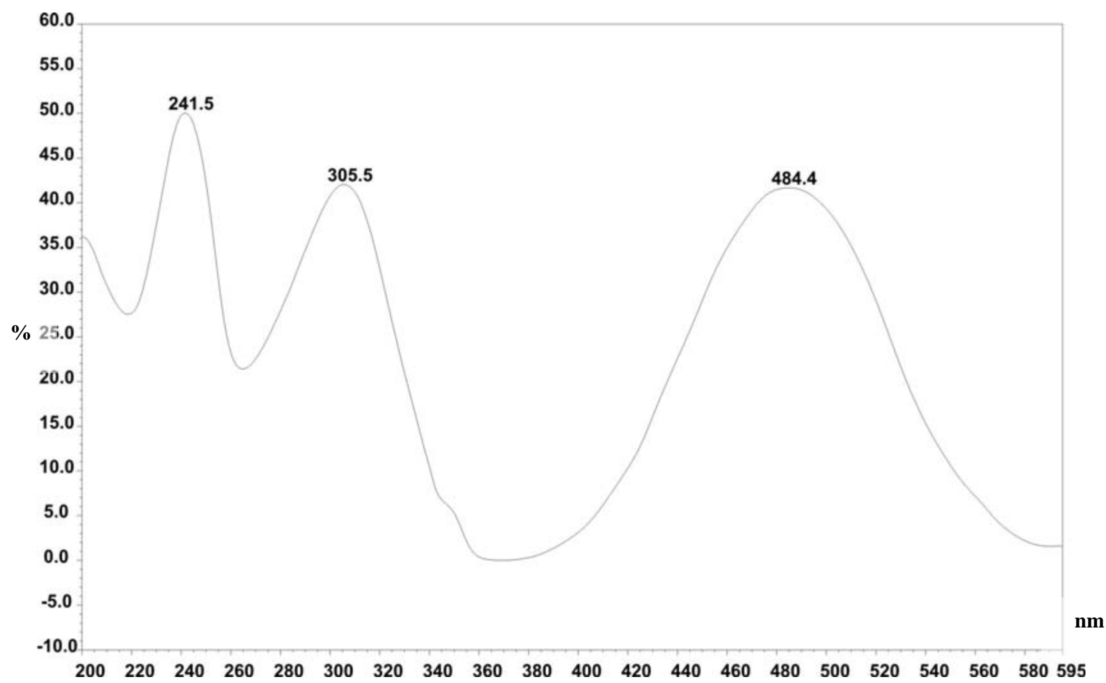


Figure 6.9 UV profile of KV9A.

The UV profile of the compound was compared against the UV library database but no match was found. An aliquot of this compound was submitted for mass spectrometry and NMR spectroscopy for further investigations.

The ^{1}HNMR spectrum of KV9A is shown in **Figure 6.10**. A search in AntiMarin database with input of 5 methyl signals of which one was a singlet methyl group (1.94 ppm), three triplet methyl groups (0.89, 0.90 and 1.04 ppm) and one methoxyl group (3.83 ppm) gave 17 hits, however, none of the hits had a similar proton NMR spectra to KV9A. This suggested that KV9A was possibly a new compound.

An aliquot of the compound was subjected to HSQC and HMBC NMR experiments to determine its structure. Poor results allowed only a fragment of the compound to be deduced.

Assignment	HNMR (δ_H)	HSQC (δ_C)	HMBC (δ_C)
1	0.90	11.4	30, 34.9
7	1.04	19.5	30, 34.9, 140
6	1.94	12.1	127.6
	3.54	54.4	94.7
	3.83	55.1	152.4

Table 6.2 Summary of HNMR, HSQC and HMBC results.

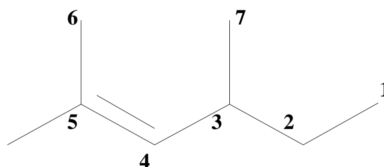


Figure 6.11 Substructure of KV9A based on the ^{1}HNMR, HSQC and HMBC results.

A search was done using the fragment of KV9A and the information from the ^{1}HNMR spectrum and it was shown that KV9A was related to laccaridiones A and B which was

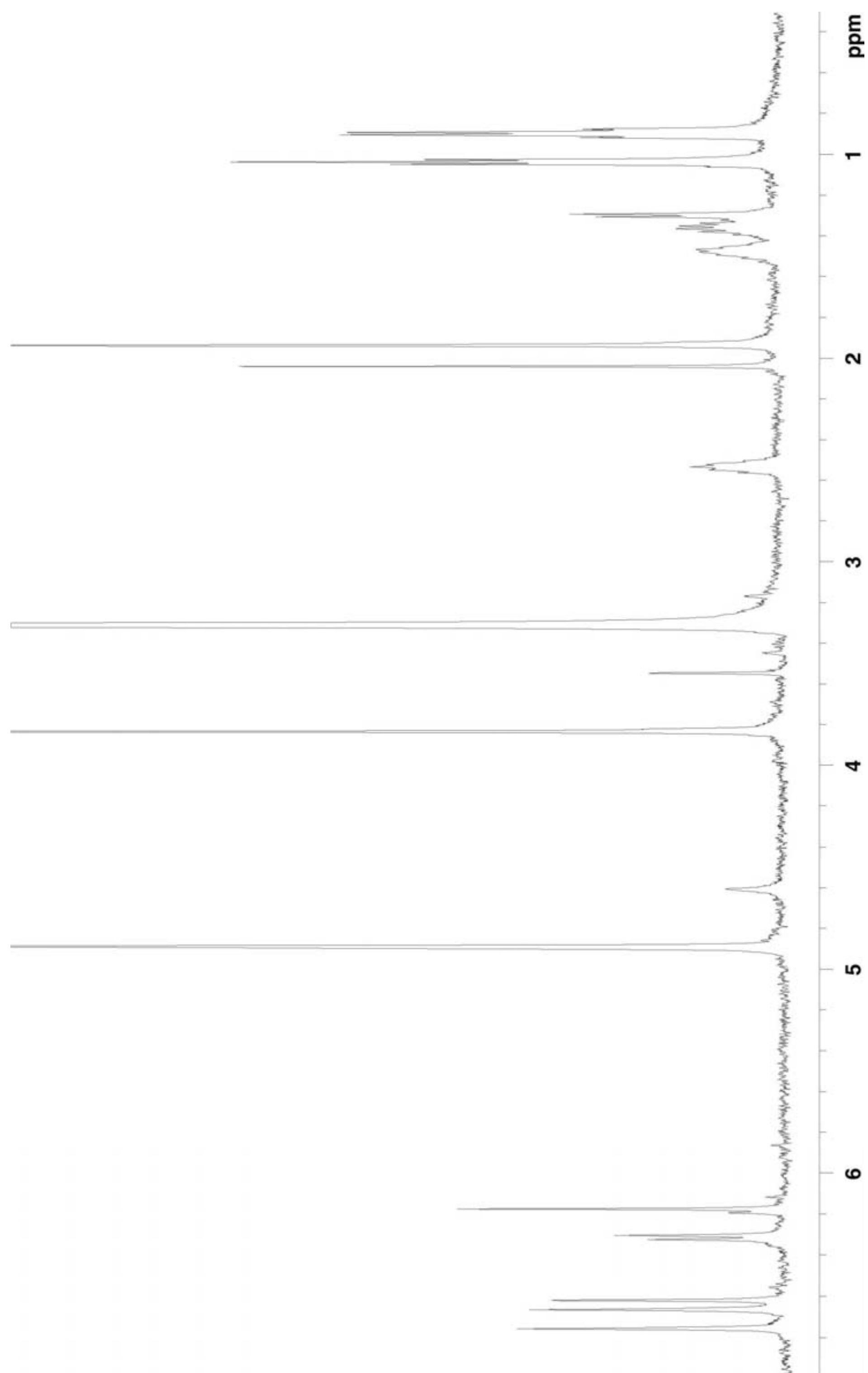
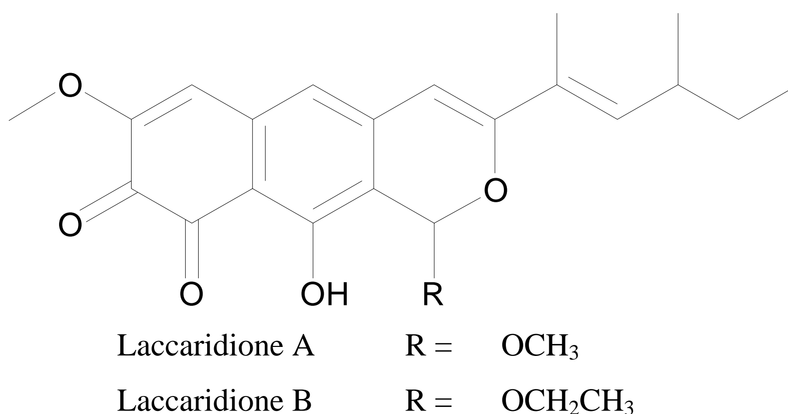


Figure 6.10 Proton NMR spectrum of KV9A.

reported by Berg *et al.*, (2000). Laccaridione A has a maximum UV absorption at 239, 298 and 481 nm. Laccaridione B has a maximum UV absorption at 239, 299 and 480 nm. The UV absorption of both laccaridiones A and B are relatively similar to KV9A (241.5, 305.5 and 484.4 nm). Laccaridiones A and B are inhibitors of a series of proteases such as trypsin, papain, thermolysin, collagenase and zinc-protease from *Bacillus subtilis*. Besides that, laccaridione B has also been reported to show strong antiproliferative effect on the murine fibroblast cell line L-929 and the human leukemia cell like K-562 (Berg *et al.*, 2000).



Laccaridione A R = OCH_3

Laccaridione B R = OCH_2CH_3

Figure 6.12 Structures of laccaridiones A and B.

The mass, however was different to laccaridiones A and B. Based on the mass spectrometry result **(Figure 6.14)**, the mass was 370 Da (major ions at 371.1 m/z $[M+H]^+$), compared to laccaridiones A (384 Da) and B (398 Da). This suggested that the R group in KV9A is a hydroxyl group which had not been previously reported. It was also observed that KV9A subsequently was methylated and changed to laccaridione A after being dissolved in methanol and stored for a few days.

From the ^{1}HNMR spectrum (**Figure 6.10**), it is apparent that KV9A is a mixture of diastereoisomers, with the hydroxyl group at the hemiacetal position occurring in two different orientations. This is evidenced from the appearance of the hemiacetal proton as two singlets at ~6.32 ppm and integrating in total for one proton. The methyl triplets and doublets at 0.90 and 1.04 ppm respectively are overlapping due to slight chemical shift

differences for these methyl signals in the two diastereoisomers. The stereochemistry of the methyl group in the side chain is not known.

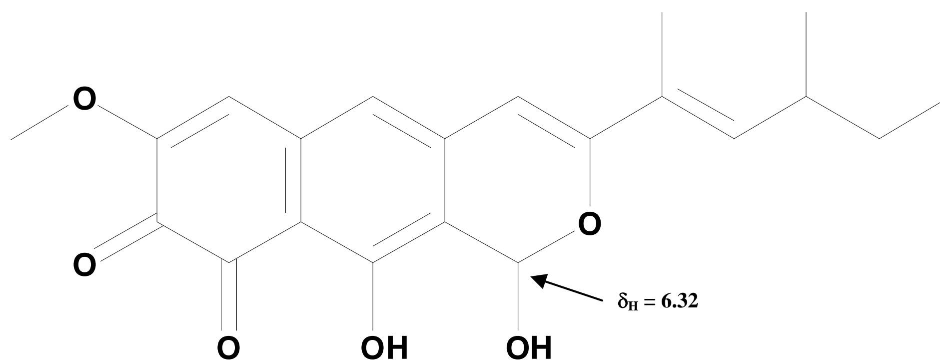


Figure 6.13 Proposed structure of KV9A.

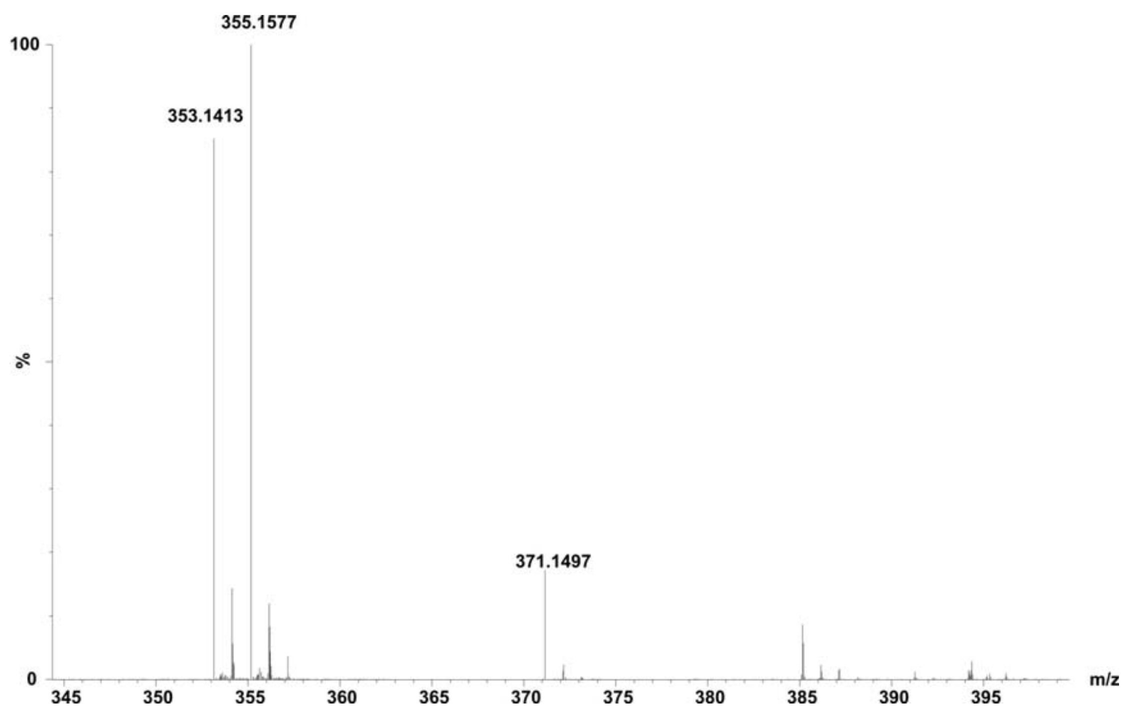


Figure 6.14 ESI-MS spectrum for KV9A.

Chapter 7

Secondary metabolites from a sterile dematiaceous fungus (RC7)

7.1 Sterile fungus (RC7)

An unidentified fungus (RC7) was isolated from leaf litter samples collected from Carew Falls (West Coast). The colony was white at first turning to dark brown. Colony on PDA floccose, developing 'tufts' of hyphae, later becoming melanized and resembling sclerotial initials. Sporulation was not observed over many months cultivation.

Surface view **Reverse view**

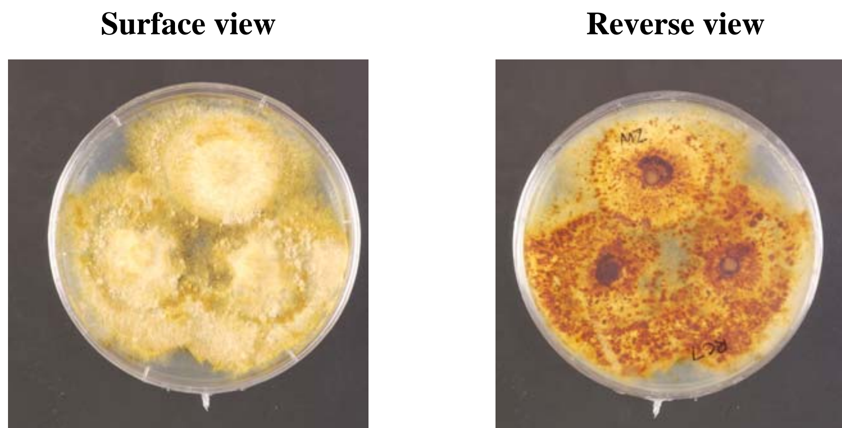


Figure 7.1 4 week old colonies of RC7 on PDA.

7.2 Compound isolated from unidentified fungus (RC7)

Based on the quick screen assays, it was shown that RC7 extract did not produce any active compounds. From the HPLC trace a single main peak, RC7A, could be detected at 12.4 minutes.

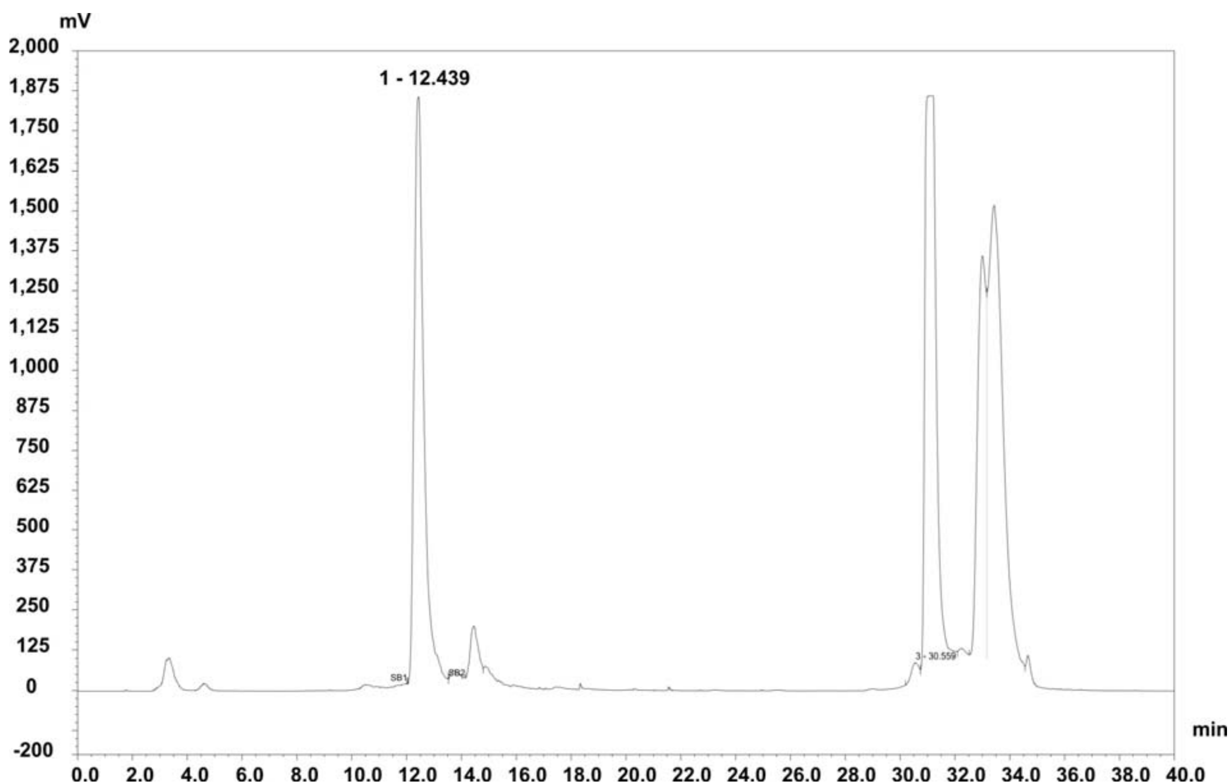


Figure 7.2 ELSD chromatogram of extract from unidentified fungus (RC7).

The UV profile of the peak showed a maximum absorbance at 226 nm which when compared with the UV library database, showed no match and thus was considered worthy of further investigation. An aliquot of RC7A was submitted for mass spectrometry and NMR spectroscopy.

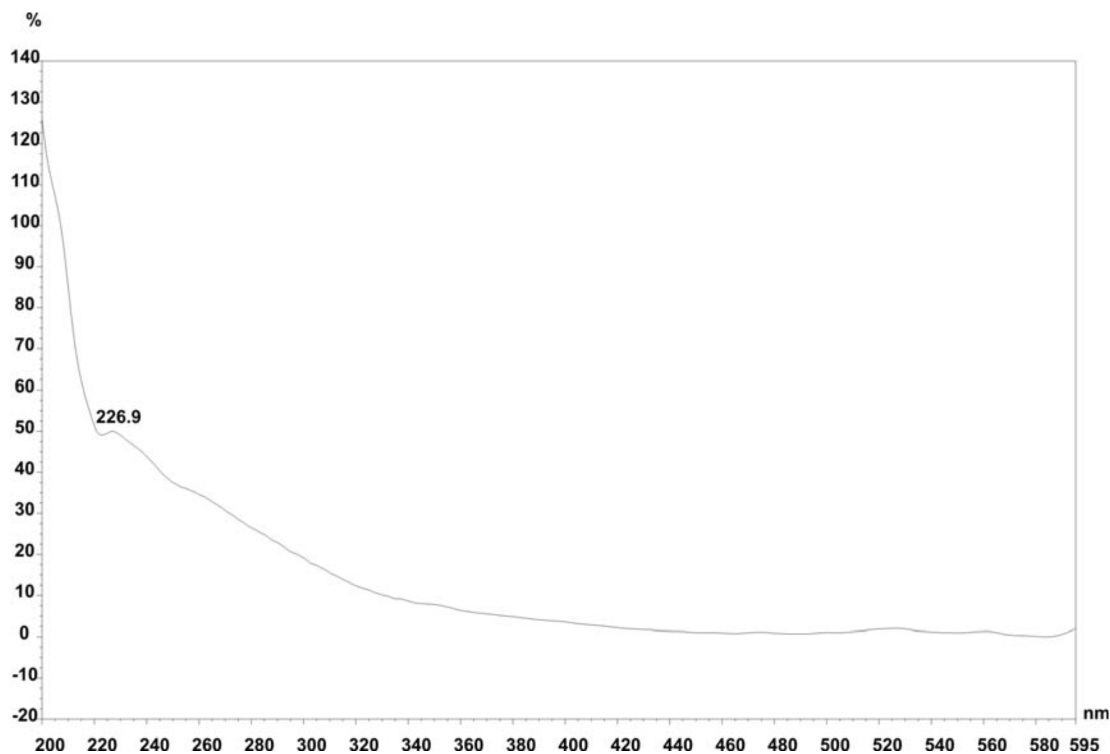


Figure 7.3 UV profile of RC7A.

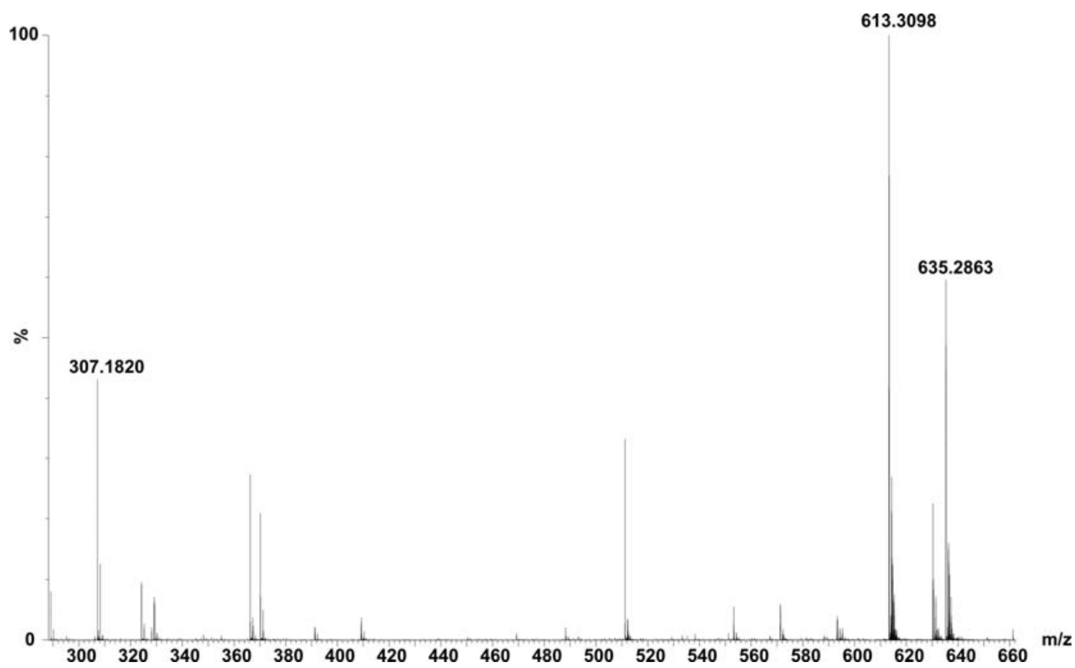


Figure 7.4 ESI-MS spectrum for RC7A.

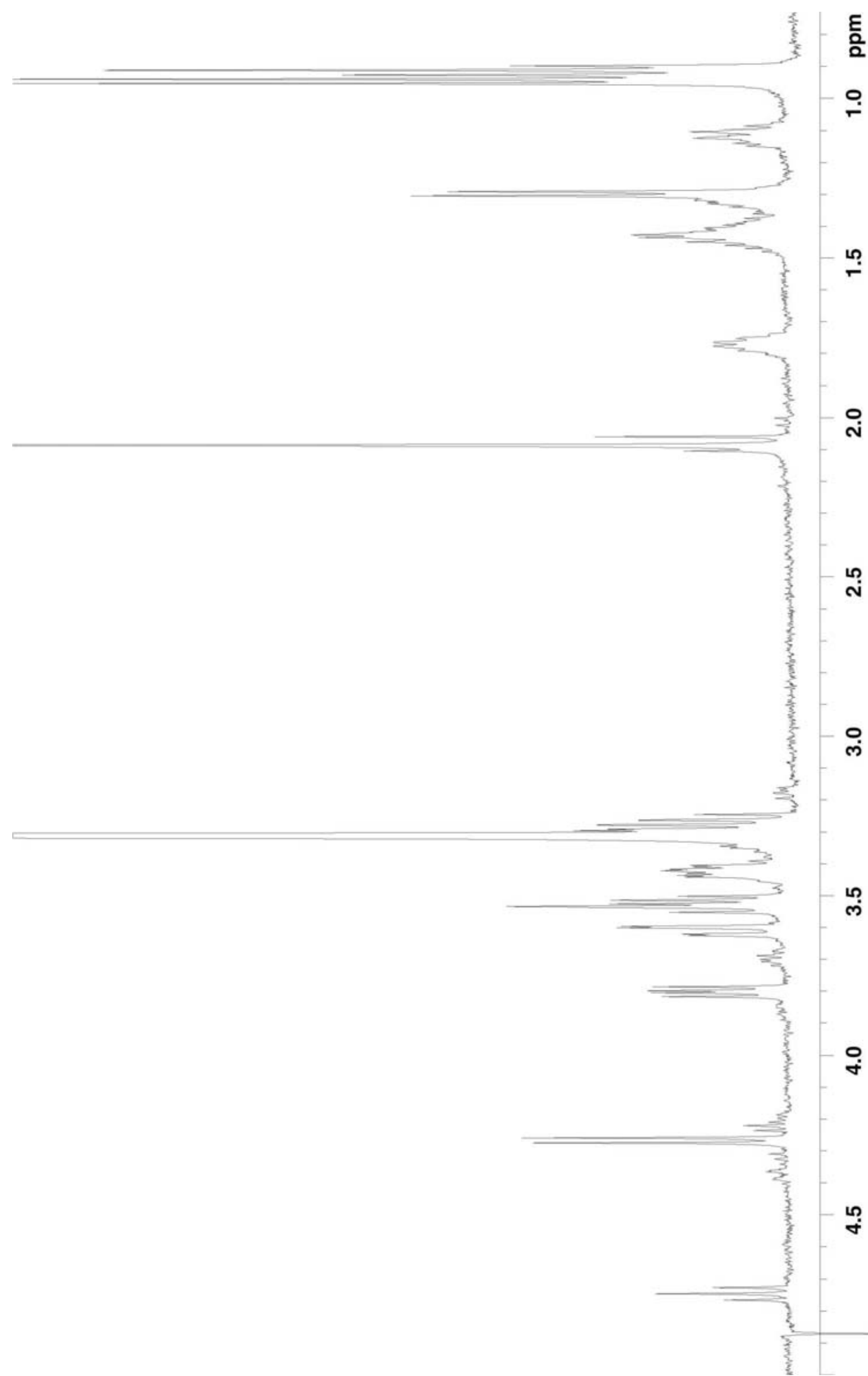


Figure 7.5 Proton NMR spectrum of RC7A.

As indicated in **Figure 7.4**, the ESI-MS showed major ions at 307.2 m/z $[M+H]^+$, 613.3 m/z $[2M+H]^+$ and 635.3 m/z $[2M+Na]^+$ suggesting a molecular weight of 306 Da.

Figure 7.5 shows the ^{1}HNMR spectrum of RC7A. A search was performed using the AntiMarin database. The information entered for the search was 3 methyl signals of which one was a singlet methyl group (2.08 ppm), one doublet methyl group (0.96 ppm) and one triplet methyl group (0.91 ppm). The molecular weight entered was 305-307 Da. The result was no hit suggesting that RC7A was possibly a new compound. An aliquot of RC7A was submitted for HSQC and HMBC spectroscopy to determine its structure. **Figures 7.6** and **7.7** show the HSQC and HMBC results for RC7A. **Table 7.1** summarizes the HNMR, HSQC and HMBC results of RC7A.

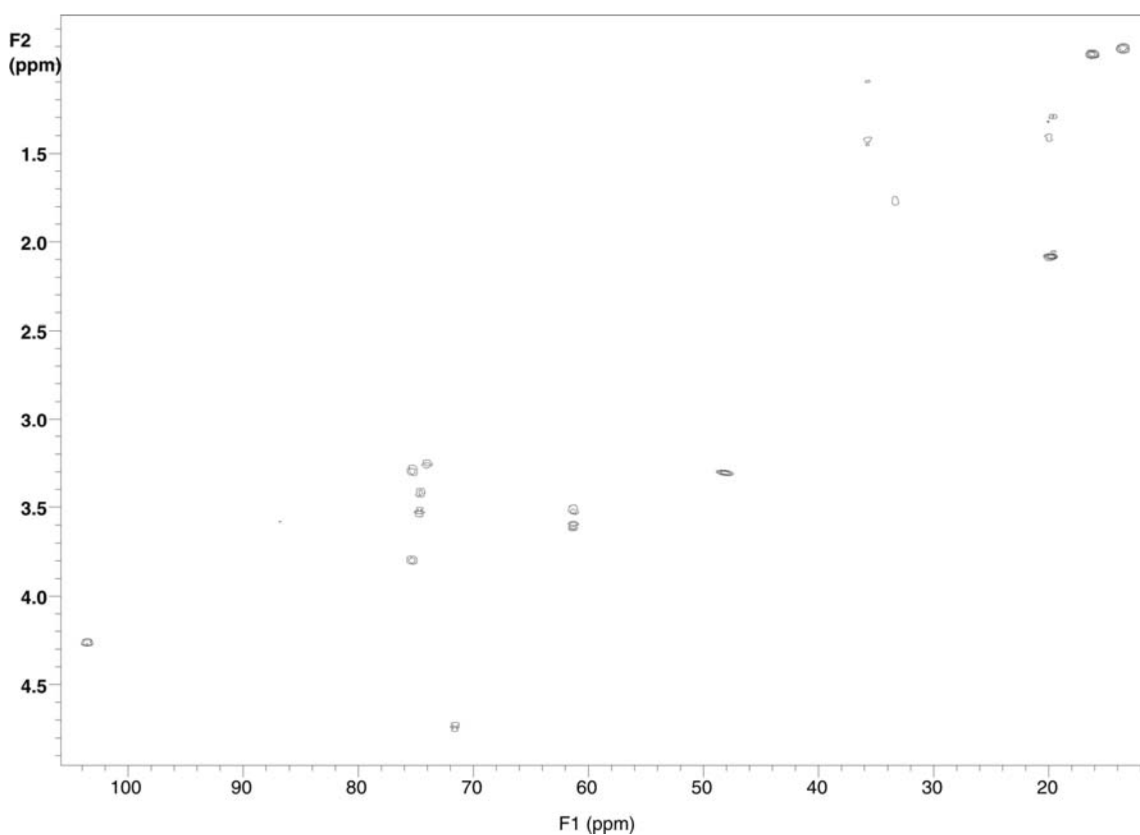


Figure 7.6 HSQC spectrum of RC7A.

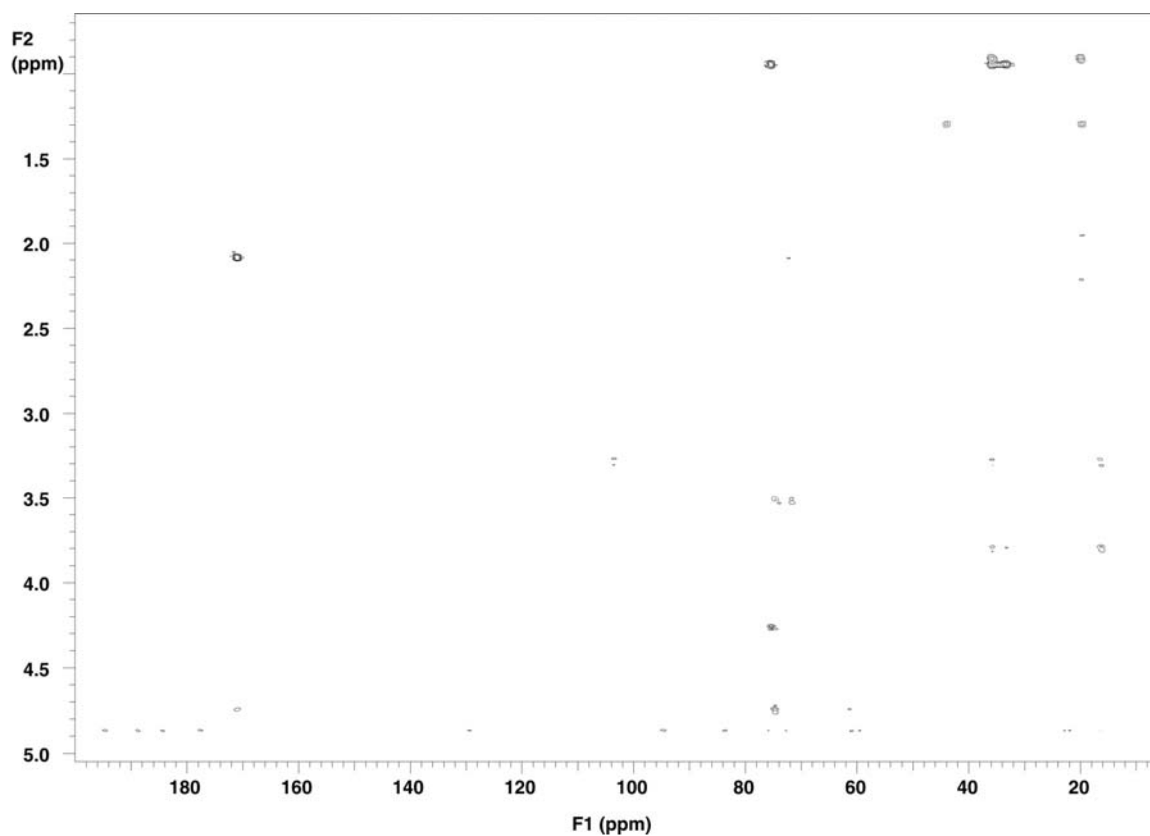


Figure 7.7 HMBC spectrum of RC7A.

Table 7.1 Summary of HNMR, HSQC and HMBC results of RC7A.

Assignment	HNMR	HSQC	HMBC
1	0.91	13.5	19.6, 35.7
2	1.4	19.6	-
2’	1.3	19.6	-
3	1.11	35.7	-
3’	1.43	35.7	-
4	1.78	33.3	-
5	3.30	75.4	-
5’	3.80	75.4	16.2
6	4.26	103.6	75.4
7	3.26	74.0	-

Table 7.1 Continued

Assignment	HNMR	HSQC	HMBC
8	3.53	74.7	71.6, 74.0
9	4.74	71.6	74.5, 171
10	3.42	74.5	-
11	0.96	16.2	33.3, 35.7, 75.4
12	3.52	61.3	-
12’	3.60	61.3	-
14	2.08	20.0	171

Based on the assignments and connectivities shown above, the compound as shown in **Figure 7.8** was constructed. The stereochemistry of the monosaccharide unit was determined from the coupling constants observed in the ^{1}HNMR spectrum. Preliminary values were extracted by inspection of the ^{1}HNMR spectrum and then these values together with the observed chemical shifts of the protons, were entered into a spin-simulation package on the NMR spectrometer. The values were adjusted manually so as to generate a calculated spectrum identical to the observed spectrum. The values finally obtained are shown in **Table 7.2**. It is seen that all of the vicinal coupling constants for the ring protons are in the range 7.7-9.6 Hz, consistent with dihedral angles between each vicinal proton pair of ~180°. This established the stereochemistry of the monosaccharide as being that of β-glucopyranose.

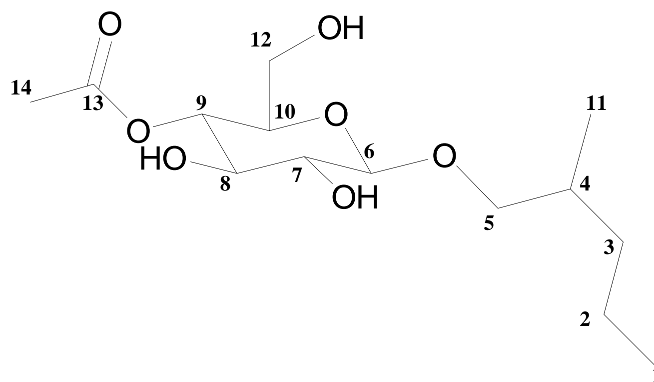


Figure 7.8 Proposed structure of RC7A.

Assignment	Coupling constant (Hz)
$J_{8,9}$	9.60
$J_{9,10}$	9.60
$J_{6,7}$	7.70
$J_{12,12'}$	12.00
$J_{10,12}$	2.50
$J_{10,12'}$	5.70
$J_{7,8}$	9.30

Table 7.2 Values of coupling constants for RC7A.

RC7A (without the acetyl group attached to C_9-O) has been reported to be used as a substrate to study the transglucosylation process of β-glucosidase from different sources (Svasti *et al.*, 2003). Kawabata *et al.* (2007) has also reported few related compounds to RC7A in their study on regioselective acylation on monosaccharides. None of these reports gave any spectroscopic data for the related compounds.

CONCLUSIONS

A total of 232 fungal cultures were isolated from leaf litter and soil samples collected in New Zealand using a particle filtration technique. In spite of many decades of research on soil and litter organisms, there still remains a large untapped potential in the discovery of new isolates with possibility of producing new bioactive compounds. The use of selective isolation techniques as studied here enables selection of slower growing and 'rarer' isolates which would not normally be recovered because of the many rapidly colonizing species obtained with many other techniques.

The bioactive screens used in this study were very limited and further screens would be highly desirable to determine bioactivities other than cytotoxicity on one cell line and activity against few microorganisms. Even given this limitation, some 45% of the selected isolates showed some bioactivity. The other 55% remain as a potential source for the future discovery of novel compounds with other bioactivity.

CapNMR has proved to be invaluable in characterizing the compounds investigated. It enabled very small quantities of compound to be fully chemically characterized. The use of these HPLC/NMR together with both UV library and AntiMarin database proved to be significant in the discovery of new compounds as well as identifying known compounds.

Nine compounds, including two new compounds (compound KV9A and RC7A), were chosen for further investigations. Five compounds were found to be active against P388 cells (sterigmatocystin, phomenone, mycophenolic acid, cycloaspeptide A and mevastatin). One compound, griseofulvin, was active against dermatophytes; another, KV9A, was active against both P388 cells and *Bacillus subtilis* while two more were inactive (compound RC7A and 3,4,6,8-tetrahydroxy-3-methyl-3,4-dihydroisocoumarin).

In conclusion, fungi remain as an important source for producing a wide range of potentially useful secondary metabolites. The investigation of unique habitats, extreme environments and utilizing selective techniques such as particle filtration should yield handsome rewards.

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