CHAPTER 7

Isolation of antifungal compounds from leaves of *Breonadia salicina*

7.1. Introduction

In chapter 6 *Breonadia salicina* was selected as the best plant species for further phytochemical investigation and isolation of antifungal compounds.

Plant extracts may contain highly polar and/or highly non-polar substances which can interfere with the separation of pure compounds during isolation if they are present in a very high concentration. Commonly known examples of polar substances are carbohydrates, glycosides and amino acids, while non-polar substances include waxes, oils, sterols and chlorophylls (Klejdus et al. 1999). Preliminary removal of inactive highly polar or non-polar substances during isolation is useful since it increases extract purity and allows more accurate determination of antifungal activity and easier isolation of active compounds.

The polarity of solvents is important when extracting plant material, in terms of targeting specific compounds from crude extracts. Various solvents such as water, alcohols, acetone and ether are used to extract bioactive substances from natural products. Ether is used to extract low polarity ingredients, such as aromatic compounds. Methanol is frequently used to extract specific bioactive ingredients from various natural resources (Kim et al. 2007).

Isolation of antifungal compounds aims at targeting pure compounds from plant material that inhibit the fungi of interest. Isolation and purification of compounds from plant extracts is often demanding and time consuming. In order to yield pure compounds, several steps need to be followed and this includes: extraction, isolation, purification, separation, detection of the active compounds and quantitative data analyses (Abidi 2001). The major disadvantage is the time taken to isolate and to characterise the active components from the extracts. The purification process is necessary since it reduces or eliminates interference of other substances. Moreover, improving diversity, quality of sample source and screen suitability and by automating and standardising early isolation steps, the effectiveness of natural products research can be enhanced (Pieters and Vlietinck 2005).

Several methods have been used to acquire compounds for drug discovery, including isolation from plants and other natural sources, and synthetic chemistry (Balunas and Kinghorn 2005). This includes column chromatography (CC), high performance liquid chromatography (HPLC), gas chromatography (GC), planar chromatography (PC) and thin layer chromatography (TLC). HPLC with a variety of columns and solvents is commonly used for the separation and quantitation of secondary compounds in plant extracts and allows the recovery of pure compounds in the 1-100 mg range (Jagota and Cheatham 1992, Bouvier and Martin 1997). However, HPLC sometimes suffers from poor peak shape, insufficient selectivity and inadequate retention control for basic compounds (Kagan et al. 2008).

A well known isolation procedure is the solvent extraction of the plant sample followed by column chromatography on different sorbents (Štěrbová et al. 2004). Column chromatography and TLC techniques are most affordable procedures and are suitable for sample purification, qualitative assays and preliminary estimates of the compounds in plant extracts (Heftmann 1995). Planar chromatography (PC) requires small amounts of solvent and provides a method for the isolation and recovery of the heaviest fractions (Lazaro et al. 1999). In this study, we follow column chromatography for isolating antifungal compound since it can purify larger samples and also use normal phase systems, i.e. a polar stationary phase (silica) eluted with organic solvents of increasing polarities.

7.2. Materials and methods

The procedure for isolation of antifungal compounds from leaves of *Breonadia salicina* is explained in a schematic representation in Figure 7-1.

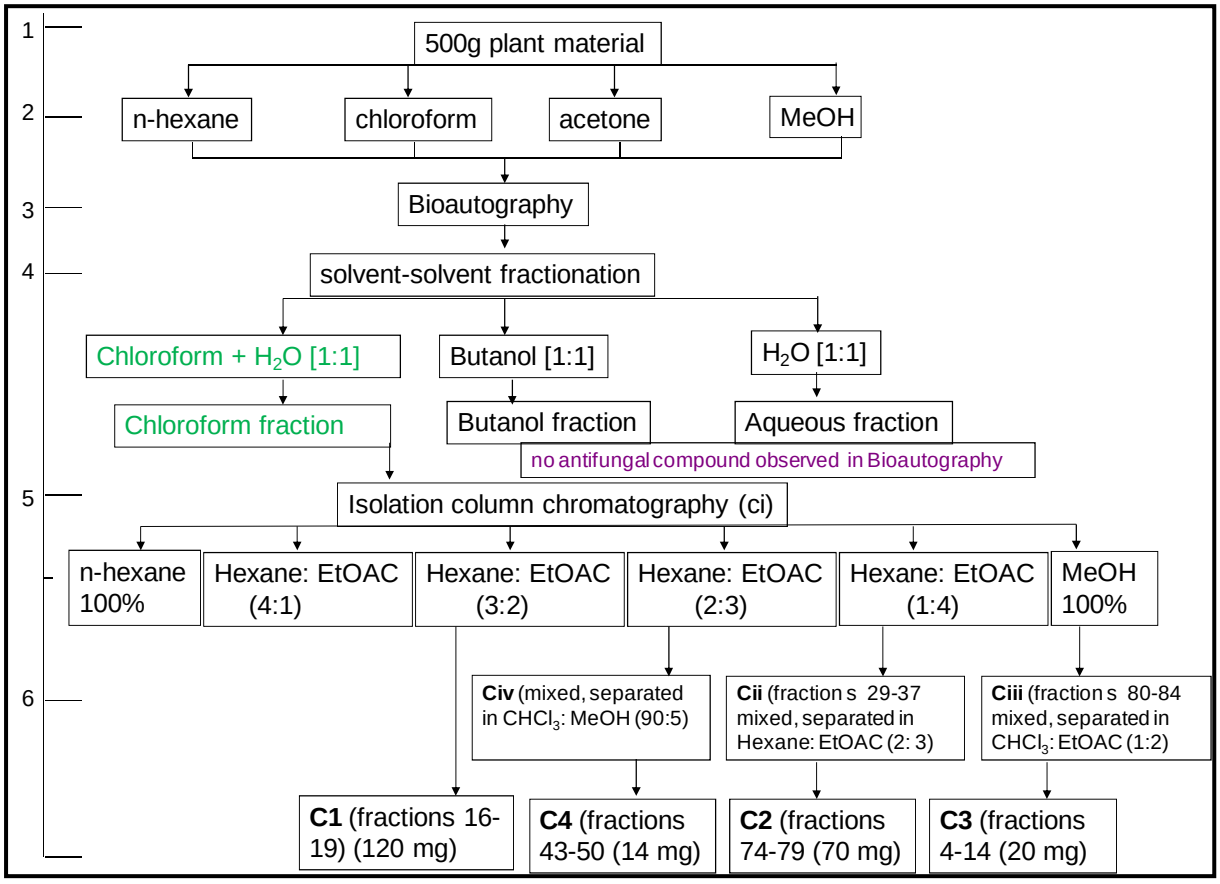

Figure 7-1 Schematic representation of bioassay-guided isolation of four antifungal compounds from the leaf extract of *B. salicina*. The isolation pathway include the following stages: (1) 500 g plant material was ground to fine powder, (2) Serial extraction was carried out using four extractants (Hexane, $CHCl_3$, Acetone and MeOH), (3) Bioautography assay was used to determine antifungal compounds, (4) Solvent-solvent fraction was carried out using chloroform fraction since antifungal compounds were present on bioautograms, (5) Isolation of antifungal compounds with column chromatography, (6) Six fractions were collected in the first column (Ci); Hexane: EtOAC (3:2) fraction yielded 120 mg of compound (C1), Further column chromatography Cii, Ciii and Civ yielded compounds C2 (70 mg), C3 (20 mg) and C4 (14 mg) respectively.

7.2.1 Isolation of antifungal compound(s)

7.2. 1.1 Column chromatography

The chloroform fraction from serial extraction was separated by solvent-solvent fractionation and the chloroform fraction was fractionated by column chromatography. Silica gel (200 g) was mixed with 500 ml hexane to form a slurry and packed to a glass column (denoted as column i) to a height of 30 cm and a diameter of 3 cm. The chloroform fraction (2 g) was dissolved in a small volume of $CHCl_3$ and mixed with 0.5 g silica gel and allowed to dry under a stream of cold air, then thinly spread on top of the column. The fraction was covered with cotton wool and a volume of 500 ml of 100% hexane was initially used to elute the column, followed by the same volume of each of the following solvent mixtures: hexane: ethyl acetate (4:1), (3:2), (2:3) and (1:4) and finally the column was eluted with 100% MeOH . Fractions of 500 ml each were collected. TLC chromatograms of the fractions were prepared in duplicate and developed in hexane: ethyl acetate (3:1). One set was sprayed with vanillin as the reference chromatograms for visualising compounds and the other was sprayed with *A. niger*, *A. parasiticus*, *C. gloeosporioides*, *T. harzianum*, *P. expansum*, *P. janthinellum* and *F. oxysporum* to locate the antifungal compounds present in the fractions.

7.2.1.1a Compound 1 (column i)

Fractions 15-19 from the first column (Ci) contained a pure compound C1. The pooled fraction was concentrated under vacuum at 45°C and transferred to a pre-weighed glass vial to dry completely.

7.2.1.1b Compound 2 (column ii)

Silica gel (13 g) was dissolved in $CHCl_3$: EtOAC (3:2) and used to pack the column ii (20 × 1.0 cm). Hexane: EtOAC (1:4) fractions (0.13 g) from column i were mixed with a small portion of silica gel and allowed to dry. The mixture was spread on top of the column and $CHCl_3$: EtOAC (2:3) was used as eluent solvent system. Fractions of 10 ml volume were collected. Fractions 75-80 contained a pure compound C2.

7.2.1.1c Compound 3 (column iii)

Silica gel (10 g) was mixed with $CHCl_3$: EtOAC (1:2) and packed in column iii (20.0 × 1.0 cm). Hexane: EtOAC (3:2) (110 mg) obtained from column ii was dissolved in $CHCl_3$, mixed with a small portion of silica gel 60, dried and loaded on the packed column. The column was eluted with 300 ml $CHCl_3$: EtOAC (1:2), (1:3) and (1:4). Fractions of 10 ml volume were collected. Fraction 4-12 contained a pure compound C3.

7.2.1.1d Compound 4

Silica gel (15 g) was mixed with $CHCl_3$: MeOH (90:5) and packed in column iv (20.0 × 1.0 cm). Hexane: EtOAC (4:1) (150 mg) obtained from column i was dissolved in $CHCl_3$, mixed with a small portion of silica gel 60, dried and loaded on the packed column. The column was eluted with 300 ml $CHCl_3$: MeOH (90:5). Fractions of 10 ml volume were collected. The fractions 43-50 containing only one compound based on TLC chromatograms were combined to yield compound 4.

7.3 Microplate dilution assay

The crude extracts and four isolated compounds were tested for antifungal activity against seven plant pathogenic fungi. The method is described in section **3.2.2.2.**

7.4 Bioautography assay

Bioautography was used to determine the number of active compounds in the crude extracts, as well as activity of the isolated compounds. The method is described in section **3.2.2.2.**

7.5 Results and discussion

Fractions 16-19 from column i contained a single spot on TLC chromatograms after spraying with vanillin and sulphuric acid and were pooled together and evaporated under reduced pressure to yield 120 mg of compound 1 (Figure 7-2). The TLC chromatograms of fractions 25-40 contained some impurities and were purified further.

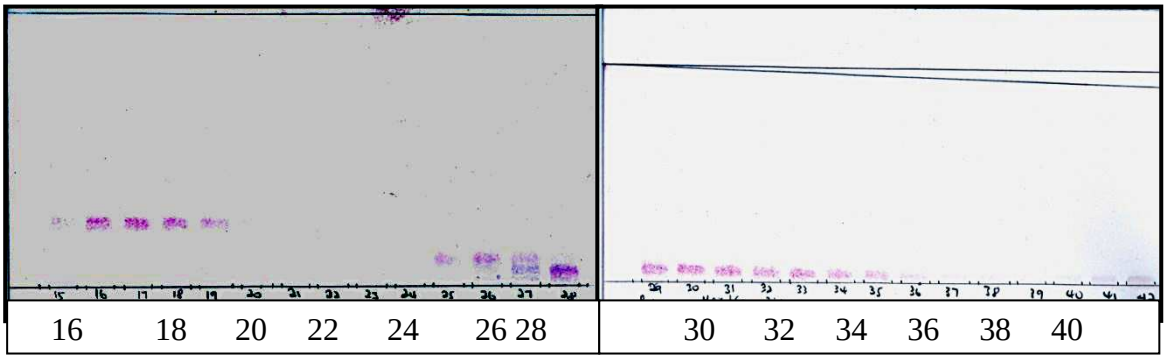

Figure 7-2 Fractions of column i developed in Hexane: EtOAC (3:1) and visualized using vanillin-sulphuric acid.

The pooled fractions were analyzed by bioautography against *F. oxysporum* (Figure 7-3). Clear zones of growth inhibition were observed on the bioautograms in fraction 15-20 and 26-40, and this indicates that the plant components inhibited the growth of fungi. The fractions were tested immediately to observe the presence of active compounds to avoid problems associated with decomposition or photo-oxidation. In bioautography, all seven plant pathogens showed sensitivity, but only the results for *F. oxysporum* are shown. Fractions 15-20 showed the presence of an active compound against *F. oxysporum* while antifungal compounds were observed against the other six plant pathogens from fractions 25-40. Bioautogram of *P. janthinellum* are shown in figure 7-4, showing the presence of active compounds from fraction 25-40.

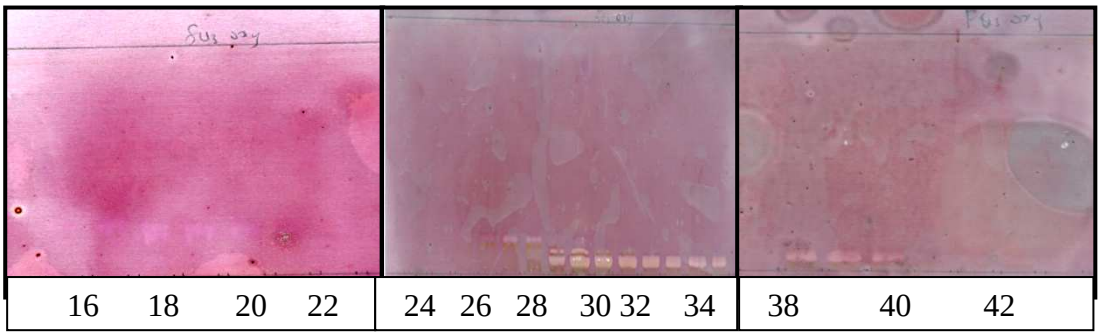

Figure 7-3 Bioautograms of fractions showing activity of fractions developed in hexane: EtOAC (3:1) and sprayed with *F. oxysporum.* White areas indicate inhibition of fungal growth on bioautograms.

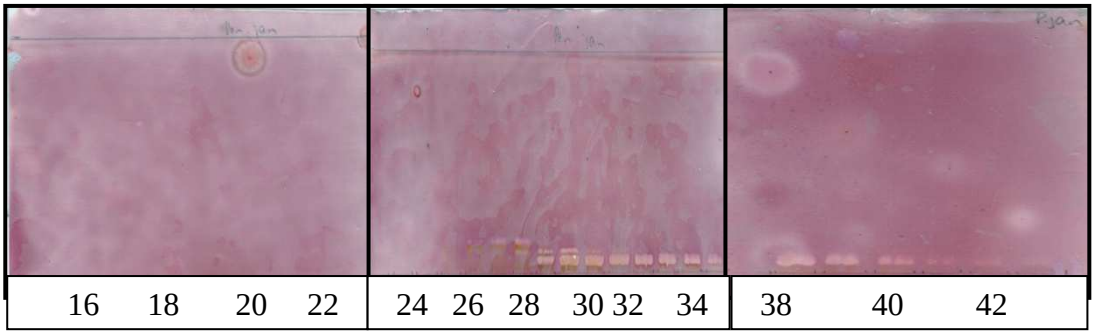

Figure 7-4 Bioautograms of fractions showing activity of fractions developed in hexane: EtOAC (3:1) and sprayed with *P. janthinellum*. White areas indicate inhibition of fungal growth on bioautograms.

Fractions 74-79 from column ii contained a single blue spot on chromatograms after spraying with vanillin spray reagent and were pooled together and evaporated to dryness to yield 70 mg of a white powder (Figure 7-5). Fractions 80-84 contained some minor impurities and were combined and evaporated under reduced pressure before being purified further.

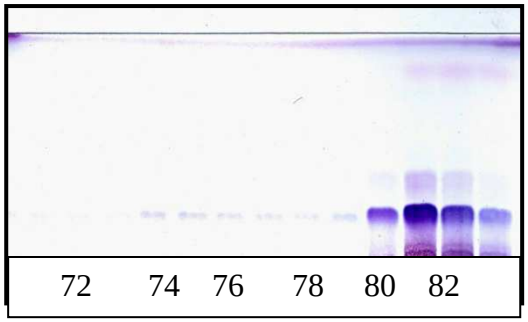

Figure 7-5 Fractions of column ii developed in hexane: EtOAC (3:2) and visualized using vanillin-sulphuric acid.

Fractions 4-14 contained a single blue compound after spraying with vanillin- sulphuric acid, and were combined and evaporated to dryness (Figure 7-6). The resultant pure compound, C3, yielded 20 mg and it was white powder.

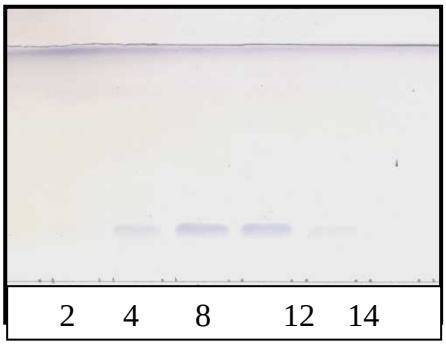

Figure 7-6 Fractions of column iii developed in $CHCl_3$: EtOAC (1:2) and visualized using vanillin-sulphuric acid.

Fractions 43-50 from column iv contained a single purple compound and were combined and evaporated to dryness (Figure 7-7). The resultant compound was a white powder, C4, and yielded 14 mg.

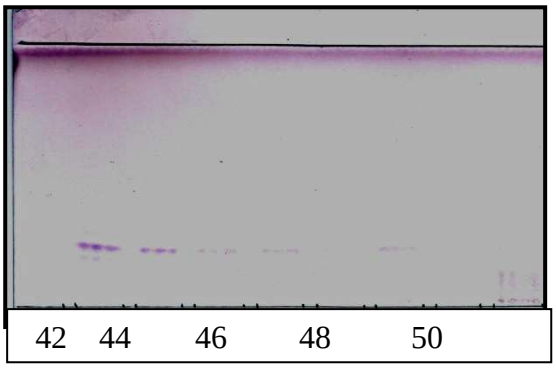

Figure 7-7 Fractions of column iv developed in $CHCl_3$: MeOH (90:5) and visualized using vanillin-sulphuric acid.

7.5.1 TLC analysis

Several compounds in the crude extract were visible after spraying the plates developed in three solvent systems with vanillin sulphuric acid (Figure 7-8 and Table 7-1). TLC chromatograms developed in BEA showed no compounds after spraying with vanillin-sulphuric acid for compounds 1 and 2. Some impurities were however visible in compounds 3

and 4 suggesting that these compound did not move in the solvent system used. Compound 1 had an R_f value of 0.82 in CEF. No visible compounds were observed in CEF with regard to compounds 2 and 4. However, compounds 1 and 3 had R_f values of 0.82 and 0.68, respectively. In TLC chromatograms developed in EMW, all compounds were observed just below the solvent front with the same R_f value of 0.92. Compound 1, C2 and C3 were not visible under UV-light at 254 and 362 nm. Visualising isolated compounds using different TLC systems helps to confirm that the compounds are sufficiently pure for structure elucidation.

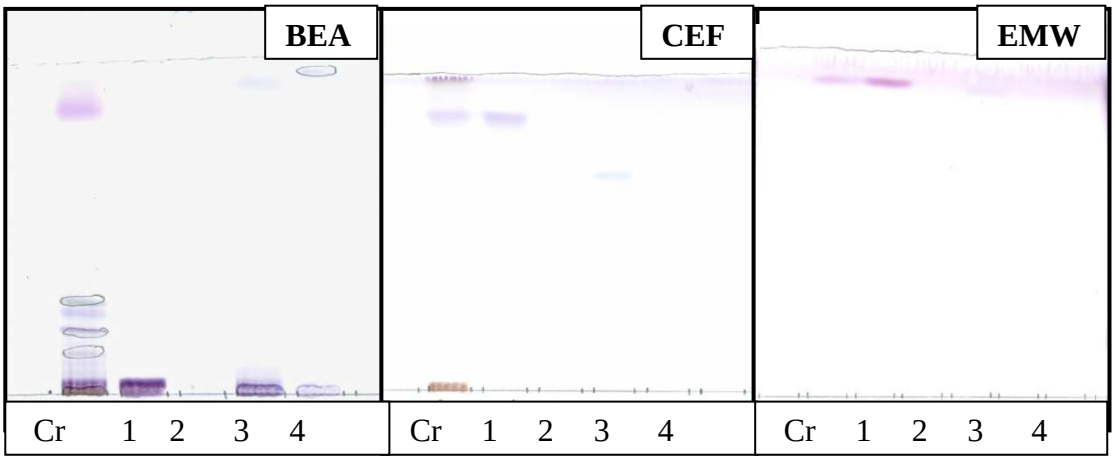

Figure 7-8 Chromatograms of 100 µg of isolated compounds developed with BEA, CEF and EMW and sprayed with vanillin-sulphuric acid. **Lanes from left to right:** Cr= Crude extract, 1= Compound 1, 2= Compound 2, 3= Compound 3 and 4= Compound 4.

Table 7-1 R_f values of compounds separated in BEA, CEF and EMW. The compounds were visualized using visible light, UV light at 254 or 365 nm, and sprayed with vanillin-sulphuric acid.

Solvent system	R_f of isolated compound (s)				
	Crude	1	2	3	4
BEA	0.12	-	-		
	0.18				
	0.29				
	0.88				
	0.90				
CEF	0.82	0.82	-	0.68	-
EMW	0.92	0.92	0. 92	0.92	-

7.6 Conclusion

Isolation of active compounds from leaves of *B. salicina* using column chromatography yielded 4 purified compounds. Compound 1 was isolated in the largest quantity (120 mg), followed by compound 2 (70 mg), compound 3 (20 mg) and compound 4 (14 mg). As expected, in the region of about 10% of plant extract was lost during isolation (packing column using silica gel and TLC analysis of the fractions). In the next chapter, the structure of the isolated compounds will be determined using NMR, EIMS and MS spectroscopy techniques.

CHAPTER 8

Structure elucidation of four isolated compounds

8.1 Introduction

Recently, more than 40% of newly registered drugs were derived from natural products (Humpf 2002, Skowroneck and Gawronski 2000). Compounds derived from natural products are mostly identified using techniques such as nuclear magnetic resonance (NMR) and mass spectroscopy (MS) that provides structural information leading to the complete structure determination of natural products.

Structural elucidation based on these techniques has been the most successful for determining both simple and complex structures (Conolly et al. 1991). Before undertaking NMR analysis of a complex mixture, separation of the individual compounds by chromatography is required (Silva-Elipe 2003). Nuclear magnetic resonance is the best method for complete structure elucidation of non-crystalline samples. When elucidating the structure of secondary natural products, ^{1}H NMR, ^{13}C NMR and 2D NMR spectroscopy are important since hydrogen and carbon are the most abundant atoms in natural products (Džeroski et al. 1998).

However, there are some difficulties encountered when using NMR because it has a very low sensitivity compared to MS and it therefore requires much larger samples for analysis. The machine can detect proton (^{1}H) sensitivity, high isotopic natural abundance and its ubiquitous presence in the organic compounds. When using NMR, all samples require signal averaging to reach an acceptable signal-to-noise level. The NMR analysis depends entirely on the size of the sample, and can range anywhere from several minutes to several days. For example, in the case of metabolites with a mass of 1-10 µg, an overnight experiment with a very powerful apparatus is required (Silva-Elipe 2003).

MS does not always provide conclusive structural information, especially when isomers of bioactive compounds are studied (Albert 2004). It can be used to determine the molecular weight and confirm the structure of the isolated compounds or natural products. In this chapter, we used NMR, MS and EIMS (electron impact mass spectrometry) to determine the

structure of four compounds isolated from leaves of *B. salicina*. The structures were elucidated from the spectroscopic data in collaboration with Dr X.K Peter of the CSIR**.**

8.2 Materials and methods

8.2.1 Structure elucidation

8.2.2.1 Nuclear Magnetic Resonance

An analytical Varian-NMR-vnmrs 600 instrument operating at proton frequency of 600 MHz was used for ^{1}H and ^{13}C. Four compounds isolated from leaves of *B. salicina* (Chapter 7) were weighed (10-30 mg) and dissolved in deuterated $CDCl_3$ since the compounds were soluble in $CHCl_3$. All of the samples were sent to the Council for Scientific and Industrial Research (CSIR) for NMR analysis. Each sample were dissolved in 0.7 ml $CHCl_3$ and transferred into NMR tubes (5 mm).

8.2.2.2 Mass Spectroscopy

An analytical THERMO electron DFS magnetic sector mass spectrometer at low resolution was used and the samples were ionized by electron impact ionization (EI). Approximately 2 mg of each isolated compound was dried, placed into a 2 ml glass vial and sent to the University of the Witwatersrand, Department of Chemistry for MS analysis.
Aliquots of the four isolated compounds were transferred into separate 1 ml HPLC vials. The samples (2 mg) were each dissolved in approximately 1 ml of DMSO (fraction 1) of which 2 µL was transferred to a direct probe crucible, and inserted into the MS. The MS source temperature was 250°C and the probe was heated from 50 to 250°C.

8.2.2.3 Electron impact mass spectrometry (EIMS)

Analytical EIMS was used to displace an electron from the organic molecule to form a radical cation known as the molecular ion**.** Compound 1 was ionized in a negative mode electron impact mass spectrometry (EIMS) with molecular ion $[M-H]^-$. This mass spectrum was used to confirm the accurate mass measurement of the isolated compound. Approximately 10 mg of compound 1 were weighed and sent to CSIR for EIMS. Before analyzing the sample, 1 mg of isolated compound was dissolved in 1 ml aceto nitrile (CH_3CN) and then direct infusion was applied.

8.3 Results and discussion

8.3.1 Structure elucidation

8.3.1.1 Compound 1

In the mass spectrum peaks were observed at *m/z*. 189.53, 207.58, 219.56 248.63 and 249.64 m/z (See p 142). The ^{13}C NMR showed the presence of 7 methyl groups at signals δ 14.1, 15.4, 15.5, 16.9, 17.0, 18.2 and 21.1 (See p 139). Furthermore, C12- C13 was identified as an olefinic group at signal δ 137.9 and 125.8 whilst an acidic group was observed at 206.9. The rest of the spectra were aliphatic CH_2 groups. The ^{1}H NMR had a signal at δ 5.19 for an olefinic proton at hydrogen 12, and hydrogen 3 was observed next to a hydroxyl group (OH) and was shifted down-field at signal δ 3.12 (See p 140). Furthermore, three hydroxyl groups were observed at signal δ 3.96. Based on ^{1}H and ^{13}C NMR spectra compound 1 was identified as the triterpenoid ursolic acid and the spectral data is in agreement with the literature (Moghaddam et al. 2006). However, our ^{13}C NMR spectrum at C28 had a peak at δ 206. 9 compared to that of Moghaddam et al. (2006) at δ 179.1. It is unlikely that the difference of the peaks could be due to the fact that we used $CDCl_3$ solvent while DMSO was used in Moghaddam et al. (2006) (Table 8-1). The structure was further confirmed by electron impact mass spectrometry (EI-MS), in a negative mode (See p 141). The spectrum displayed an accurate molecular ion peak at *m/z* 455.4 $[M-1]^+$ corresponding to the molecular formula of 456 of $C_{30}H_{48}O_3$. This was in good agreement with the ^{1}H NMR and ^{13}C NMR spectroscopic data. Previously, ursolic acid with a molecular ion peak at m/z $[M]^+$ 456 was reported (Moghaddam et al. 2006).

Ursolic acid has been isolated from *Satureja* species and also from the Lamiaceae and Oleaceae family (Escudero et al. 1985, Giannetto et al. 1979, Kontogianni et al. 2009). This compound has been previously isolated from the dichloromethane (DCM) extract of *Curtisia dentata* and stem bark of *Hyppocratea excels* (Shai et al. 2008, Cáceres-Castillo et al. 2008).

Table 8-1 ^{13}C NMR spectroscopic data for compound 1

Number of Carbon	Compound 1 ($CDCl_3$) δ ^{13}C (ppm)	Ursolic acid (DMSO) Moghaddam et al. (2006)
1	39.4	39.2
2	27.9	27.8
3	77.2	77.7
4	39.4	39.2
5	55.2	55.6
6	18.2	18.9
7	33.9	33.6
8	39.4	40.0
9	47.8	47.9
10	38.7	37.4
11	23.7	23.7
12	125.8	125.4
13	137.9	139.0
14	41.9	42.5
15	28.1	28.4
16	24.4	24.7
17	47.8	47.7
18	52.6	53.2
19	39.4	39.4
20	39.0	39.3
21	31.9	31.1
22	38.5	37.2
23	29.2	29.1
24	15.5	16.1
25	17.0	16.9
26	18.2	17.8
27	24.1	24.1
28	206.9	179.1
29	16.9	17.9
30	21.1	21.9

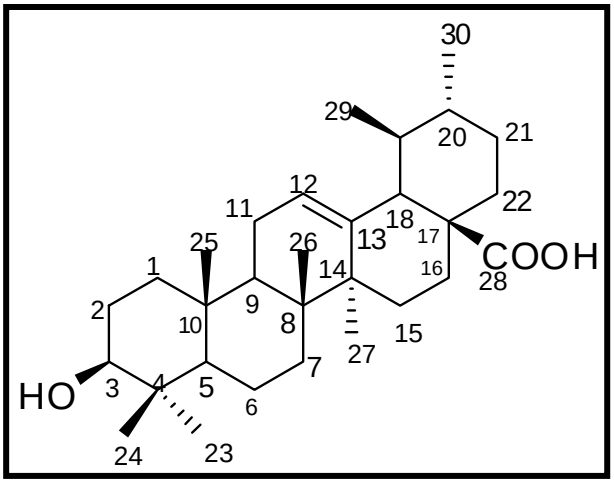

Figure 8-1 Structure of ursolic acid isolated from leaves of *Breonadia salicina*

8.3.1.2 Compound 2, 3 and 4

Compounds 2, 3 and 4 were isolated as white powders. The presence of long chain fatty acids was detected when these compounds were analysed in mass spectrometry. Despite a thorough use of different solvents to remove fatty acids from the compound, the MS still indicated the presence of long chain fatty acids among masses not explained by fatty acids. Based on the outcome of the mass spectra results and the low quantity of material available, the compound was not analysed further by NMR.

8.4 Conclusion

Four compounds were isolated from leaves of *B. salicina* and the structure of compound 1 was elucidated using NMR and MS technique as ursolic acid ($C_{30}H_{48}O_3$). With the other three isolated compounds (2, 3 and 4), only mass spectrometry was performed. To the best of our knowledge, no chemical isolation and characterization of bioactive constituents of *B. salicina* has been reported before. Ursolic acid has been previously isolated from leaves of *Curtisia dentata* (Shai et al. 2008) and stem bark of *Hippocratea excels* (Ca´ceres-Castillo et al. 2008).

Three compounds (2, 3 and 4) appeared to consist of long chain fatty acids or carboxylic acids as shown by MS. These were probably not pure and in all cases there was a significant loss of $(CH_2)_n$. In particular, there was no distinction between compound 2 and 3 from MS results.

CHAPTER 9

Antifungal and antibacterial activity and cytotoxicity of isolated compounds

9.1 Introduction

In the previous chapter, the structure of compound 1 was elucidated as the triterpenoid ursolic acid and the other three compounds consisted of long chain fatty acids. Triterpenoids form a large group of natural substances which includes steroids and consequently sterols. Steroids are one of the largest groups and a very small amount is present in bacteria but more are found in plants and animals (Connolly and Hill 1992). Various biological activities of the triterpenoid and fatty acids have been reported. Previously, an iridal triterpenoid isolated from *Iris germanica* L., has been reported to have antifungal activity against *Candida albicans* (Benoit-Vical et al. 2003). Bioassay guided fractionation led to the isolation of the active triterpenoid ergosterol-5,8-endoperoxide from *Ajuga remota* and it was active against *Mycobacterium tuberculosis* (MIC of 1 µg/ml) (Cantrell et al. 2001). Ursolic acid has been previously isolated from the dichloromethane extract of *Curtisia dentata* and was reported to have high antifungal activity against *Sporothrix schenckii* and *Microsporum canis* with MIC values of 12 and 32 µg/ml respectively (Shai et al. 2008).

Fatty acids, in particular 2-alkynoic fatty acids have been known to have antifungal activity. The activity of this compound depends on the fatty acid chain length and pH of the medium (Gershon and Shanks 1978). The optimal chain lengths of 8 and 16 carbons have been established for the 2-alkynoic fatty acid to exert maximum fungistatic effects. Another type of fatty acid, hexadecanoic acid has been reported to have antifungal, antimicrobial and cytotoxic properties (Konthikamee et al. 1982, Wood and Lee 1981).

Previously, a novel acetylenic fatty acid, known as 6-nonadecynoic acid was isolated from the ethanol extract of roots of *Pentagonia gigantifolia* (Li et al. 2003). The antifungal mechanism was due to interference of the compound with fungal sphingo-lipid biosynthesis. It was discovered to be fungitoxic to *Cryptococcus neoformans* but inactive towards *Candida albicans* (Li et al. 2008).

Crude extracts and pure compounds of medicinal plants are important in drug discovery; however their toxicity requires extensive attention since this can cause various side effects (biological implications) to human and animals. In general, cell type cytotoxic specificity of plant extracts is likely due to the presence of different classes of compounds (such as terpenes or terpenoids, and alkaloids) in the extracts. There are several types of cytotoxicity assays that can be used to determine the level of toxicity in the plant extracts, and this includes inferior organisms, biochemical assays, cell cultures and isolated organs. However, cytotoxicity with cell cultures is highly preferred because it is very common, rapid, inexpensive, and does not have ethical implications (Fernandes et al. 2005).

In this chapter I will investigate the antifungal activity of the isolated compounds against seven plant pathogens as well as against three bacteria including the Gram-positive *Staphyloccocus aureus* (ATCC 29213) and the Gram-negative *Escherichia coli* (ATCC 25922) and *Pseudomonas aureus* (ATCC 27853). I will also determine the cytotoxicity against Vero monkey kidney cells to evaluate the safety of the isolated compounds.

9.2 Materials and methods

9.2.1 TLC fingerprint

Ten milligrams of pure compounds were separately resuspended in 1 ml acetone to a known concentration (10 mg/ml) and were separated on TLC plates. The method is described in section **2.2.5.**

9.2.2 Bioassays for antifungal activity

9.2.2.1 Microdilution method

The crude acetone extracts and four isolated compounds were tested for antifungal activity against seven plant pathogenic fungi. The method is described in section **3.2.2.2.**

9.2.2.2 Bioautography assay

Bioautography was used to determine the number of active compounds in the crude extracts, as well as activity of the isolated compounds. The method is described in section **3.2.2.2.**

9.2.3 Antibacterial activity

Bioautography was used to determine the number of active compounds in the crude extracts, as well as activity of the isolated compounds. The assay was conducted as described by Eloff (1998c). The method is basically the same as the one outlined in section **3.2.2.2** the only difference is that INT (0.2 mg/ml) was added following overnight incubation of compounds with bacteria. Overnight cultures of the bacteria were diluted 1:100 with fresh Mueller Hinton (MH) broth prior to use in the assay

9.2.4 Cytotoxicity assay

9.2.4.1 Tetrazolium-based colorimetric assay (MTT)

The method described by Mosmann (1983) and slightly modified by McGaw et al. (2007) was used to determine the cytotoxicity of the crude extracts and four isolated compounds. The plant extracts and compounds were tested for cytotoxicity against Vero monkey kidney cells obtained from the Department of Veterinary Tropical Diseases (University of Pretoria). The cells were maintained in minimal essential medium (MEM, Highveld Biological, South Africa) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Adcock-Ingram). Cell suspensions were prepared from confluent monolayer cultures and plated at a density of 0.5×10^3 cells into each well of a 96-well microtitre plate. Plates were incubated overnight at 37ºC in a 5% CO_2 incubator and the subconfluent cells in the microtitre plate were used in the cytotoxicity assay. Stock solutions of the plant extracts (200 mg/ml) and isolated compounds (20 mg/ml) were prepared by dissolving them in DMSO. Serial 10-fold dilutions of each extract and isolated compounds were prepared in growth medium and added to the cells. The viable cell growth after 120 hours incubation with plant extracts and isolated compounds was determined using the tetrazolium-based colorimetric assay (3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT), Sigma) described by Mosmann (1983). Briefly, after incubation, 30 µl of MTT (5 mg/ml in phosphate buffered solution,

PBS) was added to each well and the plates were incubated for a further 4 hours. The medium was aspirated from the wells and 50 µl DMSO added to each well to solubilize the formazan produced by mitochondrial activity. The absorbance was measured on a Versamax microplate reader at 570 nm. Berberine chloride (Sigma) was used as a positive control. The intensity of colour was directly proportional to the number of surviving cells. Tests were carried out in quadruplicate and each experiment was repeated three times.

9.3 Results and discussion

9.3.1 Biological activity of the isolated compounds

9.3.1.1 Bioautography assay

Figure 9-1 shows bioautograms developed in BEA, CEF, and EMW and sprayed with *T. harzianum* (left), *A. parasiticus*, *P. janthinellum* centre and *F. oxysporum*. In TLC chromatograms developed in BEA, clear zones were observed with ursolic acid against *T. harzianum* and *A. parasiticus*, with R_f values of 0.07 and 0.15 respectively. The antifungal compounds 2 and 3 were visible at the origin ($R_f = 0$). Ursolic acid was visible at $R_f = 0.87$, and compounds 2 and 3 had the same $R_f = 0.66$ for the TLC chromatogram developed in CEF against *P. janthinellum*. For the chromatograms developed in EMW, ursolic acid, 2 and 3 showed clear inhibition zones indicating the presence of antifungal compounds against *F. oxysporum* ($R_f = 0.94$). In general, ursolic acid had a distinct active band than the other compounds. No clear visual growth inhibition was found with compound 4 against the tested microorganisms.

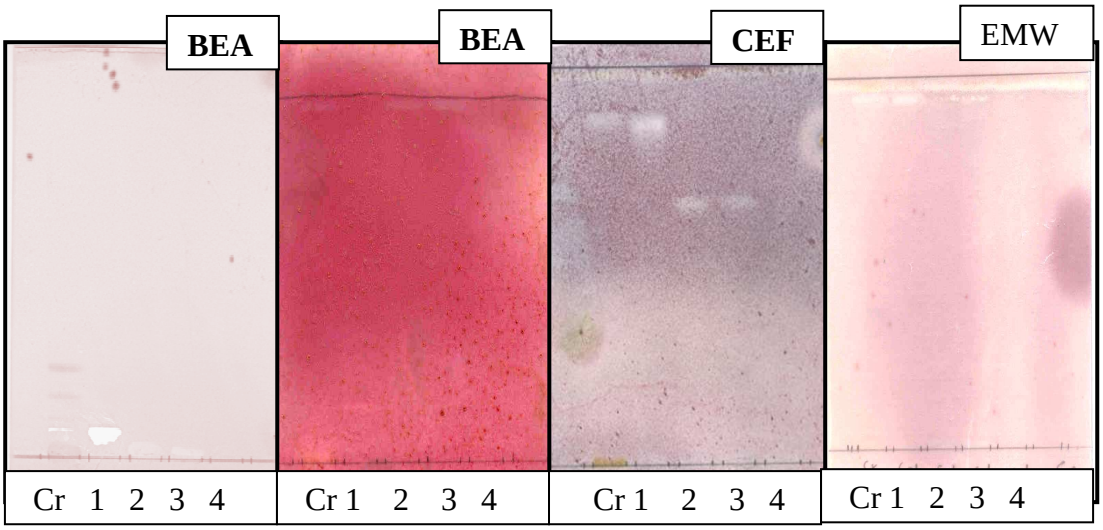

Figure 9-1 Bioautograms of 100 µg of isolated compounds, chromatograms developed with BEA, CEF and EMW and sprayed with *Trichoderma harzianum*, *Aspergillus parasiticus* (left), *Penicillium janthinellum* (centre) and *Fusarium oxysporum* (right). White areas indicate inhibition of fungal growth. **Lanes from left to right:** Cr = Crude extract, 1 = Ursolic acid, 2 = Compound 2, 3 = Compound 3 and 4 = Compound 4

Figure 9-2 shows bioautograms of ursolic acid, 2, 3, and 4 and crude extracts, with TLC chromatograms developed in BEA and EMW sprayed with *E. coli*, *P. aeruginosa*, and *S. aureus*. Ursolic acid, 2 and 3 showed active compounds against *E. coli*, *S. aureus* and *P. aeruginosa*. The results showed that the crude extract and ursolic acid had the same antibacterial compound in TLC chromatograms developed with BEA. The crude extract showed the presence of compound 1 by revealing an active band at the same R_f value as that of ursolic acid. (R_f = 0.05 against *E. coli* and *S. aureus*). Similarly, the same band in the crude extract and ursolic acid showed antibacterial compound with R_f value of 0.92 against *S. aureus* in TLC chromatograms developed in EMW. In TLC chromatograms developed in CEF, the compounds were visible below the solvent front with the same R_f value of 0.86 against *S. aureus*.

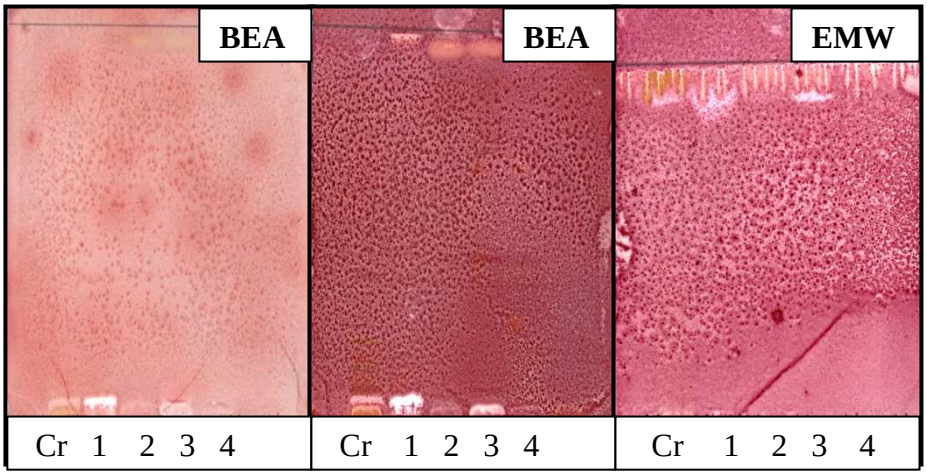

Figure 9-2 Bioautograms of compound 1, 2, 3, 4 and crude extracts, chromatograms developed in BEA and EMW sprayed with *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. White areas indicate inhibition of fungal growth. **Lanes from left to right:** Cr = crude extract, 1 = Ursolic acid, 2 = Compound 2, 3 = Compound 3 and 4 = Compound 4.

9.3.1.2 Microplate dilution assay

The crude extracts and four compounds were tested for antifungal activity against the plant pathogens. Compound 3 and 4 had good antifungal activity against *A. parasiticus* and *P. janthinellum* with MIC value of 10 and 16 µg/ml. Ursolic acid and C2 had activity with MIC values ranging between 20 and 250 µg/ml (Table 9-1). These results suggest that during isolation, 80% (crude extract= 2.5 mg/ml and compound 3 MIC = 10 µg/ml) of other impurities were removed since the compounds had low MIC values.

Table 9-1 Minimum inhibitory concentration (MIC) of four isolated compounds against seven plant pathogenic fungi. Standard deviations were 0 in all cases.

Micro-organisms	Time (hrs)	MIC (µg/ml)	MIC (µg/ml)				
		Crude extract	Ursolic acid	2	3	4	AmpB
Aspergillus parasiticus	24	630	20	20	10	30	6.4
Aspergillus niger	48	2500	120	120	120	120	1.6
Colletotrichum gloeosporioides	48	1250	30	60	20	60	3.2
Fusarium oxysporum	48	3200	50	50	50	50	3.2
Penicillium expansum	48	1250	125	32	32	125	3.2
Penicillium janthinellum	48	80	125	16	25	16	3.2
Trichoderma harzianum	48	630	125	250	125	250	3.2

9.3.2 Cytotoxicity assay

The cytotoxicity of four compounds was determined against Vero cells using the MTT assay. Berberine was used as a positive control and it was toxic with an LC_{50} of 13 µg/ml (Figure 9-3). The crude extract was less toxic than ursolic acid with LC_{50} of 82 µg/ml (Figure 9-9). Compounds 2 and 3 were not toxic at the highest concentration tested (200 µg/ml) (Figure 9-6 and 9-7) towards the Vero cells. However, C4 (compound 4) was more toxic to the cells with an LC_{50} of 35 µg/ml (Figure 9-8).

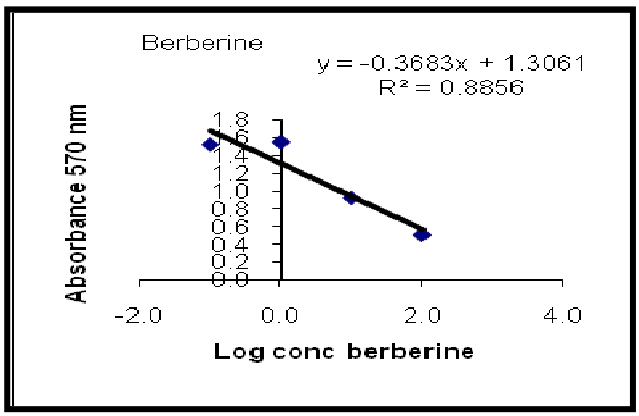

Figure 9-3 Cytotoxicity of berberine with LC_{50} = 13 µg/ml against Vero cells

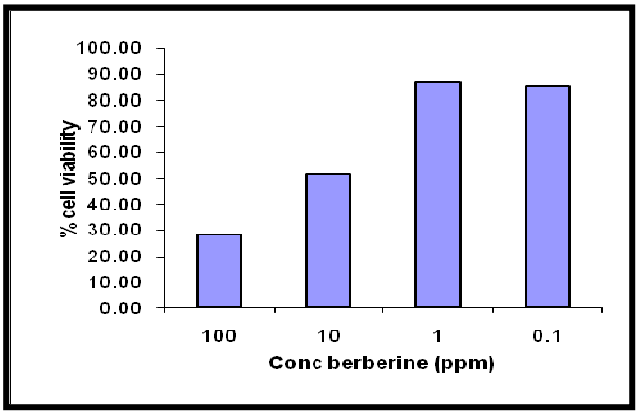

Figure 9-4 Percentage (%) cell viability of berberine

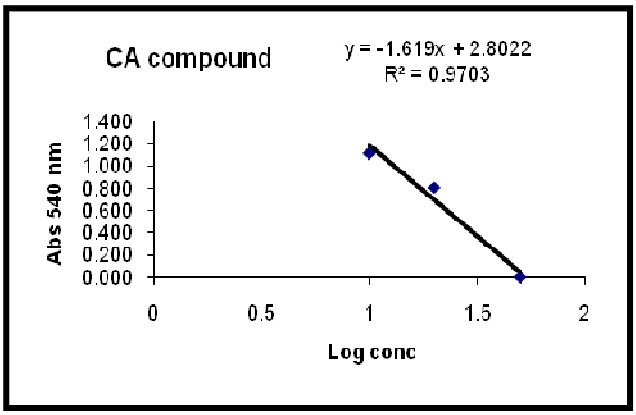

Figure 9-5 Cytotoxicity of ursolic acid with LC_{50}= 25 µg/ml against Vero cells

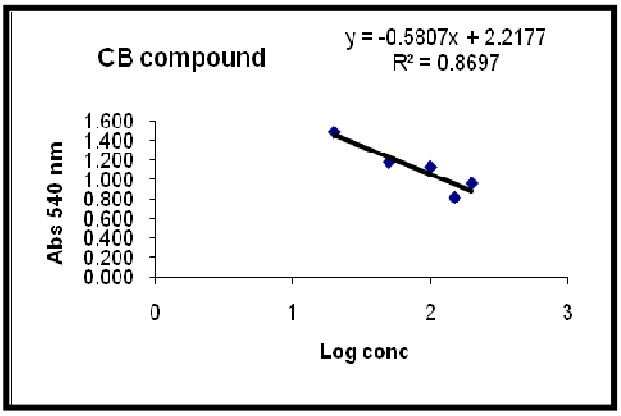

Figure 9-6 Cytotoxicity of C2 with LC_{50} = 525 µg/ml against Vero cells

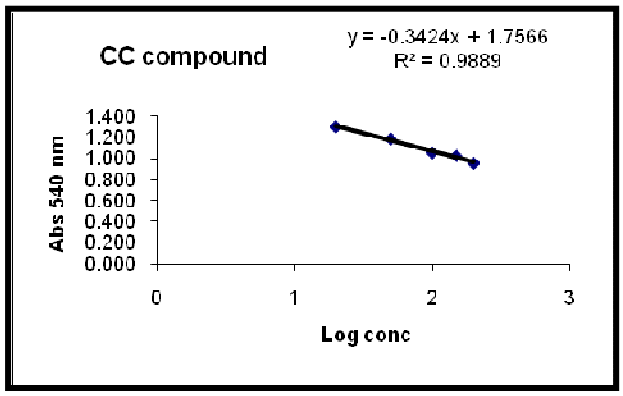

Figure 9-7 Cytotoxicity of C3 with LC_{50} = 1849µg/ml against Vero cells

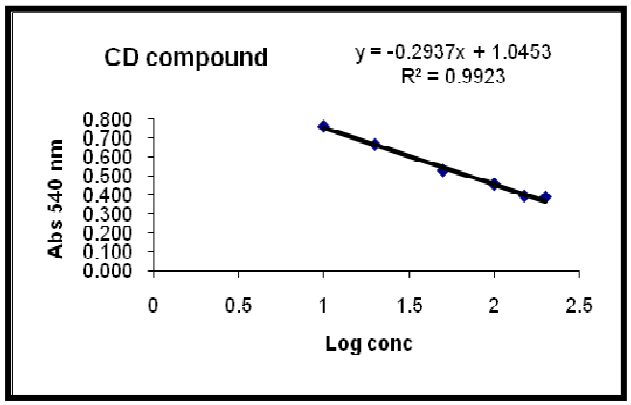

Figure 9-8 Cytotoxicity of C4 LC_{50} = 35 µg/ml against Vero cells

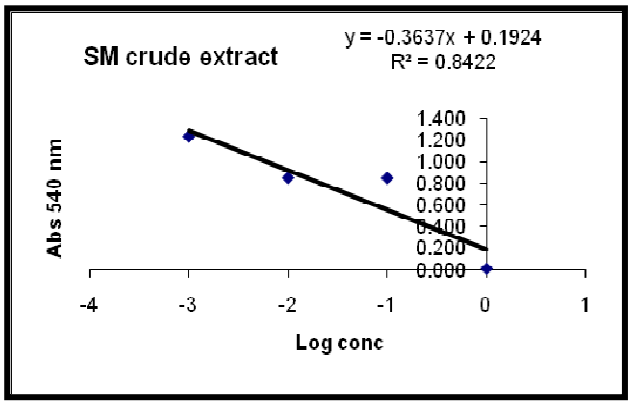

Figure 9-9 Cytotoxicity of crude extract with LC_{50} = 82 µg/ml against Vero cells

9.3.2.1 Therapeutic index of the crude extract and isolated compounds

The therapeutic index for the four antifungal compounds was calculated using the cytotoxic concentrations of the compounds.

The therapeutic index for each fungus was calculated as follows:

Therapeutic index (TI) = LC_{50} against Vero cells in mg/ml divided by the MIC in mg/ml

Table 9-3 shows the therapeutic index of four isolated compounds against different plant pathogens. Amongst the four compounds, the highest therapeutic index was observed in C3 with TI = 185 against *A. parasiticus* and the lowest was found in ursolic acid with 0.2 against *Penicillium* species and *T. harzianum*. The higher the therapeutic index the better the compounds can be considered for use in drug discovery.

Table 9-2 Cellular toxicity and minimum inhibitory concentration of crude extract and four isolated compounds against seven plant pathogenic fungi.

Micro-organisms	Time (hrs)	MIC (mg/ml)	MIC (µg/ml)				
		Crude Extract	Ursolic acid	2	3	4	AmpB
Aspergillus parasiticus	24	0.63 (0)	20(0)	20(0)	10(0)	30(0)	6.4
Aspergillus niger	48	2.50 (0)	120(0)	120(0)	120(0)	120(0)	1.6
Colletotrichum gloeosporioides	48	1.25(0)	30(0)	60(0)	20(0)	60(0)	3.2
Fusarium oxysporum	48	0.32 (0)	50(0)	50(0)	50(0)	50(0)	3.2
Penicillium expansum	48	1.25 (0)	125(0)	32(0)	32(0)	125(0)	3.2
Penicillium janthinellum	48	0.08 (0)	125(0)	16(0)	25(0)	16(0)	3.2
Trichoderma harzianum	48	0.63 (0)	125(0)	250(0)	125(0)	250(0)	3.2
Cytotoxicity (µg/ml)			25	525	1849	35	

Table 9-3 The Therapeutic Index (TI) of four isolated compounds against seven plant pathogenic fungi.

Plant pathogens				
	ursolic acid	2	3	4
Aspergillus parasiticus	1.25	26.3	185	1.2
Aspergillus niger	0.21	4.4	15.4	0.3
Colletotrichum gloeosporioides	0.83	8.8	93	0.6
Fusarium oxysporum	0.5	10.5	37	0.7
Penicillium expansum	0.2	16.4	15	0.29
Penicillium janthinellum	0.2	33.0	116	2.3
Trichoderma harzianum	0.2	4.2	7.4	0.29

9.4 Conclusion

Various compounds are present in crude extracts and this may be the reason why the MIC value was lower (0.08 mg/ml) against *P. janthinellum* than the isolated compounds. Ursolic acid had good antifungal activity against *A. parasiticus*, *C. gloeosporioides* and *F. oxysporum*. Compounds 2 and 4 had good antifungal activity against *P. janthinellum* (MIC 16 µg/ml) while compound 3 inhibited the fungus at the lowest concentration of 10 µg/ml. The inactive constituents were removed during isolation, and as a result the MIC values for all four compounds are lower compared to the crude extract as expected. The initial crude extract loaded on the first column was 2 g (20%) and ursolic acid yielded 6% followed by C2 (3.5%), C3 (1%) and C4 (0.7%). The four compounds may act additively or synergistically as the activity of the individual compounds was not as high as expected.

Amongst the four isolated compounds, only three (1, 2 and 3) had antifungal activity against the tested microorganisms. Moreover, compound 1 was most active compared to the other compounds against the plant fungal pathogens and also against the bacteria. In bioautography assay, the crude extract and compound 1 showed an active compound at the same R_f value against *A. parasiticus*, *T. harzianum* and *P. janthinellum*. This indicates that compound 1 was not an artefact of the isolation procedure.

In the cytotoxicity assay three compounds were very toxic at the concentration tested and the crude extract was less toxic than the isolated compounds. Of the four compounds tested, the highest therapeutic index was observed in C3 with 185 against *A. parasiticus* and the lowest was found in ursolic acid with a ratio of 0.2 against *Penicillium* species and *T. harzianum*. The higher the therapeutic index the safer the compounds can be considered to be in drug discovery.

From the efficacy and safety of the three unidentified compounds it may mean that the crude extract could have higher potential than the isolated compounds. It is a pity that the structure of compound 3 was not able to be elucidated because this compound had good activity and a low toxicity. In the next chapter the *in vivo* efficacy of a crude acetone extract containing a mixture of the isolated compounds and ursolic acid will be tested on fungi infecting oranges.