Chapter 6

Preliminary isolation study

6.1 Introduction

After selecting the best extractant for the extraction process, the next step was to carry out a preliminary separation procedure to simplify the complex crude extract and then to verify the antibacterial activity of the fractions before large-scale isolation. In this chapter, investigation of the activity of the root bark and leaf extracts is described, as both these extracts may be a source of bioactive compounds. Bioautography remains a useful technique to reveal compounds with antibacterial activity, and purification of the extracts and possible subsequent concentration of active compounds may reveal more plant constituents that inhibit bacterial growth.

A plant extract contains many compounds that may be seen in visible light or under ultraviolet conditions. Chromogenic spray reagents are also useful to show different types of compounds and to gain an indication of which types of compounds are present in the plant extract.

6.2 Methods

6.2.1 Extraction and preliminary column chromatography

Ten grams of dried, ground leaf material were weighed and placed in a polyethylene tube containing 100 ml acetone. The tube and contents were shaken for ten minutes. The extract was filtered using Whatman No.1 filter paper. This process was repeated three times on the marc and in each case the supernatant was filtered and combined. The extract (1.6 g) was dried and mixed with 4 g silica gel as stationary phase. The silica gel column (2.2 cm by 30 cm) was eluted with 100 ml of each of the following solvents: 100% hexane, 75:25 (hexane:acetone), 50:50 (hexane:acetone), 25:75 (hexane:acetone), 100% acetone and 90:10 (acetone:methanol). The fractions were analyzed using TLC and similar fractions were combined.

6.2.2 Solvent/solvent fractionation of root bark

This procedure simplifies extracts by fractionating the components based on polarity. The solvent/solvent group procedure used by the US National Cancer Institute as described by Suffness and Douros (1979) was applied. After extracting the root bark with acetone, the extract was dried in a Buchi RE-120 rotary evaporator under reduced pressure. This extract was dissolved in a 1:1 mixture of chloroform and water and the two phases were separated in a separatory funnel. Fig 6.1 below is a flow chart of the process used for solvent/solvent fractionation of the root bark.

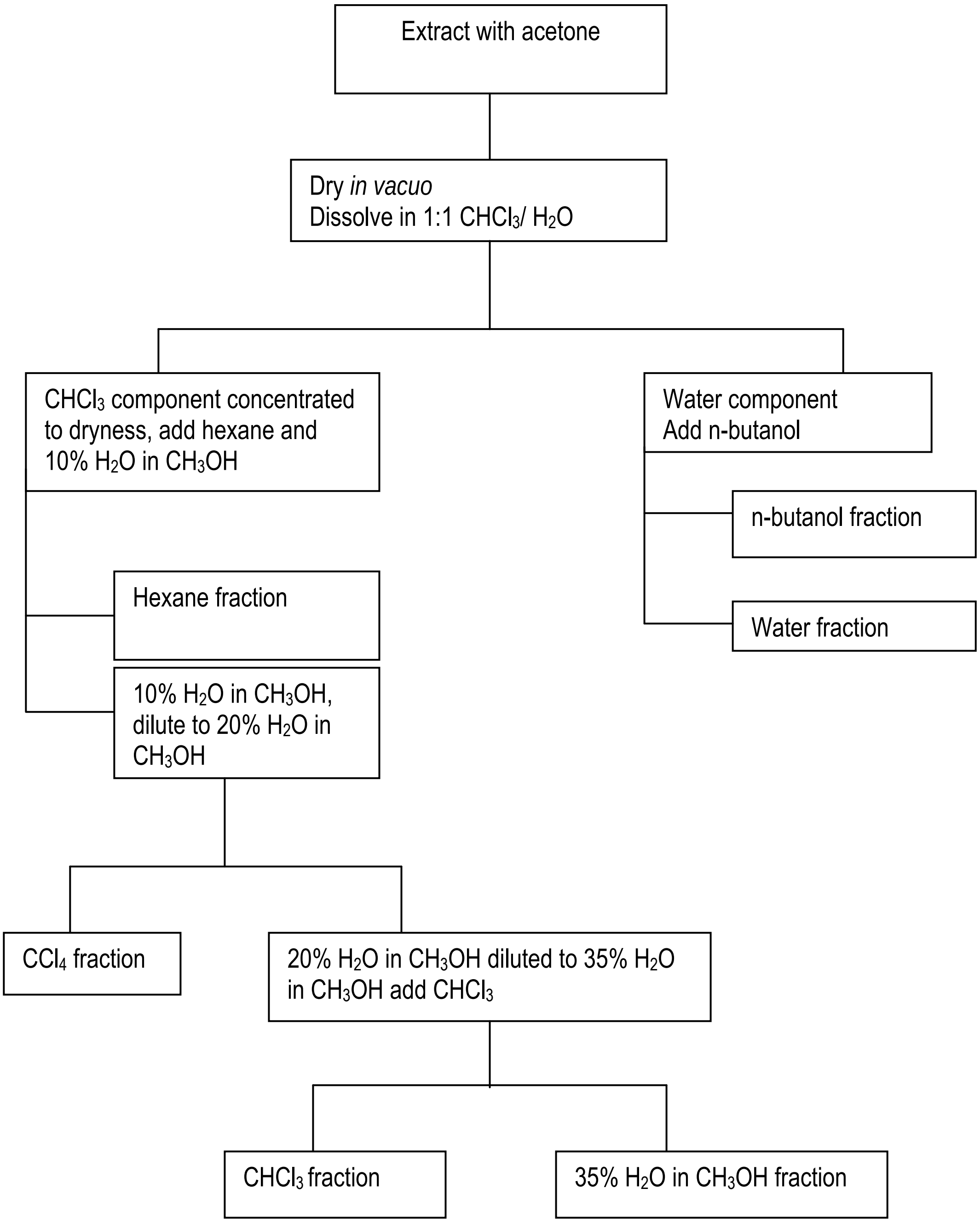

Fig 6.1. Flow chart of solvent/solvent fractionation of root bark

The water fraction was mixed with an equal volume of n-butanol in a separatory funnel to yield the water and butanol fractions. The chloroform fraction was dried in a vacuum rotary evaporator and extracted with an equal volume of hexane and 10% water:methanol mixture. This yielded the hexane fraction, and the 10% water:methanol mixture was diluted to 20% water:methanol by addition of water. This was then mixed with carbon tetrachloride in a separatory funnel giving the carbon tetrachloride fraction. The 20% water:methanol fraction was further diluted to 35% water:methanol and mixed with chloroform to yield the chloroform and water:methanol fractions. In all cases, equal volumes of solvents were used and separation repeated with small quantities of solvent to facilitate separation.

6.2.3 Bioactivity testing

All the resulting fractions from the separation of the leaf and root bark extracts were tested for antibacterial and antifungal activity (sections 3.10.1 and 3.11) as well as bioautography (section 3.10.3).

6.3 Results and Discussion

The column chromatography of the acetone extract of the leaves resulted in a reasonable separation of plant components. Fig 6.2 is a chromatogram of combined similar fractions of the leaf acetone extract separated by column chromatography. The solvent system used to develop the plate was CEF, and vanillin-sulphuric acid was used as the detection agent.

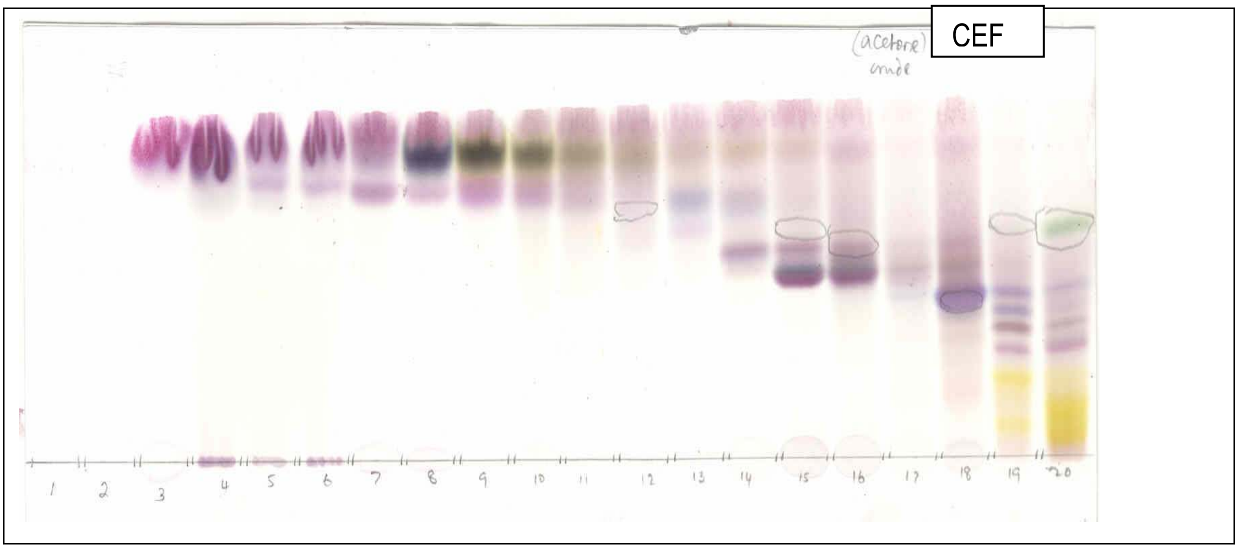

Fig 6.2. Chromatogram of combined fractions after spraying with vanillin/sulphuric acid (leaf acetone extract)

Bioautography was done with the combined fractions and compounds with bacterial growth inhibition were observed as clear zones against the purple background. Figures 6.3, 6.4, and 6.5 show bioautography using the test organisms *S. aureus, P. aeruginosa* and *E. coli* respectively.

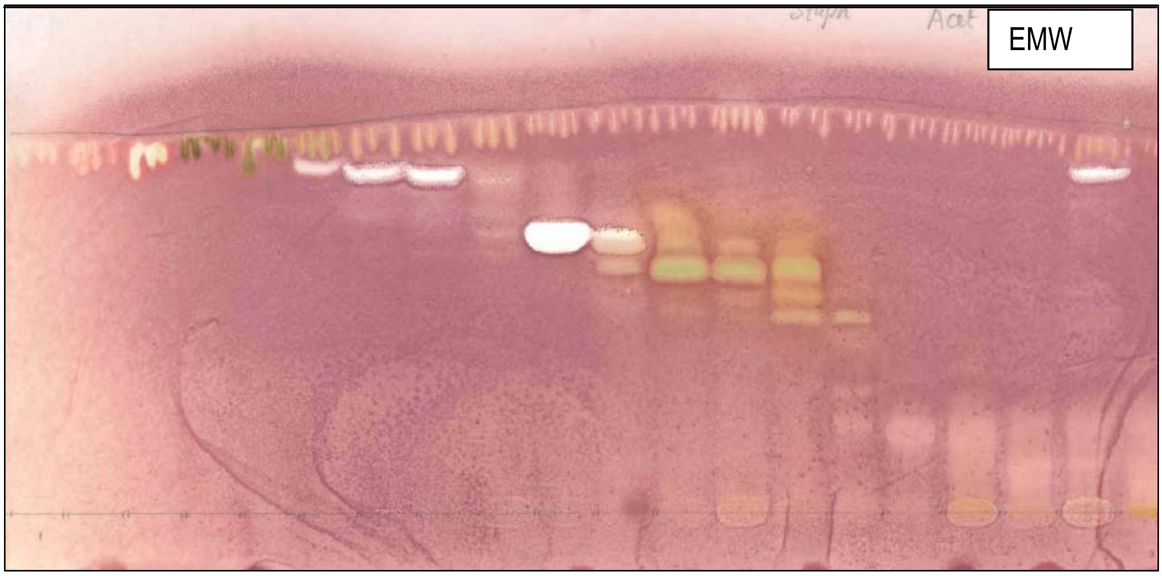

Fig 6.3. Bioautography of acetone fractions against *S. aureus* after column chromatography

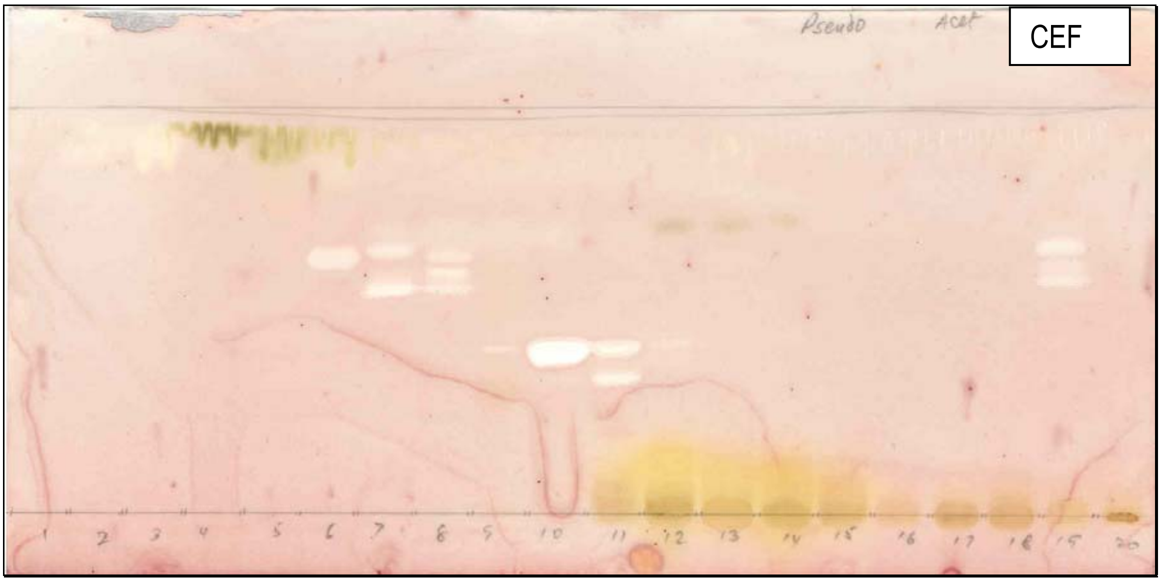

Fig 6.4. Bioautography of acetone fractions against *P. aeruginosa* after column chromatography

The acetone fraction contained compounds that were active against both Gram-positive and Gram-negative bacteria. The bioautograms confirm that purification of the acetone extract revealed many more compounds with antibacterial activity than the crude extract.

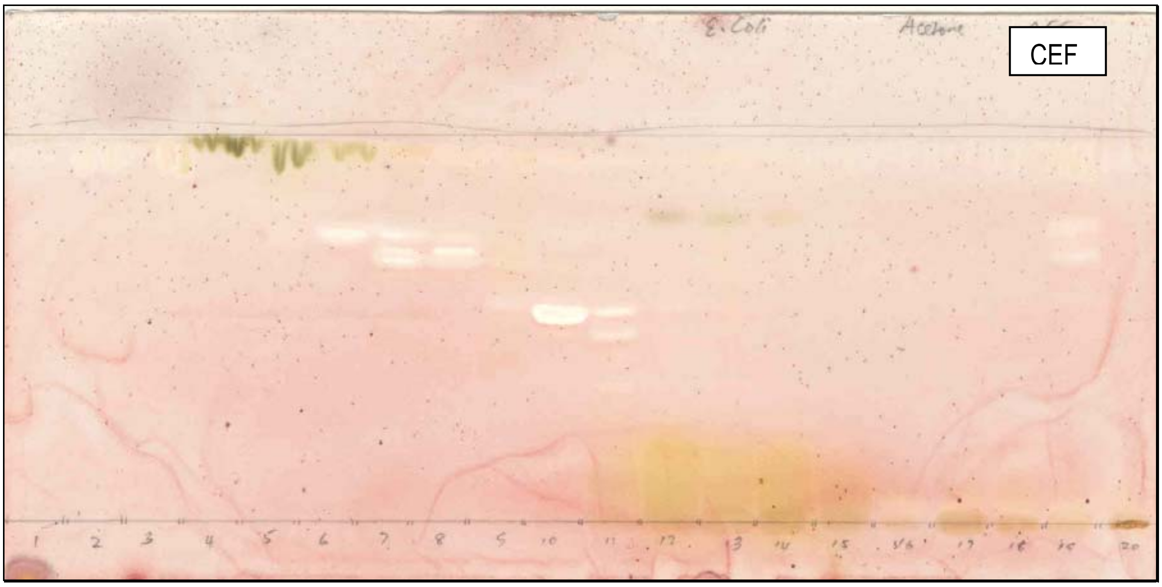

Fig 6.5. Bioautogram of combined fractions of acetone extract fractions against *E. coli* after column chromatography

From the above bioautograms, it will be observed that the compounds that inhibited *P. aeruginosa* and *E. coli* have similar R_f values. The same compounds probably cause bacterial inhibition against the two pathogens.

Fig 6.6 is a chromatogram of the root bark fractions after solvent/solvent fractionation. The chloroform and carbon tetrachloride fractions separated into many more compounds compared to the other extracts. The R_f values of components from the solvent/solvent fractionation of the root bark extract are given in Table 6.1.

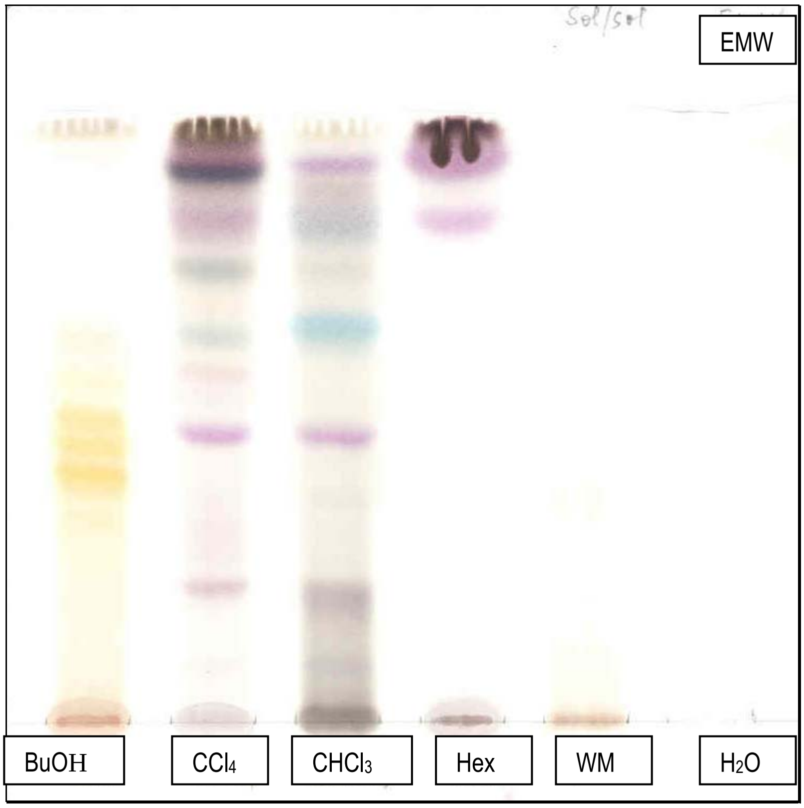

Fig 6.6. Fractions from solvent/solvent fractionation of the root bark after spraying with vanillin/sulphuric acid spray reagent

Table 6.1 R_f values of components from solvent/solvent fractionation of the root bark

Solvents	R_f values								
BuOH	0.47	0.52	0.55						
CCl_4	0.31	0.53		0.68	0.77	0.84	0.90		0.94
$CHCl_3$	0.30	0.52		0.69		0.83		0.92	
Hexane						0.84			

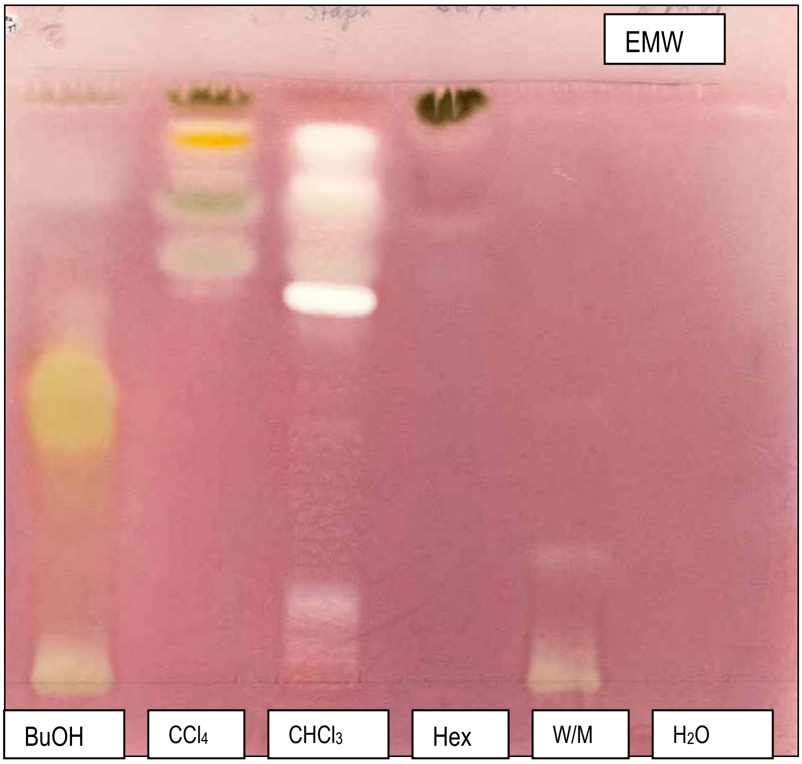

Fig 6.7. Bioautography of solvent/solvent fractions of root bark against *S. aureus*

The root bark was partitioned using six solvents and bioautography was carried out against *S. aureus*. As observed in Fig 6.7, the chloroform fraction had more compounds with antibacterial activity compared to the other fractions. The carbon tetrachloride fraction had a few areas of bacterial inhibition and the rest of the fractions did not show any inhibition of bacterial growth. The R_f values for the chloroform fraction were as follows: 0.14, 0.64, 0.81, and 0.9. For the carbon tetrachloride fraction, the values were as follows: 0.71 and 0.93. There was little activity in the butanol and water:methanol fractions.

The root bark of the plant was extracted using three solvents and the MIC and total activity values of these extracts are presented in Table 6.2. The lowest MIC value was obtained against *E. faecalis* and the highest against *E.coli.* The organisms were equally sensitive to the water and acetone extracts. The same compounds responsible for antibacterial activity are probably present in both the acetone and water extracts.

Table 6.2. MIC (mg/ml) and TA (ml/g) values of root bark extracts against four bacterial strains

Organisms	Solvents					
	Acetone		Ethyl acetate		Water	
	MIC	TA	MIC	TA	MIC	TA
S. aureus	0.63	35.4	1.25	17.8	0.31	72
P. aeruginosa	0.31	72	1.25	17.8	0.63	35.4
E. faecalis	0.31	72	0.63	35.4	0.31	72
E. coli	1.25	17.8	1.25	17.8	0.63	35.4
Average	0.62	49.3	1.09	22.05	0.63	53.7

The total activity of the water extract was generally the highest because water extracted the highest quantity of compounds from the plant material although the MIC was the highest.

The root bark fractions from solvent/solvent partitioning were tested for antifungal activity (Table 6.4 and Fig 6.8). The values obtained were not good compared to values obtained for other *Combretum* and *Terminalia* species (Masoko *et al.*, 2006). In several cases MIC values as low as 0.02 mg/ml were obtained against several fungal pathogens with these extracts, and the leaf extract was more active compared to the root bark as reported by Masoko *et al,* (2006).

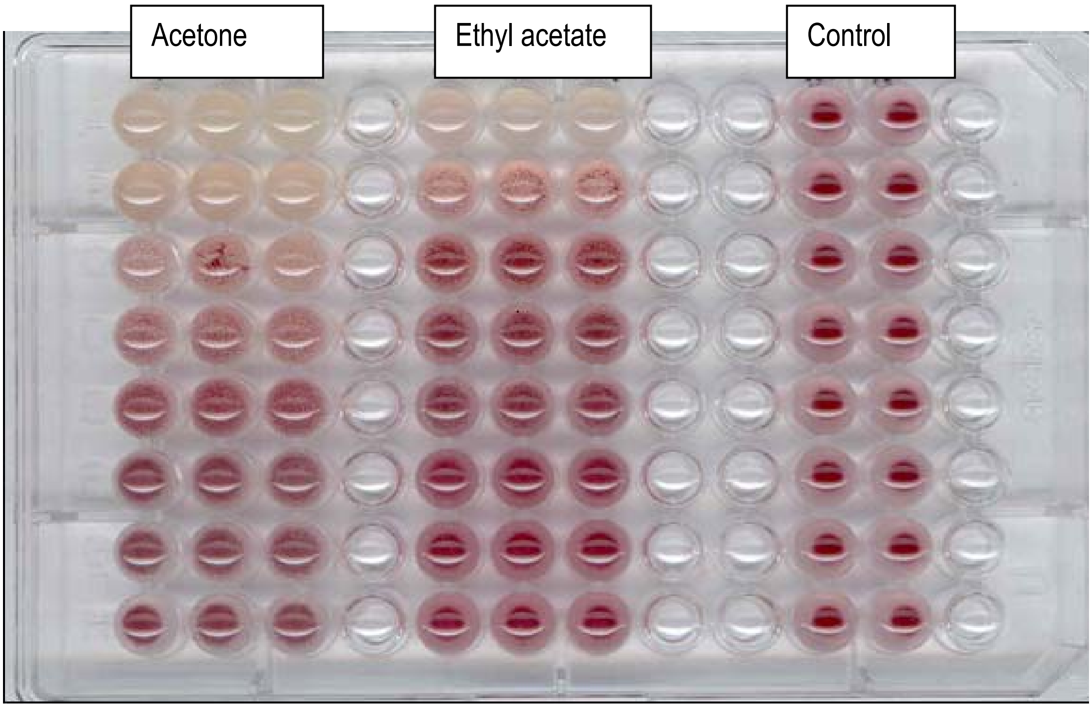

Fig 6.8. Representative microplate of root bark extracts against *Candida albicans*

Table 6.3. MIC (mg/ml) of root bark fractions obtained from solvent/solvent fractionation against *Candida albicans*, *Microsporum canis, Aspergillus fumigatus, Cryptococcus neoformans* and *Sporothrix schenckii.*

Organisms	CCl_4	H_2O (RB)[1]	BuOH	Hexane	W/MeOH	H_2O (Leaf)	$CHCl_3$
C. albicans	0.63	2.5	0.63	0.63	1.25	0.63	0.63
M. canis	0.63	0.63	0.31	0.31	0.16	0.63	0.31
A. fumigatus	2.5	2.5	0.63	2.5	1.25	2.5	2.5
C. neoformans	0.16	2.5	0.63	0.16	0.16	0.16	0.31
S. schenckii	0.16	1.25	0.16	0.63	0.16	0.63	0.16
Average	0.816	1.876	0.472	0.846	0.596	1.198	0.782

[1] RB = root bark

The butanol fraction from the solvent/solvent partitioning had the lowest average MIC value of 0.472 mg/ml while the highest was that of the water extract at 1.198 mg/ml. The lower the MIC value, the more active the extract. The extracts had good activity against *Cryptococcus neoformans* and *Sporothrix schenckii.* The lowest average MIC value was that against *Aspergillus fumigatus* with an average MIC value of 1.87 mg/ml. The MIC of the root bark for antifungal activity ranged from 0.16 to 2.5 mg/ml. The crude acetone leaf extracts of *C. paniculatum* were more active according to Masoko *et al.* (2006), with values as low as 0.02 mg/ml. This may indicate that there were synergistic effects in the crude extracts compared to the fractions generated by solvent/solvent fractionation.

The solvent/solvent fractionation of the root bark produced fractions containing 4 compounds with antibacterial activity (with R_f = 0.14, 0.64, 0.81 and 0.9) for the chloroform fraction, two for the carbon tetrachloride fraction (R_f = 0.71 and 0.91) and one for the water:methanol fraction (R_f = 0.21). The butanol fraction exhibited antibacterial activity at the origin (R_f = 0).

6.4 Conclusion

Initial extraction of the leaves using different solvents proved that acetone extracted more compounds with antibacterial activity. The acetone extract of the leaves was purified using a silica gel column and bioautography of the combined fractions revealed many more compounds with antibacterial activity.

The MIC of the leaf extracts was in the range 0.28 to 0.86 mg/ml while those of the root bark ranged between 0.42 and 1.04 mg/ml. It can be deduced from this that the leaf extract was more active compared to the root bark extract.

The root bark was subjected to solvent/solvent partitioning and the fractions evaluated for antibacterial and antifungal activity. Four antibacterial compounds were found in the chloroform fraction while the carbon tetrachloride fraction had two compounds that exhibited antibacterial activity. It appears from the retention factors that similar bioactive compounds occur in the root bark and leaves.

Antifungal activity of the root bark was disappointing, and the acetone extract of the leaf was more inhibitory to fungal growth. It is easier and more sustainable to use leaves to prepare bulk extracts for isolation of compounds, so the next phase was the large-scale extraction and isolation of bioactive compounds from leaves of *C. paniculatum*.

Chapter 7

Isolation of compounds from *C. paniculatum* leaves

7.1 Introduction

Plants have an almost limitless ability to synthesize aromatic substances, many of which are phenols or their oxygen-substituted derivatives (Geissman, 1963). Most are secondary metabolites, of which at least 12 000 have been isolated, a number estimated to be less than 10% of the total (Schultz, 1988). In many cases, these substances serve as plant defense mechanisms against predation by microorganisms, insects, and herbivores (Cowan, 1999).

The separation of plant constituents is generally performed using one or a combination of chromatographic techniques including column chromatography, thin layer chromatography and high-pressure liquid chromatography. The choice of technique depends largely on the solubility properties and volatilities of the compounds to be separated. The complexity of plant extracts can be simplified using different separation techniques based on size or polarity. For preparative work, column chromatography coupled with an automatic fraction collector is frequently used. This procedure can yield purified components in larger amounts.

7.2 Methods

7.2.1 Extraction and isolation

The air-dried leaf material (1100 g) was pulverized and extracted using 70% acetone in water. The extract was concentrated in a rotary evaporator and placed in a column containing XAD-16 as stationary phase. The column was sequentially eluted with 2 500 ml each of acetone, methanol and water. The fractions were concentrated in a rotary evaporator and analysed using TLC. The acetone fraction contained the green pigment. Sixty grams of the methanol extract was mixed with same amount of silica gel and placed in a column (3.0 by 45 cm) containing XAD-16 as stationary phase.

The dried fraction eluted by 100% methanol was placed onto a silica gel column after concentration and eluted with varying ratios of dichloromethane in methanol beginning with 100% dichloromethane. The column was eluted with 2 000 ml of each of the solvents. Ten fractions were collected and fraction 2 was concentrated and run in a column packed with silica gel and eluted with hexane:ethyl acetate (9:1). This yielded compound 1. The column was further eluted with a 20:1 dichloromethane:methanol (2 000 ml) and 12 fractions were collected. Fraction 15 was dried and separated in another silica gel column and eluted in a 30:1 dichloromethane:methanol mixture (1 000 ml) and compound 2 was obtained.

Further elution of the column with 10:1 dichloromethane:methanol (2 000 ml) gave 12 fractions from which fraction 29 was dried and fractionated in a Sephadex column using 4:1 dichloromethane:methanol (2 000 ml) together with a few drops of acetic acid. Thirty-three fractions were collected and fraction 13 was dried and run in a silica column using 15:1 dichloromethane:methanol (1 000 ml) as eluent. This resulted in the isolation of compound 5. In each case, the fractions were analyzed by TLC and fractions showing a similar profile of compounds were combined.

The objective was to isolate antibacterial compounds, but when pure compounds were found in some fractions, they were also analyzed for possible antiviral activity. A flow chart of the process that led to the isolation of compounds 1, 2, 5, and 6 is presented in Fig 7.1.

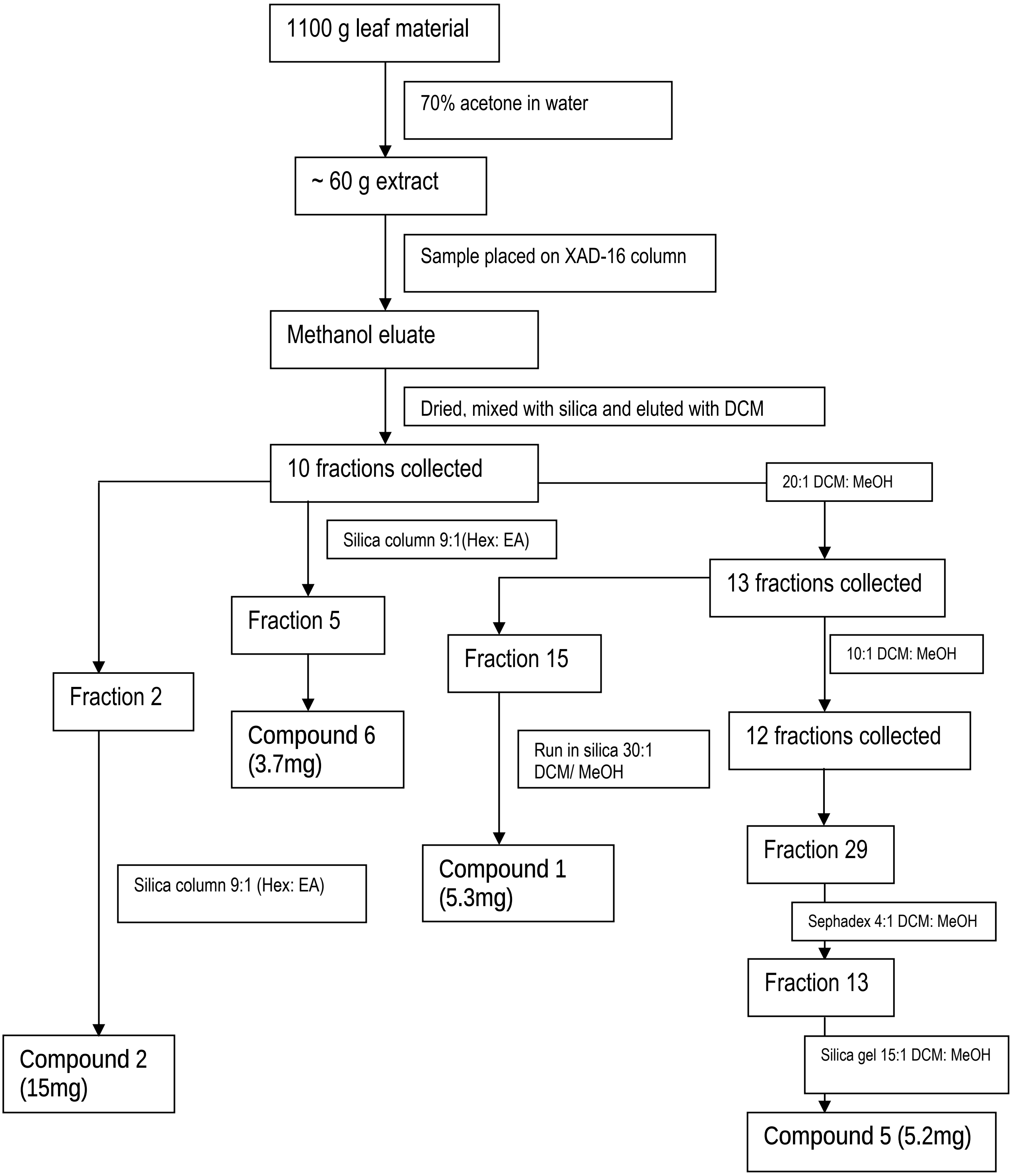

Fig 7.1. Flow chart for isolation of compounds 1, 2, 5 and 6

A portion of the methanol extract (50 g) was partitioned in ethyl acetate (60 ml) and water (60 ml). The ethyl acetate fraction was evaporated under reduced pressure and a mixture of 20 g sample and 20 g silica gel was placed in a silica gel column. The column was eluted with 100% dichloromethane (2 000 ml) and 11 fractions were collected. Elution was continued with a 20:1 DCM:MeOH (2 000 ml) mixture and 13 fractions were collected. Fraction 16 was dried and fractionated in a Sephadex column using methanol (1 000 ml) as eluent, and fractions were collected and separated using TLC. Fractions 9 and 10 were dried, combined and separated in a silica gel column eluting with a 40:1 DCM:MeOH (1 000 ml) mixture. This resulted in the isolation of compound 4. Elution was continued with a 10:1 DCM:MeOH (1 000 ml) mixture. After TLC analysis, fractions 25, 26 and 27 were combined, dried and fractionated by preparative HPLC with a mixture of acetonitrile and water (HPLC grade, 400 ml of each) as eluents. Purification was carried out in a Merck-Hitachi apparatus (Germany). An ODS column (250 X 4.0 mm I.D., 5 µm) was employed at a temperature of 30ºC, flow rate of 1.0 ml/min and wavelength of 254 nm. The fractions were dissolved in methanol and injected into the HPLC column. This resulted in the isolation of compounds 3, 7, 8 and 9. A flow chart of the isolation process resulting in the isolation of compounds 3, 4, 7, 8 and 9 is presented in Fig 7.2.

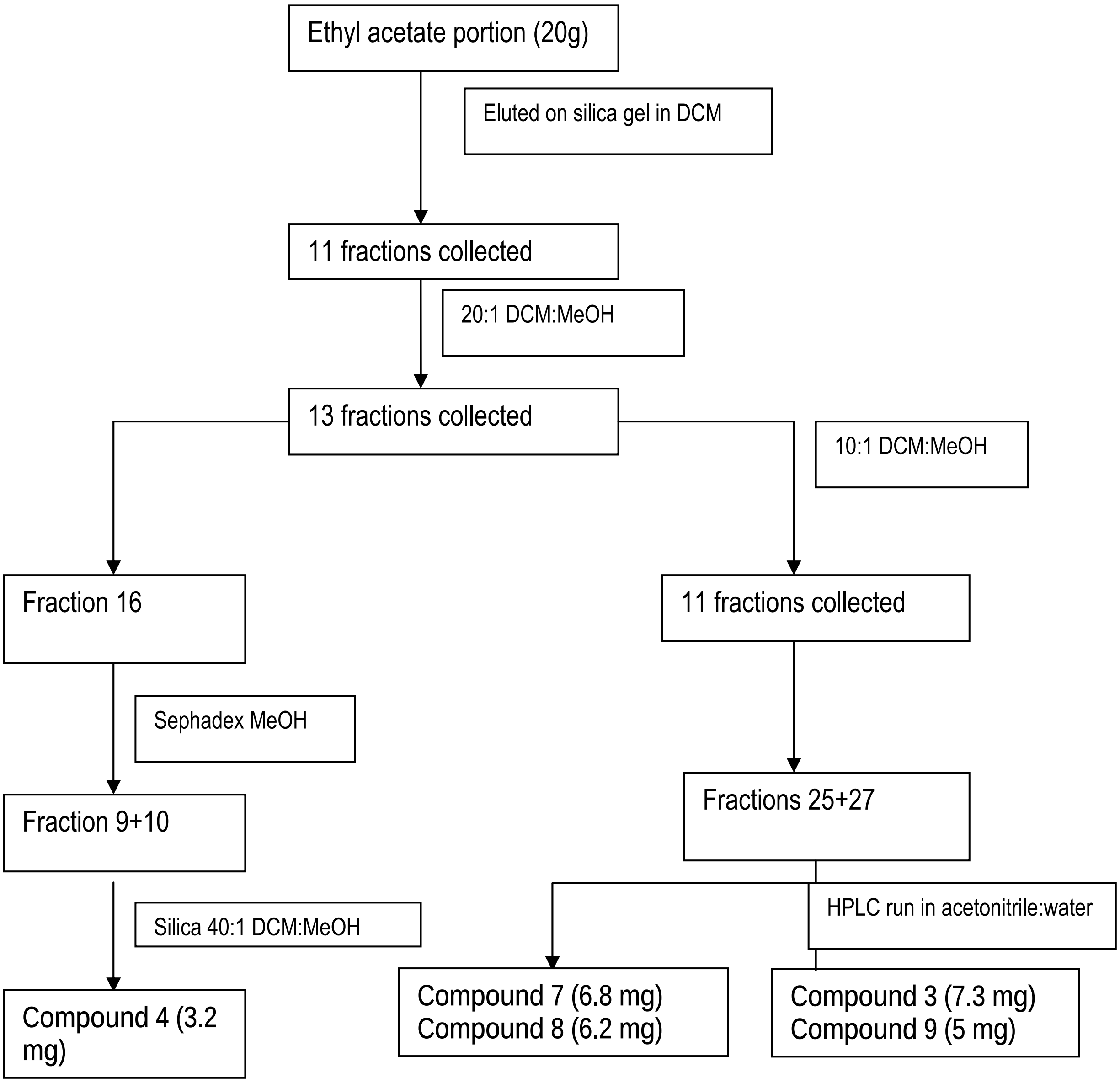

Fig 7.2. Flow chart of isolation of compounds 3, 4, 7, 8 and 9

7.3 Results and Discussion

Initial extraction using different solvents proved that acetone extracted the most components with antibacterial activity. Bioautography supported the conclusion and therefore acetone was used to extract the bulk sample for the isolation process. Water was mixed with acetone (30:70) because in the extraction process water extracted the largest quantity. A combination of acetone and water was therefore used to maximize the number of antibacterial compounds and amount of material extracted from the plant.

The compounds were isolated via initial antibacterial bioassay-guided extraction. Compounds that crystallized after column chromatography were also collected. The isolation process was continued without determining whether other fractions collected thereafter were active or not. This was because the facilities for bioassay-guided isolation were not available at that stage of the project. In bioassay-guided fractionation, fractions are subjected to bioautography or other bioassay in order to target compounds that exhibited antibacterial activity. At the HKI, where this portion of the research was performed, biological assays are carried out in another facility by other staff and sometimes one had to wait for a week to get results. This would have slowed down the isolation process and it was therefore decided to aim to isolate as many compounds as possible.

Nine compounds were isolated using column chromatography and HPLC. R_f values for some of the compounds are shown in Table 7.1. Compounds 3, 7 and 8 did not migrate from the bottom of the TLC plate.

Table 7.1. R_f values of the isolated compounds

Compounds	R_f values	Solvent system used for elution
1	0.26	BEA
2	0.43	Hexane:chloroform (8:2)
3	0.46	EMW
4	0.62	CEF
5	0.75	CEF
6	0.65	Hexane:DCM (8:2)
7	0	EMW
8	0	EMW
9	0	EMW

Compounds 7, 8 and 9 were polar and were isolated by the use of HPLC apparatus with water and acetonitrile as eluent. The R_f values of the isolated compounds were not compared to those occurring in the crude extract because they were developed in different solvent systems.

7.4 Conclusion

Using column chromatography and HPLC, nine compounds were isolated from the acetone:water extract of *C. paniculatum* leaves. All the compounds reacted with *p*-anisaldehyde-sulphuric acid spray reagent. Many other compounds were present but the quantity of fractions was too small to enable characterization of these compounds.