CHAPTER 5

PRELIMINARY SEPARATION AND ISOLATION OF BIOACTIVE COMPOUNDS

5.1 Introduction

Separation techniques leading to the isolation of bioactive compounds are important in the natural product isolation process. The complexity of a plant extract can be simplified through different separation techniques. In this chapter the use of solvent-solvent fractionation and vacuum liquid chromatography (VLC) processes as important techniques in the preliminary separation and isolation of natural product compounds were compared. Separation and isolation of the bioactive compounds was performed according to **Fig. 5-1.**

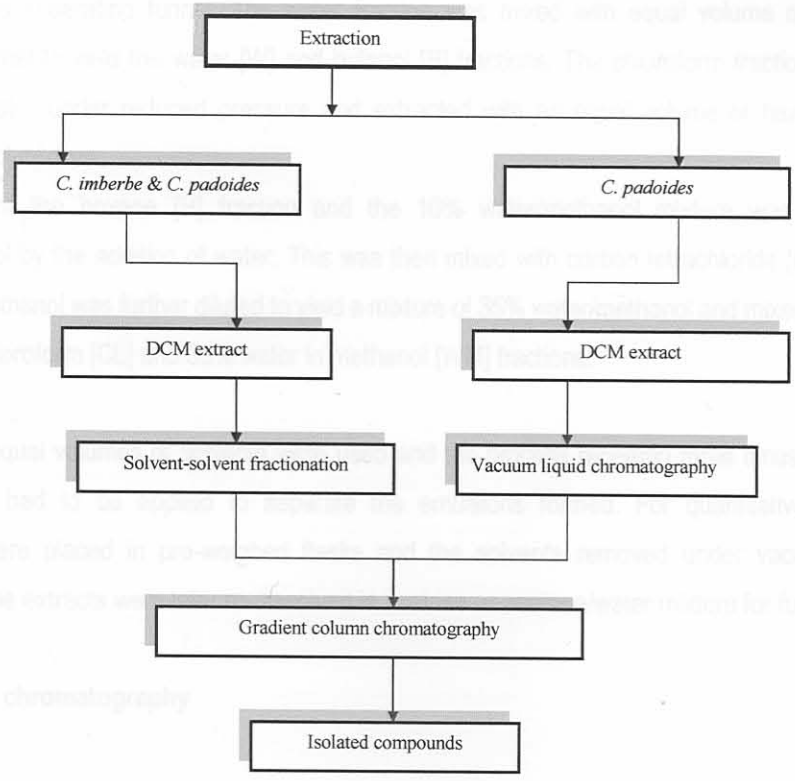

Figure 5-1: Diagrammatic summary of the isolation routes

In order to determine the fraction(s) containing antibacterial compounds to quantify the activity of the solvent-solvent and VLC fractions, bio-autography and the MIC of the fractions were determined. The bioautography method was based on Begue and Kline (1972) and the MIC method was in accordance with Eloff, (1998) as described in sections **4.2.2** (page 51) and **4.2.3** (page 52).

5.2 Materials and methods

5.2.1 Solvent-solvent fractionation

The purpose of solvent-solvent partitioning is to simplify extracts by fractionating the chemical compounds into broad groups based on their solubility (Suffness and Dous, 1979). The procedure is summarised in **Fig. 5.2.** Three g of *C. imberbe* and 10 g *C. padoides* DCM extracts obtained from serial exhaustive extraction were used. These extracts were dissolved in 1:1 mixture of chloroform and water and the two phases were separated in a separating funnel. The water fraction was mixed with equal volume of n-butanol in a separating funnel to yield the water [W] and butanol [B] fractions. The chloroform fraction was dried in a rotary evaporator under reduced pressure and extracted with an equal volume of hexane and a 10% water/methanol mixture.

The yielded of the hexane [H] fraction and the 10% water/methanol mixture was diluted to 20% water/methanol by the addition of water. This was then mixed with carbon tetrachloride [CT] fraction. The 20% water/methanol was further diluted to yield a mixture of 35% water/methanol and mixed with chloroform to yield the chloroform [CL] and 35% water in methanol [WM] fractions.

In all cases, equal volumes of solvents were used and the process repeated three times. In some cases centrifugation had to be applied to separate the emulsions formed. For quantitative determination, extractants were placed in pre-weighed flasks and the solvents removed under vacuum in a rotary evaporator. The extracts were later re-dissolved in acetone or acetone/water mixture for further analysis.

5.2.2 Column chromatography

5.2.2.1 Vacuum liquid chromatography

In an attempt to compare solvent-solvent fractionation and vacuum liquid chromatography (VLC) routes in isolating antibacterial compounds, 10 g of the DCM extract of *C. imberbe* was fractionated on a 10 x 30 cm VLC column. The extracts were separated by TLC using 3 solvent systems [hexane:ethylacetate (1:4), (3:2),

(1:1)] to select the best system necessary to start the VLC gradient elution. Hexane:ethylacetate (3:2) indicated the best separation required to start the gradient elution. One thousand grams silica gel 60 was placed into a 10 x 30 cm diameter column in which the mobile phase was sucked through by reduced pressure. A separation funnel was connected to the column via a T-piece adaptor, with the side arm connected to the vacuum line; fractions were collected as the column was developed. Eluents were applied in a stepwise gradient (**Table 5-1**).

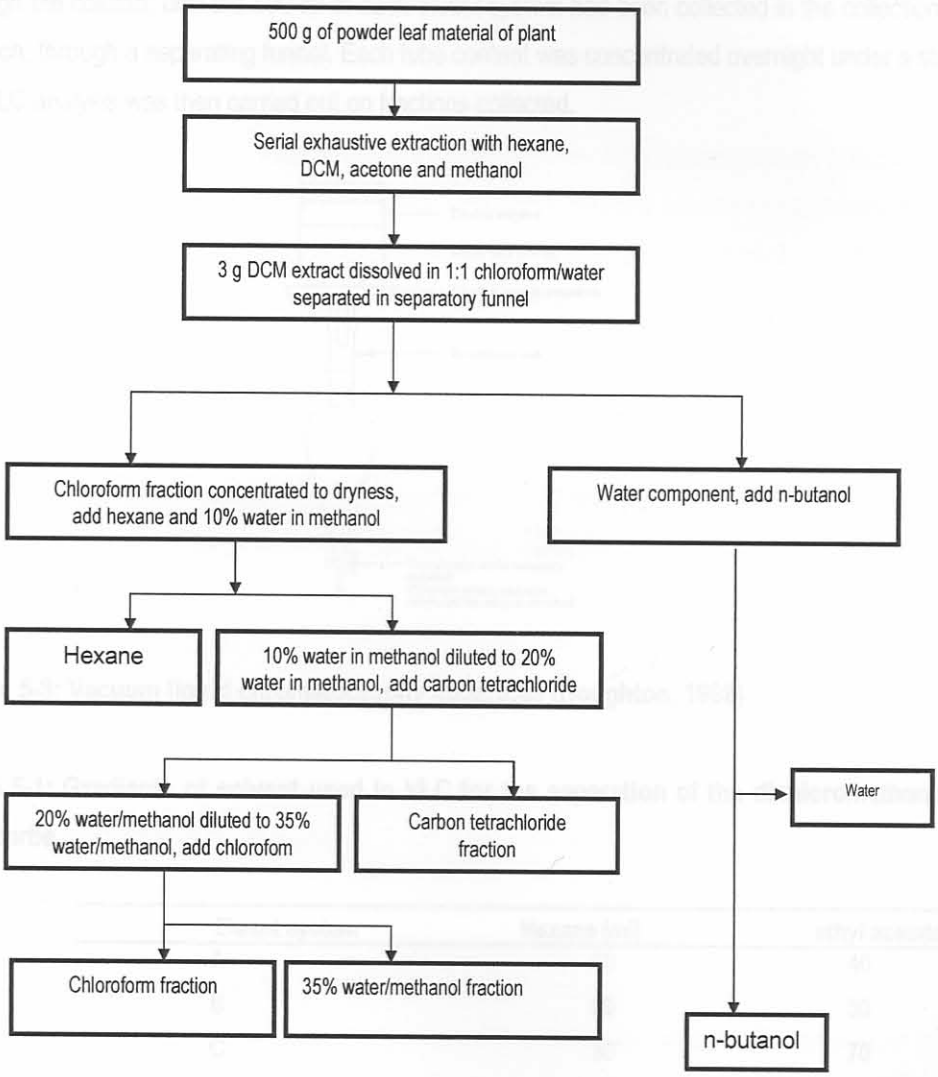

Figure 5-2: The protocol used for the solvent-solvent fractionation of the components in the DCM extract of *C. imberbe* and *C. padoides*.

The dry method for packing of chromatographic columns was used. Approximately 1000 g of silica gel 60 was poured slowly into the column, on top of a small amount of cotton wool. Silica gel (5 g) was mixed with 10 g of dichloromethane extract (dissolved in a small amount of DCM) and allowed to dry. The dry sample was then placed neatly on top of the silica in the column. Filter paper was neatly placed on top of the sample to prevent disturbance at the surface during solvent introduction. With the stepwise addition of each eluent system (500 ml) onto the column, the vacuum was switched on. The solvent was allowed to run through the column; until the 500 ml of each eluent system had been collected in the collection tubes of 50 ml each, through a separating funnel. Each tube content was concentrated overnight under a stream of cold air. TLC analysis was then carried out on fractions collected.

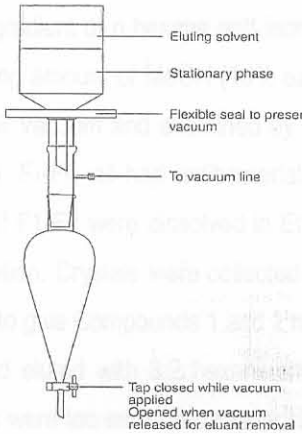

Figure 5-3: Vacuum liquid chromatography apparatus (Houghton, 1998)

Table 5-1: Gradients of solvent used in VLC for the separation of the dichloromethane extract of *C.imberbe*

Eluent system	Hexane (ml)	ethyl acetate (ml)
A	60	40
B	50	50
C	30	70
D	10	90
E	0	100
	Ethyl acetate (ml)	**methanol (ml)**
F	90	10
G	80	20
H	60	40

The solvents used were hexane, ethyl acetate and methanol. The gradient began with 3:2 (hexane: ethyl acetate)

5.2.2.2 Gravity gradient column chromatography

The DCM extract of *C. imberbe* (16.5 g), having the highest antibacterial activity was subjected to liquid-liquid fractionation process that afforded six fractions [chloroform (6.5 g), water (0.125 g), n-butanol (0.476 g), hexane (0.8), CCl_2 (1.356 g), 35% H_2O in MeOH fractions].

The high activity antibacterial fraction [chloroform (6.5 g)] was chromatographed on a 2x30 cm silica gel 60 open column using a stepwise gradient of n-hexane and increasing amount of ethyl acetate (EtOAc) (20% each step); EtOAc with increasing amount of MeOH (10% each step); and finally at 40% MeOH. Collected fractions were evaporated under vacuum and examined by TLC. Homogeneous fractions were pooled to give 12 major fractions (F_1-F_{12}). F_1-F_{12} all had antibacterial activity according to bioautography (Section **5.3.3**). The dried components of F1-F2 were dissolved in EtOAc, allowed to evaporate under a stream of cool air to encourage crystallisation. Crystals were collected from F_2 and F_6 and washed with hexane and 1:1 hexane: chloroform mixture to give Compounds **1** and **2** respectively. F_1 (0.78 g) was separated on a 1x 20 cm silica gel 60 column and eluted with 3:2 hexane:chloroform to yield 7 mg of Compound **3.** The quantities of the others fractions were too small to consider chromatographic processing.

The DCM (3 g) extract that was the most active extract from *C. padoides* according to bioautography (8 antibacterial compounds) and MIC (0.08 mg/ml), was subjected to liquid-liquid fractionation process that afforded six fractions chloroform (1.69 g), water (0.28 g), n-butanol (0.78 g), hexane (0.321 g), CCl_4 (0.283 g), 35% H_2O in MeOH (1.25 g) fractions.

The high activity antibacterial fraction (chloroform 1.69 g) (**Table 5-2**) according to bioautography (8 antibacterial compounds) and MIC (0.037 mg/ml) results was chromatographed on a 2x30 cm silica gel 60 open column using a stepwise gradient of n-hexane and increasing amount of ethyl acetate (EtOAc) (20% each step), with increasing amount of MeOH (10% each step) and ending up at 50% MeOH. Collected fractions were evaporated under vacuum or cold air stream and examined by TLC. Homogeneous fractions were pooled to give 13 major fractions (A-K). Re-grouped fractions resulted in seven more distinct fractions (F_1-F_7) and were all found to have antibacterial activity.

Crystals were observed in tube containing fraction F4 and were carefully washed with hexane and acetone to yield 7 mg of Compound **8** with R_f value of 0.5 in EMW at room temperature. Precipitated sediment was filtered out in large quantity from fraction F6 and washed with 100% hexane and a combination of hexane and chloroform with 10% increase amount of chloroform at each wash. The result of this gave 53 mg of Compound **7** with R_f value of 0.3 in EMW and appeared to be the major antibacterial compound in the extract of *C. padoides*.

5.2.2.3 Sephadex LH-20

One of the commonest problems in phytochemical and biochemical research is to separate the many components, frequently macromolecules, in the plant cell extracts. Methods for separating the components of a mixture exploit differences in size, electrical charge and solubility in different solvents of the molecules in the extract. Two examples are; electrophoresis, which separates such macromolecules as proteins and DNA by their charge (and sometimes size as well), and gel filtration using e.g., Sephadex LH-20 that separates mainly by virtue of size. The porosity of the gel can be selected to exclude all molecules above a certain size. Known as size exclusion chromatography, Sephadex and Sepharose are trade names for gels that are available commercially in a broad range of porosities. In this work, Sephadex LH-20 was used for the separation of very polar fractions of the extracts. A column was filled with semi-solid beads of a Sephadex gel that admits ions and small molecules into their interior but not large ones. When a mixture of molecules dissolved in a solvent is applied to the top of the column, the smaller molecules are retarded more than larger molecules. Consequently, the large molecules move more rapidly through the column, and in this way the mixture components can be separated.

Fractions F_9-F_{12} were combined to give (F_{13}) that was dried in a rotary evaporator to yield 3.2 g of the combined fractions. The combined fraction F_{13} was filtered and then applied to the Sephadex L-H20 (3x120) cm column and eluted with methanol as eluent to yield six sub-fractions $F_{13.1}$-$F_{13.6}$. Sub-fractions $F_{13.4}$ and $F_{13.5}$ were combined (0.3 g) and subjected to a silica gel 60 2x30 cm closed column and eluted with a mixture of chloroform and methanol (9:1, 1200 ml) to yield pure Compounds **4** and **5**. From *C. padoides*, 150 mg of fraction F7 was further chromatographed on a 3 x 120 cm Sephadex LH-20 and eluted with methanol. This yielded 5 mg of Compound **6** with R_f value of 0.22 in EMW. This appeared to be a minor compound as indicated by its low concentration on the TLC chromatogram compared to other compounds (**Fig. 5-17**).

5.2.3 Conventional Preparative TLC

Preparative TLC was used to purify limited quantities of (< 50 mg) semi-pure fractions of *C. imberbe* with about 2 or 3 compounds on preparative TLC. Preparative TLC is one of the simplest and cheapest methods available for the isolation of a component or compounds from a mixture, only small amounts can be obtained from each fractionation procedure. Fraction A (30 mg) of *C. imberbe* resulting from the gravity based chromatographic column (F2) was applied in a form of a band on a TLC plate. The plates used in this method were 0.5-1 mm thick (analytically TLC uses plates of 0.25 mm thickness). This allowed a greater amount of sample to be loaded on to the plate. The plates were developed in a solvent (EMW) system known to separate the components. A Non-destructive detection method was used in detecting the separated compounds. The most common method of visualizing developed chromatograms was the use UV light, to detect all quenching compounds. However, there are a large number of naturally occurring compounds that do not fluoresce or quench, so other detection methods were used. A simple method for non-water-soluble compounds is to spray the layer with a fine mist of water so that it was wet enough to become transparent. Non-water-soluble compounds appeared as dark areas with transmitted light and as lighter areas when the plate is viewed in reflected light. Chromatograms were also sprayed with vanillin sulphuric acid to view vanillin active compounds. At least 90% of the plate was covered while the exposed part was sprayed. The active fraction was scraped off the TLC plates and eluted from the silica with ethanol. The active compound in ethanol was filtered through Millipore filters (0.45 µm and 0.22 µm) to remove the silica and this yielded more of Compound **2**.

5.2.4 Analysis and Grouping of fractions

Following vacuum liquid chromatography (VLC) or gravity-based chromatography, test tubes were placed under a stream of air to facilitate concentration of the fractions for TLC analysis and bioassays. After about 50% of the volume of the eluent had been evaporated, 5 ml was collected from each test tube into a pre-weighed pill vial and allowed under a stream of air to dry rapidly. The mass of each was measured and the concentration (10 mg/ml) determined. Fractions were analyzed by TLC as described in Section **3.2.4**
For the isolation work done at Hans-Knoll institute (HKI), fractions (5 ml each) collected from the fraction collector machine were analyzed directly by TLC without further concentration. The solvent systems used in analysis were, chloroform: methanol (C:M) 9:1, and (C:M) 20:1. Combined fractions were concentrated immediately using a rotary evaporator at 40^{0}C

5.2.4.1 Combination of fractions

From TLC results, fractions were combined according to the similarity of their chemical profile. Combined fractions (A, B, C, D, E, F and G) were placed under air current a slowly blowing fan to facilitate drying and crystallization. Once dry, the fractions were weighed to calculate the total mass fractionated and the crystallized fractions were washed with a combination of solvents to obtain pure compounds. Active fractions, Sections 5.2.1 and 4.2.2 were further chromatographed 3 x 35 cm silica gel column to obtain the active compounds.

5.2.5 Dereplication

A system was established to identify isolated compounds from the crude extract. The dereplication method relies on the R_f value, UV active and vanillin sulphuric acid active colours and the activity of the compounds as indicated on the bioautogram. These parameters of the pure compounds were compared with that of crude extract to confirm the identity of the isolated compounds.

5.3 Results and discussions

5.3.1 Solvent-solvent fractionation of the DCM extracts.

The main aim of the solvent - solvent fractionation process was to determine the extent to which the extracts can be fractionated without losing antibacterial activity. Different solvents of varying polarities were used to simplify the complex DCM extracts *of C. imberbe* and *C. padoides* hence facilitating the isolation of antibacterial compounds. The quantity in each solvent-solvent fraction was calculated after drying off the solvent in a pre-weighed glass flask. The highest percentage of the DCM extract from *C. imberbe* was in the chloroform (65.5%) and carbon tetrachloride fractions (13.6%). Water and 35% water in methanol fractions yielded the lowest percentages (1.33% and 1.25%). This result was similar to an earlier study carried out on *C. woodii* in our laboratory (Eloff *et al.*, 2005a). With the *C. padoides* DCM extract, the highest percentage fraction was in hexane (32.15%) and chloroform (28.23%). The lowest mass was in water (2.8%) and n-butanol (7.86%). This result differed from that obtained with *C. imberbe* (**Table. 5-2**). The leaves of *C. imberbe* and *C. padoides* were harvested in November during the high rainfall period that is when the leaves were young, fresh and potentially with a high content of chlorophyll that will most likely appear in the hexane fraction, giving it a higher percentage.

Table 5-2: Quantity (g) and percentage of initial mass (10 g) of *C. imberbe* and *C. padoides* DCM extracts, extracted by different solvents in a solvent-solvent fractionation process

Fractions from DCM	Mass from *C. imberbe*	% of initial mass	Mass from *C. padoides*	% of initial mass
Hexane (H)	0.8	8,05	3.211	32,11
Water (W)	0.125	1,25	0.28	2,8
n-butane (B)	476	4,76	0.786	7,86
35% WM	0.133	1,33	1.256	12,56
CCL_4	1.356	13,56	2.832	28,32
$CHCL_3$	6.551	65,51	1.698	16,98

5.3.1.1 Complexity of fractions

TLC analysis was used to ascertain the complexity of the fractions. The analysis was done in three solvent systems (see Section **3.2.4**) developed in our laboratory for the separation of components of Combretaceae. The hexane fraction had the largest concentration of non- polar compounds, which were very well separated by BEA. The carbon tetrachloride fraction also had many nonpolar compounds. As expected very few non-polar compounds showed up in the chloroform, 35% water in methanol, butanol and water fractions, because these compounds were already separated into hexane and carbon tetrachloride fractions. The 35% water in methanol and chloroform fractions had the most complex mixture of compounds. But the complexity was far less than that of the crude DCM extracts that is made up of a mixture of mostly intermediate polarity compounds. Most of the compounds that appeared in these fractions seemed to be of intermediate polarity separated effectively in EMW and most effectively in CEF (intermediate polarity solvent system). A few compounds were present in the water fraction. It was also observed that the carbon tetrachloride fraction resulting from *C. padoides* had a highly complex mixture of components of intermediate polarity. Generally, the solvent-solvent fractionation process assists in reducing the complexity of a crude extract that makes it easier to isolate the pure compounds using gradient column chromatography. In this study, solvent-solvent fractionation was more successful than the VLC process.

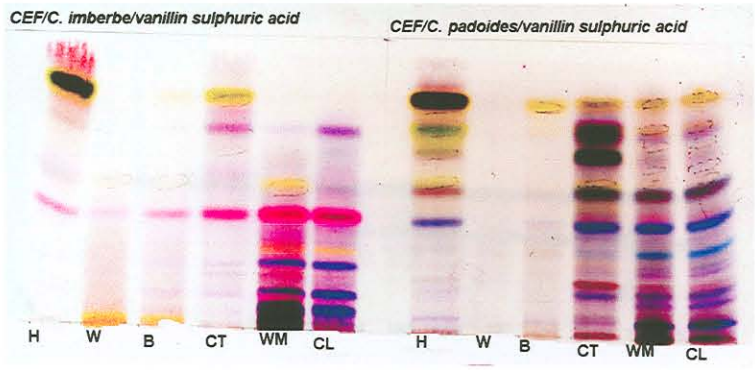

Figure 5-4: Separation of components present in the different fractions obtained by solvent-solvent extraction with CEF and sprayed with vanillin-sulphuric acid. Lanes from left to right: hexane, water n-butanol, carbon tetrachloride, 35% water in methanol and chloroforms fractions. In each case 50 μg of 5 mg/ml stock solution was chromatographed.

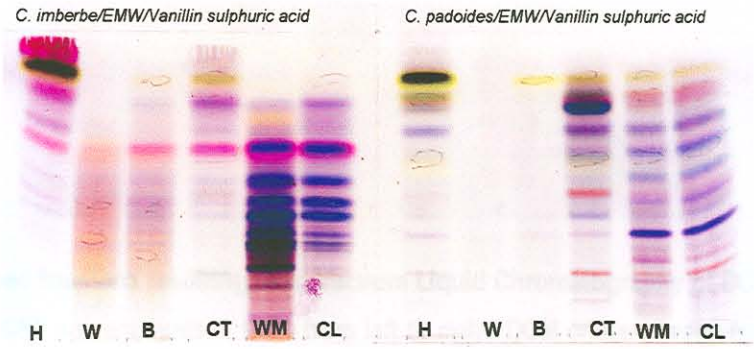

Figure 5-5: Separation of components present in the different fractions obtained by solvent-solvent extraction by EMW and sprayed with vanillin-sulphuric acid. Lanes from left to right: hexane, water, n-butane, carbon tetrachloride, 35% water in methanol and chloroform fractions. In each case 50 μg of 5 mg/ml stock solution was chromatographed.

5.3.2 Vacuum liquid chromatography

Vacuum liquid chromatography was carried out in this study with a similar quantity of DCM extract (10 g) of *C. imberbe* in order to compare the isolation process with that of the solvent-solvent fractionation. This approach was taken only with the *C. imberbe* DCM fraction. In the VLC process, 198 test tubes (50 ml each) were collected and grouped according to their similarity in TLC chemical profile into 7 fractions A, B, C, D, E, F and G **(Fig. 5-7)**

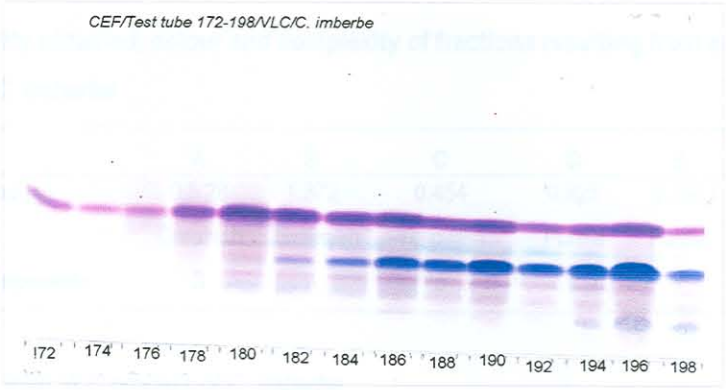

Figure 5-6: Chromatogram ran in CEF indicating number of test tubes collected in the VLC process

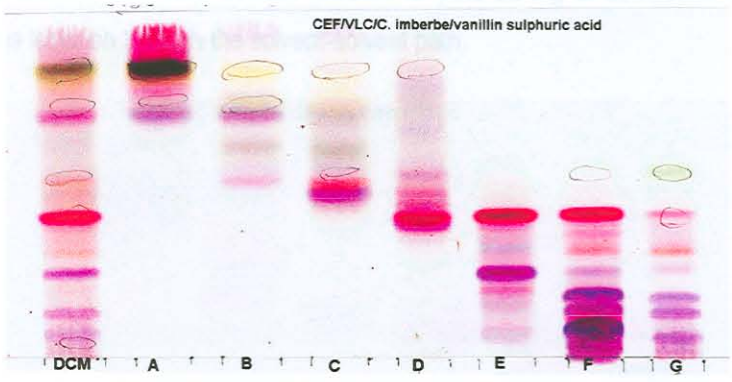

Figure 5-7: Grouped fractions resulting from Vacuum Liquid Chromatography of DCM extract of *C. imberbe* ran with CEF solvent system. Lane from left to right: DCM crude extract, fractions A, B, C, D, E, F and G

To indicate the extent of fractionation in the VLC, TLC of grouped fractions (A-G) was run along with the DCM crude extract, Fig 5-7. Fractions A, B, C and D were consisted mostly of non-polar and intermediate polarity compounds. While fractions E, F and G were made up of intermediate polarity and polar compounds. The quantity fractionated and the complexity of each fraction resulting from the VLC process is indicated in Table 5-3. Fractions E (2.294 g), F (1.564 g) and G (1.777 g) consisted of mostly polar compounds in higher quantities as compared to fractions A (0.528 g), B (1.372), C (0.453 g) and D (0.825 g) which contained less polar compounds in low quantities. Based on the TLC chemical profile of the fractions, the more polar fractions (E-G) were more complex than the less polar fractions A-D.

Table 5-3: Quantity obtained, colour and complexity of fractions resulting from a VLC process of the DCM extract of *C. imberbe*

Fractions	A	B	C	D	E	F	G
Quantity obtained (g)	0.5 28	1.372	0.454	0.825	2.294	1.564	1.777
Colour	Greenish	Greenish	Yellowish	Purple	Purple	Brown	Dark brown
No of visible compounds	3	4	5	8	7	8	8

5.3.2.1 Fractions from VLC process of C. imberbe

Fractions E, F and G were combined (5.635 g) and 2.5 g separated on 2 x 35 cm silica gel 60 under a 20% gradual increase of hexane and ethyl acetate gradient to yield 22 mg of compound **1** which had been isolated in a previous isolation through the solvent-solvent path.

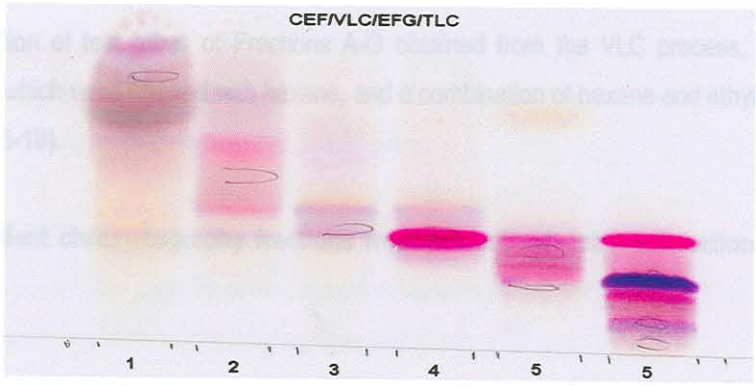

Figure 5-8: Chromatogram from the combined fractions E, F and G ran on CEF solvent system Fractions 1, 1a, 1b, 2, 2a, 3a and 4 were combined to yield 22 mg of Compound 1

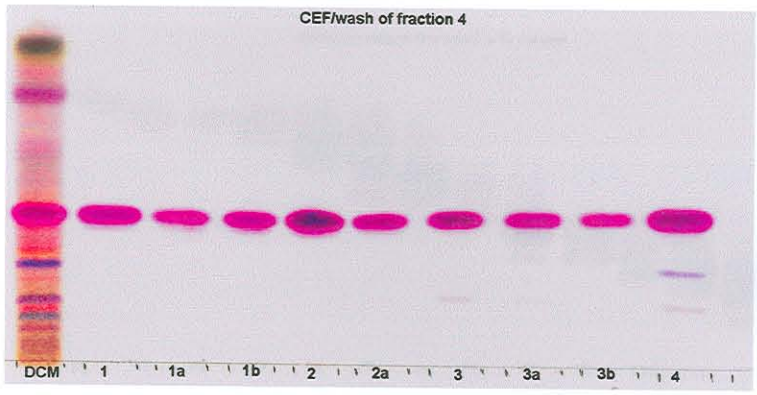

Figure 5-9: Chromatogram of fractions resulting from the wash of the crystallized test tube of Fraction 4 run along the crude DCM extract. (a and b indicates fractions from other separations).

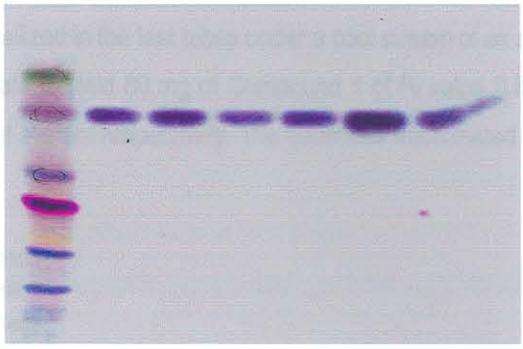

Figure 5-10: Chromatogram of fractions resulting from the wash of crystallized test tubes of fraction A and B run along the crude DCM extract. (1, 2,3,4,5, and 6 indicate fractions from other separations).

Based on observation of test tubes of Fractions A-G obtained from the VLC process, tubes A and B contained crystals, which were washed with hexane, and a combination of hexane and ethyl acetate to yield Compound **2 (Fig. 5-10).**

5.3.3 Gravity gradient chromatography fractions from solvent-solvent fractionation process of *C. imberbe*

The antibacterial chloroform fraction (6.55 g) obtained by solvent-solvent fractionation was chromatographed over silica gel 60 3 x 35 cm column and eluted with 20%, 40%, 60% 80%, 100% ethyl acetate in hexane and 10%, 20%, 30% and 40% methanol in ethyl acetate to yield 12 major fractions (F1-F12).

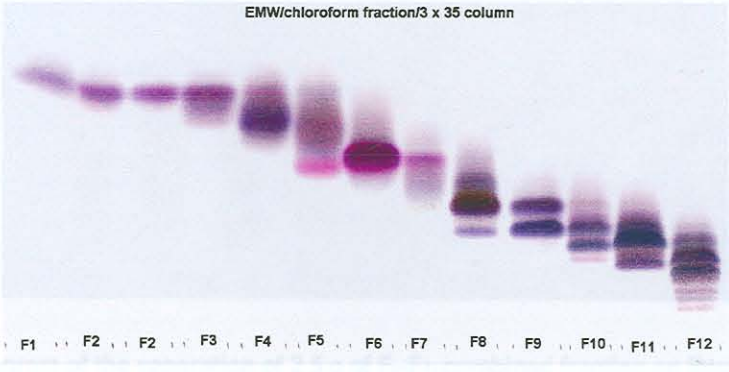

Figure 5-11: Separation of components present in 50 μg of 12 different fractions resulting from 3 x 35 cm silica gel 60 column using EMW as eluent and vanillin-sulphuric acid spray reagent.

Fractions F2 and F6 crystallized in the test tubes under a cool stream of air and were washed with a mixture of hexane and ethyl acetate to yield 80 mg of Compound **1** of R_f value 0.81 and 7 mg of Compound **2** R_f value 0.57 in EMW solvent system respectively. The quantities fractionated are indicated in **Fig 5-12.**

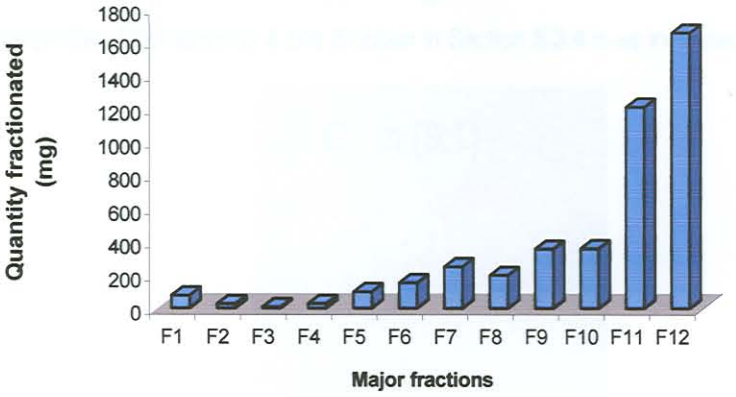

Figure 5-12: Quantities obtained from 6.55 g of chloroform fraction with the solvent-solvent fractionation process of the DCM extract of *C. imberbe*.

It was observed that the quantity fractionated from 6.55 g of the chloroform fraction increased with the polarity of the compounds. The quantity of non-polar fractions F_1-F_7 extracted ranged from 7 mg to 251 mg and quantity of the polar fractions varied ranging from 199 mg to 1657 mg.

5.3.4 Sephadex LH-20

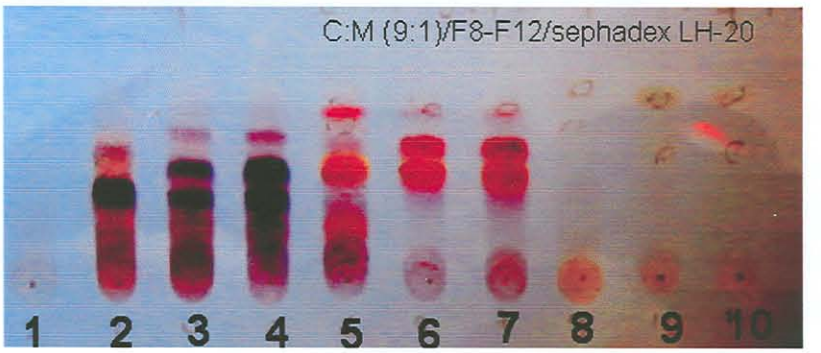

Figure 5-13: Chromatogram of the separation of 2.5 g of F_8-F_{12} combined fraction on Sephadex LH-20 separated with chloroform/methanol (9:1) solvent system and sprayed with vanillin sulphuric acid spray reagent.

5.3.5 Conventional preparative TLC

The conventional preparative TLC method applied was successful. Bands appeared to be single after spraying with vanillin sulphuric acid reagent on the preparative chromatogram but when collected and then analyzed on normal TLC, compounds did not appear single.

The TLC chemical profile of Compounds **4** and **5** obtain in Section **5.3.4** is as indicated in **Fig. 5-14**

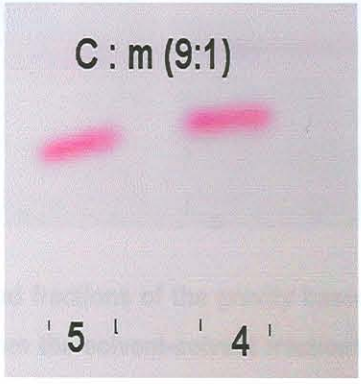

Figure 5-14: Compounds 4 and 5 isolated from *C. imberbe* by Sephadex LH-20 column separated by TLC (c:m, 9:1)

5.3.6 Gravity gradient chromatography

5.3.6.1 Fractions from solvent-solvent fractionation process of Combretum padoides

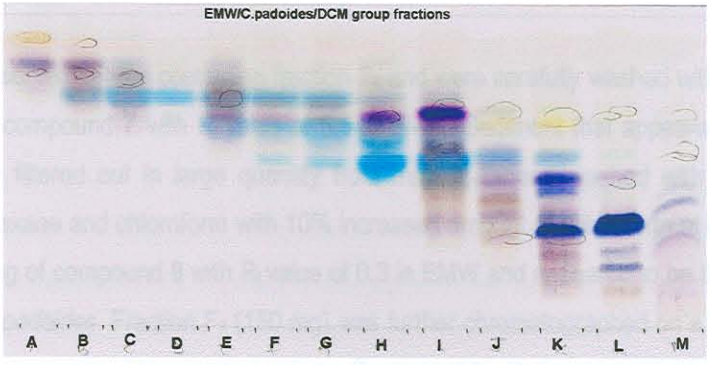

Figure 5-15: Gravity based separation of components of the chloroform fraction resulting from the solvent-solvent fractionation of the DCM fraction of *C. padoides* separated in EMW and sprayed with vanillin sulphuric acid.

TLC analysis of the fraction A-M indicated that some of the fractions still had similar chemical profiles. The fractions were again re-grouped in to seven major fractions (F1-F7) as indicated in **Fig. 5-16**

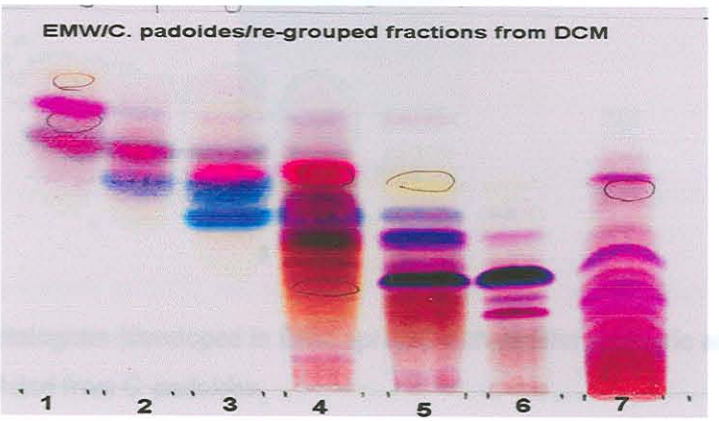

Figure 5-16: Separation of re-grouped fractions of the gravity based separation of components of the chloroform fraction resulting from the solvent-solvent fractionation of the DCM fraction of *C. padoides*

Table 5-4: Quantity of fractions derived through gravity gradient column chromatography of the chloroform fraction obtained from solvent-solvent fractionation of the DCM extract of *C. padoides*.

Fractions	F_1	F_2	F_3	F_4	F_5	F_6	F_7
Quantity fractionated (mg)	19	74	52	453	153	153	156
No of vanillin active compounds	3	4	6	8	7	5	8

Crystals were observed in tube containing fraction F_4 and were carefully washed with hexane and acetone to yield 7 mg of compound **7** with R_f value of 0.5 in EMW. Sediment that appeared to be a precipitated compound, were filtered out in large quantity from fraction F_6 and washed with 100% hexane and a combination of hexane and chloroform with 10% increased amount of chloroform at each wash. The result of this gave 53 mg of compound **8** with R_f value of 0.3 in EMW and appeared to be the major compound in the extract of *C. padoides*. Fraction F_7 (150 mg) was further chromatographed on a 3 x 120 cm Sephadex LH-20 eluted with methanol. This resulted in 5 mg Compound **6** with R_f value of 0.22 in EMW and appeared to be minor compound as indicated by its low concentration on the chromatogram compared to other compounds (**Fig. 5-17).**

Figure 5-17: Chromatogram (developed in EMW sprayed with vanillin sulphuric acid spray reagent) of compounds isolated from *C. padoides*.

5.3.7 Bioautography and MIC

The bioautography result of the solvent-solvent fractionation process of *C. imberbe* indicated in **Fig. 5-18.**

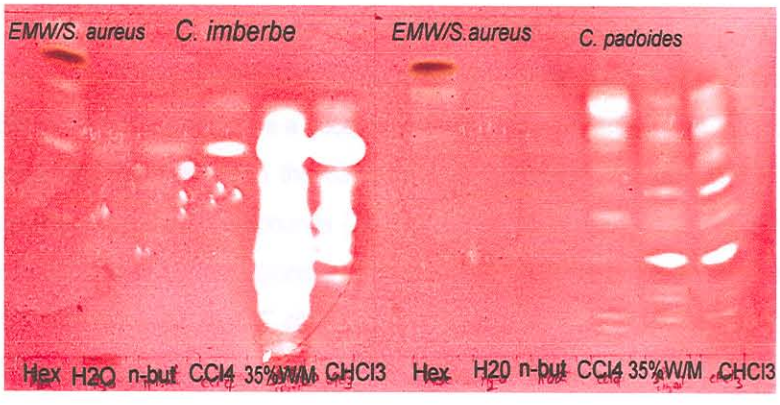

Figure 5-18: Bioautogram of DCM extracts of *C. imberbe* (left) and *C. padoides* (right) leaves separated into different fractions by solvent-solvent extraction. TLC plate developed in EMW and sprayed with *S. aureus* culture incubated overnight and then sprayed with INT. Growth inhibition is indicated by colourless zones on TLC plates. Lanes from left to right: hexane, water, n-butanol, carbon tetrachloride, 35% water in methanol, chloroform fractions.

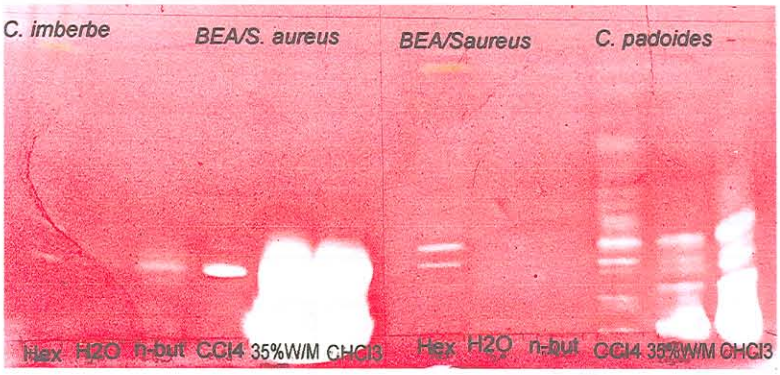

Figure 5-19: Bioautogram of DCM extracts of *C. imberbe* (left) and *C. padoides* (right) leaves separated into different fractions by solvent-solvent extraction. TLC plate developed in BEA and sprayed with *S. aureus*. Lanes from left to right: hexane, water, n-butanol, carbon tetrachloride, 35% water in methanol, chloroform fractions.

In *C. imberbe*, most of the compounds in the 35% water in methanol (15) and chloroform (11) fractions showed antibacterial activity against *S. aureus*. This is indicated by the white areas present on the bioautogram **(Fig. 5-18)**. Few compounds in the carbon tetrachloride and hexane fractions had antibacterial activity. The EMW solvent led to a better separation of antibacterial compounds compared to the BEA solvent system where most of the antibacterial compounds (**Fig. 5-19**) were found at the base of the bioautogram. The EMW solvent system gave a good separation of components from *C. padoides*. Most of the antibacterial compounds were found in the 35% water in methanol (7), chloroform (8) and carbon tetrachloride (6) fractions. Few active compounds (2) were observed in the hexane fraction (**Table 5-5**). Both the 35% water in methanol and chloroform fractions from *C. imberbe* had a good activity against *S. aureus*, with an MIC 0.03 mg/ml and an MIC of 0.02 mg/ml against *E. faecalis*. Activity against the Gram-negative bacteria (MIC > 2.5 mg/ml) was poor. Fractions that were not active on the bioautogram were not tested for MIC.

Similar fractions from *C. padoides* also had similar activity. The 35% water in methanol, chloroform fractions from *C. padoides* had a good activity against *S. aureus* (0.08 mg/ml each) and *E. faecalis* (0.02 mg/ml each). The two fractions did not have an activity against the Gram-negative pathogens (MIC >2.5 mg/ml each). From the bioautography and MIC results, the chloroform and 35% water in methanol fractions were the most active fractions that could be used for further investigation. From the bioautogram, the active compounds found in these two fractions were similar; therefore, the chloroform fraction from each of the plant species was chosen for further analysis because of its higher number of active compounds and higher activity.

Table 5-5: Number of antibacterial compounds and MIC values of fractions resulting from the solvent-solvent fractionation process of the DCM fraction of *C. imberbe* and *C. padoides*

Fractions	Quantity (g) fractionated	Approximate no of antibacterial Compounds	MIC (mg/ml) SA	EF	EC	PA
C. imberbe						
Hexane	0.8	3	nt	nt	nt	nt
Water	0.1	0	nt	nt	nt	nt
n-butane	0.5	1	nt	nt	nt	nt
35% W/M	0.1	15	0.01	0.02	> 2.5	> 2.5
CCL4	1.4	2	nt	nt	nt	nt
Chloroform	6.6	11	0.01	0.02	> 2.5	> 2.5
C. padoides						
Hexane	3.2	2	nt	nt	nt	nt
Water	0.3	0	nt	nt	nt	nt
n-butane	0.8	0	nt	nt	nt	nt
35% W/M	1.3	7	0.08	0.02	> 2.5	> 2.5
CCL4	2.8	6	nt	nt	nt	nt
Chloroform	1.7	8	0.08	0.02	> 2.5	> 2.5

S. aureus (SA), *E. faecalis* (EF), *E. coli* (EC), *P. aeruginosa* (PA), MIC test not done because of no activity of these (nt) fractions on bioautography.

The chloroform fraction (6.55 g) of *C. imberbe* was chromatographed by gravity-based silica gel 60 column to give 12 fractions. All the fractions F_1-F_{12} had activity against *S. aureus* (**Fig. 5-20**). Since all the fractions had activity on bioautogram, most of the fractions were used for further work in isolating the individual compounds responsible for the activity.

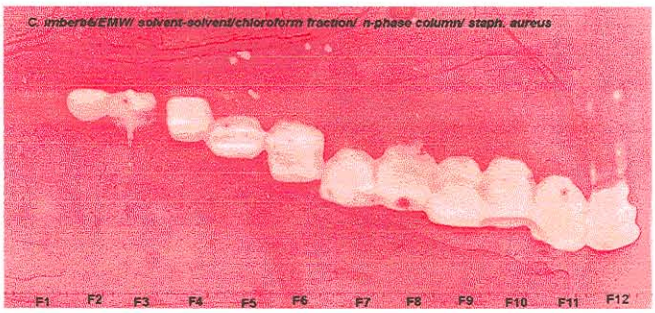

Figure 5-20: Bioautogram of the group separation (silica gel 60) of 6.55 g of the chloroform resulting from the solvent-solvent separation of DCM extract of *C. imberbe* on EMW.

5.3.8 Bioautography and MIC of VLC fractions

The bioautography result of the VLC fractions is indicated in **Fig. 5-21**. The VLC process resulted in seven major fractions (A-G) with quantities fractionated as 0.5 g (A), 1.4 g (B), 0.5 g (C), 0.8 g (D), 2.3 g (E), 1.6 g (F) and 1.8 g (G). All the fractions indicated at least one compound active against *S. aureus* (**Fig. 5-21**).

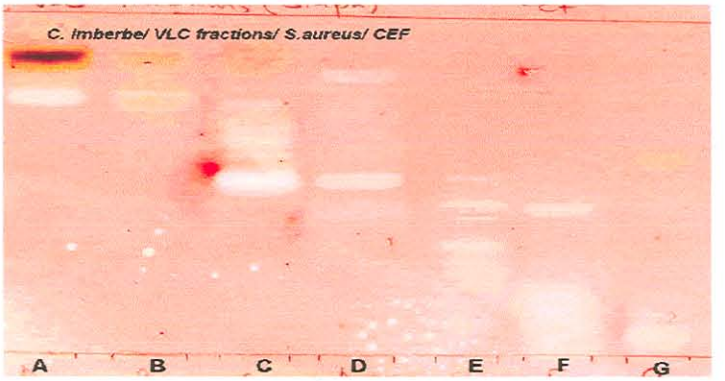

Figure 5-21: Bioautogram of the DCM extract of C. *imberbe* separated into different fractions in a Vacuum Liquid Chromatography process. TLC plate developed in CEF (as best separating system) and sprayed with *S. aureus*. Lanes from left to right: fractions A, B, C, D, E, F and G.

Most of the fractions from the VLC, fractions (B-G) also indicated a good activity against both Gram-negative and Gram-positive bacteria. Fraction E and G indicated the best activity (0.02 mg/ml) against *E. faecalis* and *E. coli* respectively.

Table 5-6: Quantity obtained, no of compounds, and MIC of VLC fractions of DCM extract of *C. imberbe*.

				MIC (mg/ml)			
Fractions	**Colour**	**Quantity (g)**	**No of compounds**	***SA***	***EF***	***EC***	***PA***
A	Greenish	0.528	5	0.08	0.06	> 2.5	> 2.5
B	Greenish	1.372	6	0.16	> 10	0.3	0.3
C	Yellowish	0.454	7	0.08	0.3	0.3	0.3
D	Purple	0.825	7	0.16	0.3	0.6	0.6
E	Purple	2.294	7	0.02	0.08	> 10	0.02
F	Brown	1.564	9	0.02	0.02	> 10	0.3
G	Brown	1.777	10	0.04	0.02	> 10	0.3

S. aureus (SA), *E. faecalis* (EF), *E. coli* (EC), *P. aeruginosa* (PA)

5.3.9 Dereplication of compounds

The available compounds (1,3,4,5) isolated from *C. imberbe* were separated along with the crude DCM extract. **Fig. 5-22** indicates that compound (**1**) R_f 0.597, (3) R_f = 0.746, (4) R_f = 0.417 and (5) R_f = 0.433 were found on the crude with the same R_f values. **Table 5-7** indicates the R_f values of isolated compounds in diferrent solvent systems.

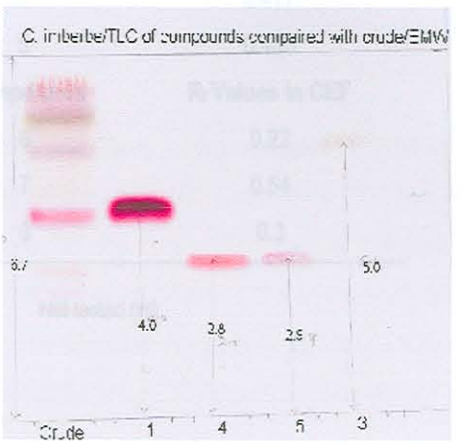

Figure 5-22: Chromatogram of the crude extract of *C. imberbe* compared with isolated compounds to dereplicate isolated compounds from the crude extract. Lanes from left to right: Crude extract of *C. imberbe*, compunds 1, 4, 5 and 3.

This is an indication that the isolated compounds were not denatured during the isolation process. The same dereplication process was carried out with compounds isolated from *C. padoides* and compounds (6) R_f = 0.22, (7) R_f = 0.54 and (8) R_f = 0.30 all on CEF solvent system could be found with similar R_f values on the crude extract. This again is an indication that oxidation or any chemical or physical processes had not changed the compounds during the isolation **(Fig. 5-23).**

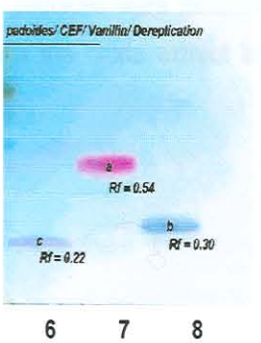

Figure 5-23: Identification of isolated compounds from the crude extract of *C. padoides*

Table 5-7: Dereplication, R_f values of isolated compounds

Compounds	R_f Value in EMW
1	0.597
2	nt
3	746
4	0.417
5	0.433
Compounds	**R_f Values in CEF**
6	0.22
7	0.54
8	0.3

Not tested (nt)

5.4 Summary

Fractions resulting from the solvent-solvent fraction process of the DCM extract of *C. imberbe* and *C. padoides* had many antibacterial compounds based on bioautography. The 35% water in methanol and chloroform extracts from both plant species had higher number of antibacterial compounds with MIC values of 0.02 mg/ml against both *S. aureus* and *E. faecalis* for *C. imberbe* fractions. MIC values of 0.08 mg/ml and 0.02 mg/ml against *S. aureus* and *E. faecalis* were observed from the same fractions from *C. padoides* respectively. Isolation through the solvent-solvent fraction process was more successful than the VLC process. Five antibacterial compounds were isolated from *C. imberbe* through the solvent-solvent process as compared to two compounds isolated from the same species through the VLC process.

Isolation of compounds from *C. padoides* was through the solvent-solvent fraction approach only. Three antibacterial compounds from *C. padoides* were isolated through this process. A comparison of the R_f values of isolated compounds with the crude extract indicated that the compounds isolated were not artifacts of the isolation process.