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CHAPTER 3

PLANT COLLECTION, EXTRACTION AND ANALYSIS

3.1 Introduction

Fresh or dried plant material can be used as a source for the extraction of secondary plant components. Most scientists have opted to use dry material for several reasons:
Traditional healers frequently use dry plant material.
The time delay between collecting plant material and processing it makes it difficult to work with fresh material.
There are fewer problems associated with the large-scale extraction of dry plant material. Freshly harvested and dried material is more commonly used since old dried material stored for a period of time may undergo some qualitative changes.

In this chapter dry leaves were used for direct extraction with acetone for the preliminary screening step. Serial exhaustive extraction using hexane, dichloromethane (DCM), acetone, and methanol as successive extractants was employed for the extraction leading to isolation of antibacterial compounds.

3.2 Material and Methods

The standard procedures for extracting and testing for antibacterial activity to be used in the present study [sections **3.2.3.1, 2.2.3.2** and **4.2.2** (page 51)] have been developed to such a degree in the University of Pretoria Phytomedicine laboratory that few problems were encountered. Nevertheless, there is such a difference in anti-microbial activity and chemical composition between different plant species, that much innovation and modification were required in the isolation.

3.2.1 Experimental design for bioassays

Four treatments were used in bioassays.

Group 1: Test Group: Consisted of the organism (ATCC strains of *Staphylococcus aureus, Eneterococcus faecalis, Escherichia coli* and *Pseudomonas aeruginosa*) plus different concentrations of the extracts (this group determined if the extracts are effective as antibacterial agents).

Group 2: Positive control: Organisms plus a known antibiotic (This ensured that utilized organisms were susceptible to common chemotherapeutics and were not resistant strains).

Group 3: Pure cultures: Only the organism in the absence of antibiotic or plant extract. This was to ensure that the organism was growing properly under the defined laboratory conditions. This was necessary to distinguish poor growth from inhibition of growth.

Group 4: Negative controls: Organisms plus the pure extraction solvent (this was necessary to prove that the extraction solvent had no inhibitory action of its' own). All determinations where quantitative data are important were carried out in duplicate.

3.2.2 Plant collection

C. imberbe, C. padoides, C. celastroides ssp*. orientale* and *C. celastroides* ssp. *celastroides* leaves were collected from the National Botanic Garden in Nelspruit, South Africa in September of 2002. The plant label identified the trees and Prof. JN Eloff of the University of Pretoria, confirmed the identity. The origin of each tree was recorded in the database of the botanical garden and the Garden's Herbarium, contain voucher specimens

3.2.3 Plant preparation and extraction

Leaves were carefully examined and old, insect damaged, fungus-infested leaves and twigs were removed. Healthy leaves were spread out and dried in the laboratory at room temperature until they broke easily by hand. Leaf material was ground to a fine powder using a Jankel and Kunkel Model A10 mill once dried completely. Large quantity was ground using a Wiley mill to fine powder of c1 mm diameter.

3.2.3.1 Direct extraction with acetone

Direct extraction with acetone following the method of (Eloff, 1998) was used as an extraction method for the purpose of preliminary screening of the Hypocrateropsis species. Acetone will extract a wider variety of

constituents (Elff, 1998) making it the best extractant to be used in the extraction of species of the Combretaceae (Eloff, 1998).

In this method, finely ground plant material (0.5 g) was extracted with 5 ml of technical grade (Merck) acetone in a centrifuge tube while shaking. The extracts were decanted into pre-weighed glass vials after centrifuging at c. 3000-x g for 5 minutes. The process was repeated three times on the same plant material but using fresh solvent. The solvent was removed by placing the extracts in front of a stream of air in a fume hood at room temperature. The extracted residues were weighed and re-dissolved in acetone to yield 10 mg/ml solutions ready for further analysis

3.2.3.2 Serial exhaustive extraction

A number of factors were taken into consideration in choosing solvents that were to be used in the serial exhaustive extraction. The choice of solvent also depended on what was planned with the extract. The effect of solvent on subsequent bioassay was an important factor. From previously published work, where authors screened plant material for anti-microbial properties, various extractants, from 80% ethanol, methanol (Taylor *et al*., 1995), petroleum ether, chloroform, ethanol and water were used. Eloff (1998) found that acetone extracted a greater number of inhibitors (14) than other solvents used. The defatting process by hexane is of importance in the isolation process since nonpolar compounds were extracted rapidly in this process. Therefore serial exhaustive extraction was used with hexane as a starting solvent, followed by dichloromethane (DCM), acetone and methanol as extractants. The process was repeated three times for each solvent. The polarity of solvents gradually increased and ranged from a non-polar solvent (hexane) to a more polar solvent (methanol). This was to ensure that a wide polarity range of compounds could be extracted in the process.

Extraction was initially performed on a Labotec Model 20.2 shaking apparatus with a 10 ml: 1 g solvent to dry weight ratio. With large quantities of plant material, the ratio was raised on a proportional scale. Dried leaves (500 g) of the various plants were exhaustively extracted in serial manner with solvents of increasing polarity. Finely ground plant material (500 g) was initially extracted with 5000 ml of hexane. The solvent was allowed to extract for 1 hour while shaking before being decanted. The same quantity of solvent was added to the marc and shaken for an hour again. The process was repeated six times. The marc was allowed to dry and the process of extraction was repeated with dichloromethane, acetone and finally methanol. The extracts were filtered through Whatman (no. 2) filter paper using a Büchner funnel, and solvent was removed by vacuum distillation in a Büchi rotary evaporator at 60^0C. Once concentrated to a small volume,

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the extracts were placed in pre-weighed beakers and allowed to dry completely in front of a cool stream of air. The mass extracted with each solvent was measured. To determine the TLC chemical profile, 20 mg of each extract was weighed into a pill vial and made up to a concentration of 5 or 10 mg/ml by re-dissolving in acetone.

3.2.4 Analysis of plant extracts for preliminary screening

The chemical profile of extracts was determined by TLC using aluminum backed thin layer chromatography plates [ALIGRAM ^{R} SIL g/UV 254 – MACHEREY – NAGEL]. In each case 50 µg was chromatographed. The following three solvent systems were used to develop the plates: Ethyl acetate/methanol/water (40:5.4:4) [EMW] (polar), Chloroform/ethyl acetate/formic acid: 5:4:1 [CEF (intermediate polarity/acidic) Benzene/ethanol/ammonium hydroxide: 90:10:1 [BEA] (non-polar/basic). The solvent systems have been optimized to separate components of each extracts of Combretaceae members (Eloff, 1998c). Development of the chromatogram was done in closed tanks in which the atmosphere had been saturated with eluent vapour by wetting a filter paper lining. Samples were applied rapidly and developed without delay to minimize the possibility of oxidation or photo-oxidation of constituents. The separated components were visualized under visible and ultraviolet light [254 and 360 nm, Camac Universal UV lamp TL-600]. The TLC plates were subsequently sprayed with vanillin sulphuric acid spray reagent (2 mg of vanillin in 28 ml of methanol plus 1ml of concentrated sulphuric acid) and heated for 4-5 minutes at 100°C to allow for development of colour.

For the isolation work done at Hans-Knöll Institute (HKI) Jena, Germany, the solvent systems used for the analysis were different; the most frequently used solvent systems were chloroform: methanol (CM) 20:1; chloroform: methanol (CM) 9:1; chloroform: methanol (CM) 4:1 and cyclohexane:chloroform (CyC) 9: 1.

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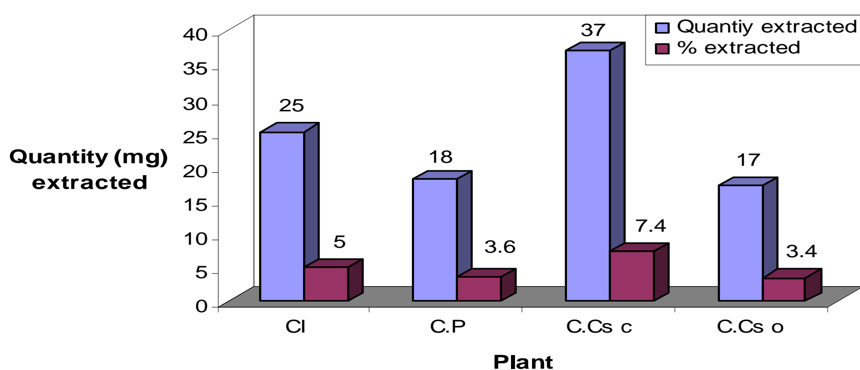
3.3 Results

3.3.1 Extraction

3.3.1.1 Direct extraction with acetone

Direct extraction with acetone was used to prepare extracts from the 4 plant species for TLC analysis and subsequent Bioautography and MIC for preliminary screening purposes. The quantity of material extracted, and the percentage extracted from *C. imberbe, C. padoides, C. celastroides* ssp. *orientale and C. celastroides* ssp. *celastroides,* differed substantially (**Fig. 3-1** and **3-2**).



C. imberbe (C.I*), C. padoides* (C.P*), C. celastroides* ssp. *celastroides (*C.Cs.c) and *C. celastroides* ssp*. orientale* (C.Cs.O).

Figure 3-1: Quantity (g) and percentage of material extracted from 500 g of *C. imberbe, C. padoides, C. celastroides* ssp. *orientale*, *C. celastroides* ssp. *celastroides* in a direct extraction with acetone process.

3.3.1.2 Serial exhaustive extraction

Based on the Bioautography results of the preliminary screening to be discussed later *C. imberbe* and *C. padoides* were selected for isolation purposes. Extracts of these two plants had many antibacterial compounds on their bioautogram compared to *C. celastroides* ssp. *orientale* and *C. celastroides* ssp. *celastroides*, which had a single active spot each in the highly non-polar region of the bioautogram. This indicated that the compound responsible for activity may be a fatty acid. The MIC results of these two

species were also very low giving an indication of poor activity of the single less polar compound (discussed latter). Consequently *C. celastroides* ssp. *orientale* and *C. celastroides* ssp. *celastroides* were not investigated further. Serial exhaustive extraction was then carried out on *C. imberbe* and *C. padoides*. The result of the serial exhaustive extraction *for C. imberbe* and *C. padoides* are summarised in **Table 3-1** and **Table 3-2** respectively.

Table 3-1: Quantity in g extracted at each batch (A, B, C, D, E, and F) in a serial exhaustive extraction from 500 g of *C. imberbe* leaves

Extractant	Quantity extracted in each batch						
	A	B	C	D	E	F	Total
Hexane	6.5	2.7	1	0.6	0.26	0.2	11.26
DCM	10.1	4.1	2.4	1.4	0.6	0.39	18.99
Acetone	5.6	2.6	1.1	1	0.5	0.3	11.1
Methanol	29.9	9	4.9	2	1.1	0.3	55.3

Table 3-2: Quantity in g extracted at each batch (A, B, C, D, E and F) in a serial exhaustive extraction from 500 g of *C. padoides* leaves.

Extractant	Quantity extracted in each batch						
	A	B	C	D	E	F	Total
Hexane	6.6	3.6	1	0.5	0.2	0.18	12.08
DCM	5.4	3.2	1.6	0.8	0.5	0.3	11.8
Acetone	10.1	4.5	2.2	1	0.5	0.4	19.6
Methanol	57.1	27.4	7.4	3	1.6	0.7	97.2

Methanol extracted the highest quantity of material from *C. imberbe* (55.3 g, 11.06 %) and *C. padoides* (97.2 9, 19.4 %) (**Table 3-1) and (Table 3-2).** Acetone extracted the lowest quantity of material for *C. imberbe* (11.1 g, 2.2 %) and the lowest quantity of material was extracted from C*. padoides* by DCM (11.8 g, 2.36 %).

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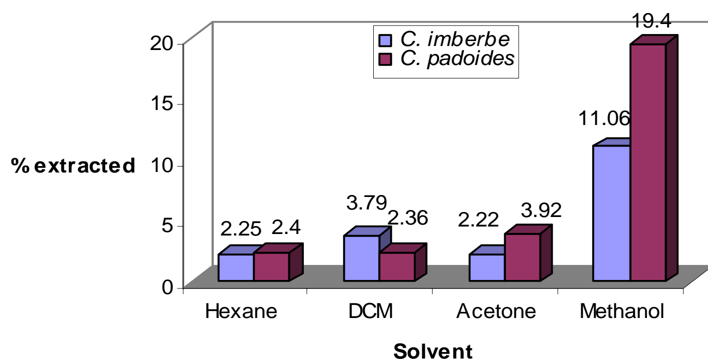

Figure 3-2: Percentage of material extracted from *C. imberbe* and *C. padoides* by each solvent in the serial exhaustive extraction process.

3.3.2 TLC analysis of plant extracts for preliminary screening

Extracts of the four plant species were analyzed by TLC and viewed under UV 365 nm (left) and also sprayed with vanillin sulphuric acid spray reagent (right). The chemical profile of the chromatograms is indicated in **(Fig. 3-3)**. All chromophoric compounds were not vannilin active.

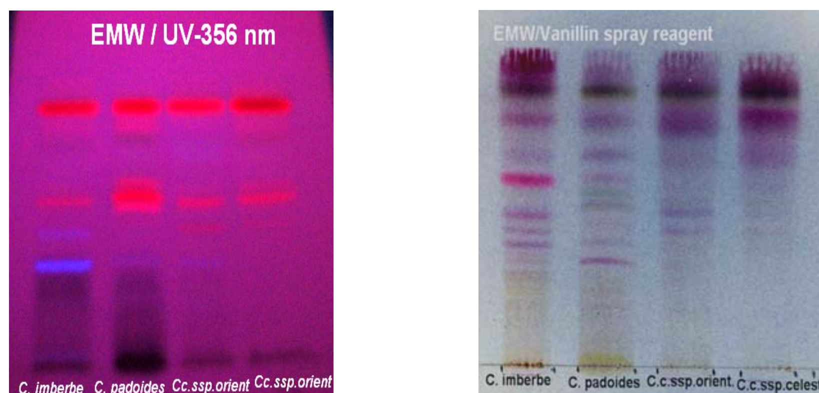

Figure 3-3: TLC chromatograms (viewed under UV 365 nm and vanillin sulphuric acid spay reagent) of extracts from direct extraction with acetone in preliminary screening process

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Table 3-3: Total number of UV and vanillin spray reagent active compounds resulting from direct extraction with acetone of *C. imberbe* and *C. padoides*

Plant species	*C. imberbe*	*C. padoides*	*C. celestroides* **ssp.** *orientale*	*C. celestroides* **ssp.** *celestroides*
No of UV (254) active compounds	4	4	2	1
No of UV (356) active compounds	5	6	4	3
No of Vanillin active compounds	11	10	8	9
Total No of compounds	**20**	**20**	**14**	**13**

C. imberbe and *C. padoides* had the highest number of vanillin and UV active compounds (20 each), and *C. celestroides* ssp *celestroides* had the lowest number of vanillin and UV active compounds (**Table 3-3**).

3.3.3 TLC analysis of plant extracts from serial exhaustive extraction.

Five µl of 10 mg/ml plant extract (50 µg) were spotted on the TLC plate and developed with three solvent systems (EMW, CEF and BEA). Separation was more effective in EMW solvent system (**Fig. 3-4, Fig. 3-5** and **Fig. 3-6** respectively).

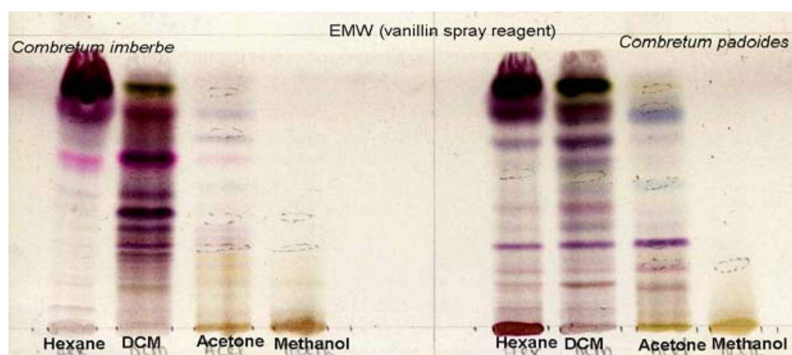

Figure 3-4: TLC chromatogram (viewed after spraying with vanillin sulphuric acid spray reagent) of serial exhaustive extraction extracts of C*. imberbe* and *C. padoides* developed with EMW. Areas on the chromatogram cycled indicate UV active compounds

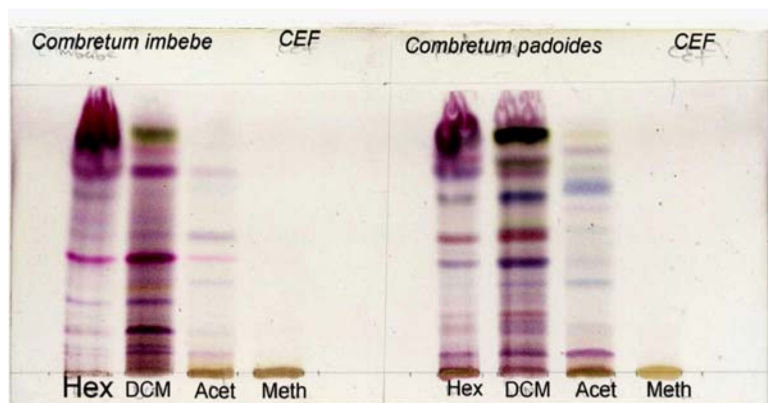

Figure 3-5: TLC chromatogram (viewed after spraying with vanillin sulphuric acid spray reagent) of serial exhaustive extraction extracts of C*. imberbe* and *C. padoides* developed with CEF. Areas on the chromatogram cycled indicate UV active compounds.

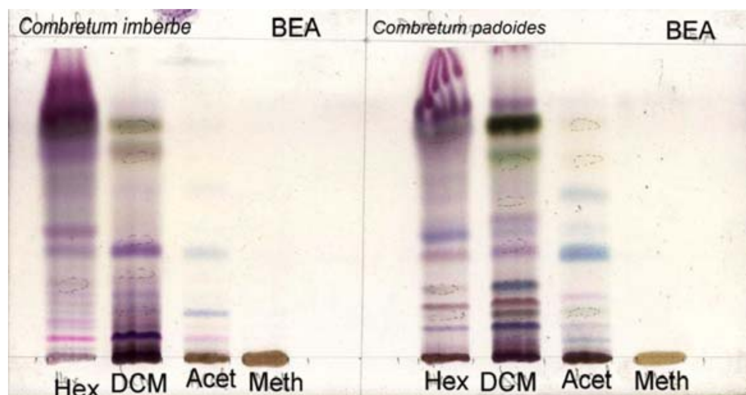

Figure 3-6: TLC chromatogram (viewed after spraying with vanillin sulphuric acid spray reagent) of serial exhaustive extraction extracts of C*. imberbe* and *C. padoides* developed with BEA. Areas on the chromatogram cycled indicate UV active compounds.

In the serial exhaustive extraction, DCM had extracted the highest number of vannilin and UV active compounds for *C. imberbe* (14) and for *C. padoides* (15). In the same process, methanol had extracted the lowest number of vanilin and UV active compounds for *C. imberbe* (5) and for *C. padoides* (3) (**Table 3-4).**

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Table 3-4: Total number of UV and vanillin sulphuric acid spray reagent active compounds resulting from the serial exhaustive extraction extracts of *C. imberbe* and *C. padoides* in EMW solvent system.

Combretum imberbe

Solvents	**Hexane**	**DCM**	**Acetone**	**Methanol**
Aproximate No of UV (254) active compounds	0	1	0	0
Aproximate No of UV (356) active compounds	0	1	3	2
Aproximate No of Vanillin active compounds	11	13	9	3
Aproximate total No of active compounds	**11**	**14**	**13**	**5**
Combretum padoides	**Hexane**	**DCM**	**Acetone**	**Methanol**
Apriximate No of UV (254) active compounds	1	0	0	0
Apriximate No of UV (356) active compounds	1	1	3	1
Aproximate No of Vanillin active compounds		14	10	2
Aproximate total no of active compounds	**14**	**15**	**13**	**3**

3.4 Discussion and conclusion

In the direct extraction experiment, acetone extracted a broad range of compounds thus, allowing an effective preliminary screening for bioactive compounds (Eloff, 1998). Other solvents such as hexane, and methanol might extract a wide range of compounds but acetone will always extract compounds with a wider range of polarities. In this process, acetone extracted the highest quantity of extract from *C. celastroides* ssp. *orientale* (7.4%), followed by *C. imberbe* (5%). The lowest quantity extracted was from *C. celastroides* ssp. *celastroides* (3.4%) and (3.6%) from *C. padoides.* The TLC chemical profile of the 4 plant species indicated differences in the number of compounds extracted. The same numbers of compounds (20) were extracted from *C. imberbe* and *C. padoides* followed by *C. celastroides* ssp. *orientale* (14) and *C. celastroides s*sp. *celastroides* (13) (**Table 3-3**). Although many compounds were extracted from *C. imberbe* and *C. padoides* the numbers of compounds extracted were not directly related to the quantity extracted. A greater quantity of material was extracted from *C. celastroides* ssp. *orientale* but fewer compounds were visible compared to *C. imberbe* and *C. celastroides ssp. celastroides.* This could be as a result of the fact that some of the compounds extracted might have been glycosides that do not move from the origin with the system used.

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In the serial exhaustive extraction (SEE), methanol extracted the highest quantity of material, 8% from *C. imberbe* and 10.54% from *C. padoides*. DCM extracted the lowest quantity (2.2 %) from *C. imberbe* while the lowest quantity (1.84%) extracted from *C. padoides* was by hexane. The TLC chemical profile of both plants (*C. imberbe* and *C. padoides*) indicated that DCM extracted the highest number of visible compounds (14 and 15 respectively). Most of the compounds present in the hexane were non-polar while those in DCM and acetone extracts were of varying polarity. This was seen from their effective separation in non-polar BEA and intermediate polarity CEF solvent systems respectively. Compounds that appeared in the chemical profile of methanol were seen to be mostly polar since most of them showed up at the lower part of the chromatogram developed with the polar solvent system (EMW). EMW is a polar solvent system which could effectively separate polar compounds, compounds seated at the lower part of a chromatogram developed with EMW most therefore be of higher polarity.

In this process, (serial exhaustive extraction) methanol extracted the largest quantity of material in both plants but the least number of compounds (3) showed up in this extract (**Table 3-4**). More compounds showed up in DCM but less material was extracted compared to methanol. DCM could be a useful solvent to extract selectively material from *C. imberbe* and *C. padoides.*

3.5 Summary

Leaf material of *C. imberbe, C. padoides, C. celastroides* ssp. *orientale and C. celastroides* ssp. *celastroides* was collected from the National Botanic Garden in Nelspruit (South Africa), dried under standard conditions and ground to powder. The material was extracted directly with acetone as well as exhaustively with hexane, DCM, acetone and methanol in a serial fashion. This was necessary for the isolation process since hexane will first of all defat the plant material by extracting non-polar compound paving the way for the subsequent solvents to extract intermediate polarity antibacterial compounds. The quantity of material extracted from each plant species varied with the different solvents used and also when extracted with the same solvent.

Different numbers of chemical components were detected in the different plant species analysed by TLC. From these results, it appears that the DCM extract resulting from the serial exhaustive extraction could be the best extract to be used for isolation of phytocompounds since it contained the highest number of visible compounds than any other extract. The antibacterial activity of the different extracts is the most important factor and this is addressed in chapter 4.

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CHAPTER 4

BIOLOGICAL ASSAYS FOR PRELIMINARY SCREENING

4.1 Introduction

Natural products isolated from higher plants and microorganisms have provided novel, clinically active drugs. The key to the success of discovering naturally occurring therapeutic agents rests on bioassay-guided fractionation and purification procedures. The route involved in isolating bioactive compounds is an important factor towards rapid attainment of biologically active natural compounds. Much time is involved in isolating a compound first before determining whether the compound is active or not. In this chapter, the importance of bioassay-guided isolation of antibacterial compounds is highlighted. The use of bioautography and the minimum inhibitory concentration (MIC) assay are the most important in preliminary screening.

4.2 Material and Methods

4.2.1 Antibacterial assay

4.2.1.1 Test organisms

The following test organisms were used for the bioautography and MIC assay of the plant extracts *Staphylococcus aureus* (Gram-positive) [American Type Culture Collection ATCC number [29213], *Pseudomonas aeruginosa* (Gram-negative) [ATCC 27853], *Escherichia coli* (Gram-negative) [ATCC 25922] and *Enetrococcus faecalis* (Gram-positive) [ATCC 29212]. All these organisms are important nosocomial pathogens widely used in screening tests and are reference isolates recommended by the National Committee for Clinical Laboratory Standards, USA [NCCLS, 1992]

4.2.2 Bioautography of preliminary screening

Bioautography is a rapid aid in the bioassay-guided isolation and fractionation of antibacterial compounds and fractions. In this approach, the activity of plant extracts against bacteria is determined on chromatograms, in accordance with the bioautography procedure of Begue and Kline (1972)

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Developed chromatography plates of 50 µg extracts of *C. imberbe, C. padoides, C. celastroides* ssp. *orientale and C. celastroides* ssp. *celastroides* were dried overnight, sprayed with a suspension of actively growing cells of Gram-positive or Gram-negative bacteria, and incubated at 37°C in a chamber at 100% relative humidity for 18 hours. Plates are sprayed with 0.2 mg/ml p-iodonitrotetrazolium violet. Clear zones on the chromatogram indicate inhibition of growth after incubating for 1 hour at 37°C. This method was chosen for its simplicity, low cost, accuracy and rapid result that makes it ideal for bioassay-guided isolation (Eloff, 1998a).

***p*-iodonitrotetrazolium violet (INT) reaction**

The INT reaction is based on the transfer of electrons from NADH; a product of for example the threonine dehydrogenase [TDH] catalyzed reaction, to the tetrazolium dye [p-iodonitrotetrazolium violet]. Threonine dehydrogenase [TDH] from bacteria catalyses the NAD-dependent oxidation of threonine to form 2- amino-3-ketobutyrate and NADH. During the active growth of bacteria, an electron is transferred from NADH [which is transparent in the visible range] to p-iodonitrotetrazolium violet, a formazan dye which is purple-red in colour. Therefore, the clear zones (s) on the chromatogram indicate areas of inhibition [zones where no active growth of bacteria has taken place].

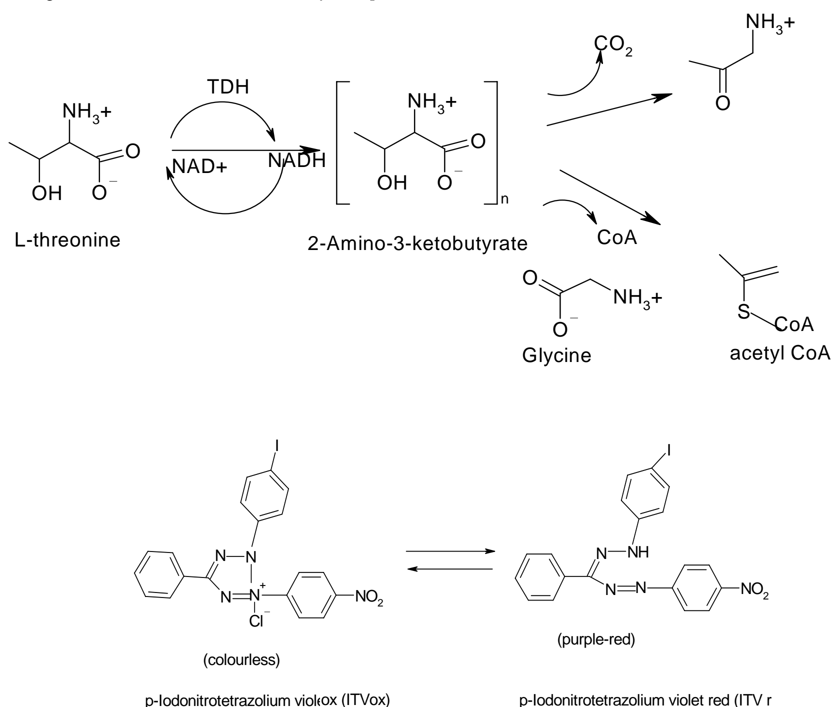

Figure 4-1: INT, coupling reagent for the colorimetric assay (reaction pathway for the assay of TDH)

4.2.3 Microplate dilution assay for preliminary screening

Agar diffusion assay is used widely to determine the antibacterial activity of plant extracts. The technique works well with defined inhibitors (Hewit and Vincent, 1989). However, when examining extracts containing unknown components, there are problems leading to false positive and false negative results (Eloff, 1998).

In this study, with the aim of quantifying the activity of the extracts, the microplate dilution method was used to determine the Minimum Inhibitory Concentration (MIC) values of the extracts against each test bacterial species (Eloff, 1998). This was determined by 2-fold serial dilution of extracts beyond the level where no inhibition of growth of *S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853, *E. faecalis* ATCC 29212 and *E. coli* ATCC 25922 was observed (Eloff, 1998). Plant extracts or fractions were reconstituted to 10 mg/ml with acetone and 100 µl of the fractions were serially diluted 50% with water in 96-well microplates. Muller-Hinton [MH] broth culture was inoculated (1%) with the test bacteria and was incubated at 37^0C overnight, and 100 µl of the resulting culture were added to each well. Neomycin was used as a reference antibiotic and two wells were used as sterility and growth controls respectively with the sterility control containing only Oxoid MH broth, while the negative growth control contained both MH broth as well as test organism. The microplates were sealed and incubated at 37 ^{0}C at 100% relative humidity for 18 hours. As an indicator of bacterial growth, 40 µl of 0.2 mg/ml solution of p-iodonitrotetrazolium violet [INT] dissolved in water were added to the microplate wells and incubated at 37^0C for 30 minutes. The MIC was recorded as the lowest concentration of plant extract at which bacterial growth was inhibited. The colourless tetrazolium salt acted as an electron acceptor and was reduced to a red coloured formazan product by biologically active organisms **(Fig. 4-1).**

4.2.4 Determination of total activity

Total activity is a measure of the amount extracted from a plant in relation to the MIC of the extract, fraction or compound isolated. There are several reasons for screening studies: to find new lead biologically active compounds for developing pharmaceuticals, and to confirm the ethnomedicinal use of plants to develop phytomedicines for use as herbal medicine. In many screening studies, activities are reported non-quantitatively. Even if extracts data are expressed in quantitative terms such as antibacterial activity in MIC, it is usually not possible to compare different plants with the result presented. To compare plants however, the quantity extracted from the plant should be brought into the equation (Eloff, 2004). In mathematical terms it can be expressed as:

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$$\text{Total activity (ml)} = \frac{\text{Amount extracted from 1 g (mg)}}{\text{MIC (mg/ml)}}$$

The units are adjusted to ml and indicate the degree to which the active extracts, fractions or compounds in one gram of plant material can be diluted and still inhibit the growth of the test organisms (Eloff, 2004).
The same approach can be used to determine the total activity of different fractions to determine if any activity has been lost (Eloff, 2004). In this case the values are expressed in ml/fraction and indicate to what volume the fraction can be diluted and still kill the bacterial. In this work, to compare the activity of extracts or fractions, the total activity of each extract was determined using the formula stated above.

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4.2 **Results**

4.3.1 Antibacterial assays

4.3.1.1 Bioautography

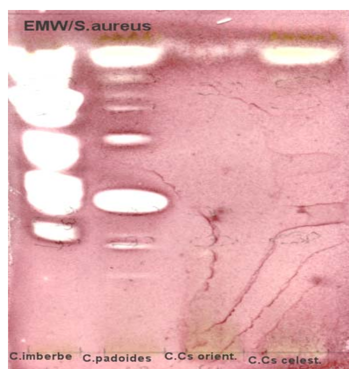

Figure 4-2: Bioautograms of acetone extracts of *C. imberbe*, *C. padoides*, *C. celastroides* ssp. *orientale* and *C. celastroides* ssp. *celastroides*. TLC developed in EMW and sprayed with actively growing *S. aureus* cultures and later sprayed with INT. White areas indicate zones of growth inhibition.

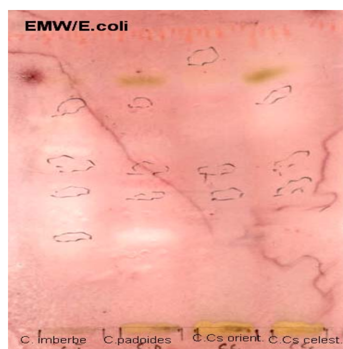

Figure 4-3 Bioautograms of acetone extracts of *C. imberbe, C. padoides, C. celastroides* ssp. *orientale* and *C. celastroides* ssp. *celastroides*. TLC developed in EMW and sprayed with actively growing *E.coli* cultures and later sprayed with INT. White areas indicate zones of growth inhibition

In this screening, when chromatograms were developed with EMW (**Fig. 4-2**), the *C. celastroides* extracts exhibited only a limited activity. They all indicated one active compound each in the non-polar region of the chromatogram. *C. imberbe* indicated the highest number of active compounds followed by *C. padoides* (**Fig. 4-2**). This solvent system yielded good separation of compounds against *S. aureus* as indicated on the bioautogram. When chromatograms were developed with the less polar acidic solvent system BEA, all active compounds remained on the base of the chromatogram without any proper separation. With BEA, more compounds seem to show activity against *E. coli* than with EMW solvent system (**Fig. 4-5**). This indicates the importance of the right choice of solvent system when developing chromatograms for antibacterial activity in a bioassay-guided process. However, this system did not give a good separation for *S. aureus* since most of the active compounds were so polar that they hadly moved from the origin **(Fig. 4-4).**

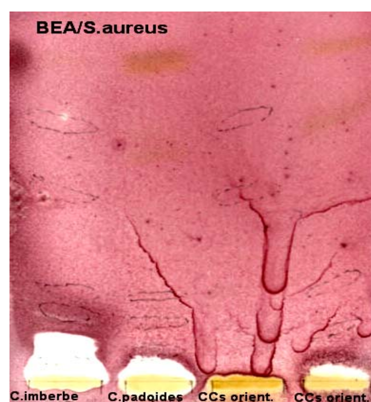

Figure 4-4: Bioautograms of acetone extracts of *C. imberbe*, *C. padoides* *C. celastroides* ssp. *orientale* and *C. celastroides* ssp. *celastroides*. TLC developed in BEA and sprayed with actively growing *S. aureus* cultures and later sprayed with INT. White areas indicate zones of growth inhibition.

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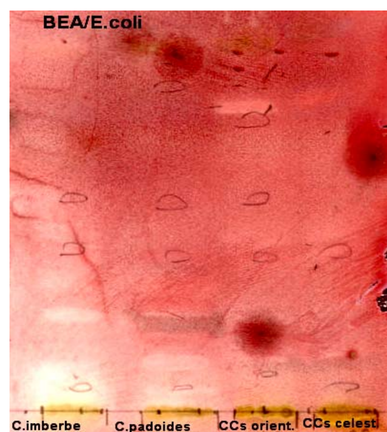

Figure 4-5: Bioautograms of acetone extracts of *C. imberbe*, *C. padoides*, *C. celastroides* ssp. *orientale* and *C. celastroides* ssp. *celastroides*. TLC developed in BEA and sprayed with actively growing *E. coli* cultures and later sprayed with INT. White areas indicate zones of growth inhibition.

Table 4-1: Bioautography (TLC in EMW) and Minimum Inhibitrory Concentration (MIC) results of preliminary screening of *C. imberbe*, *C. padoides*, *C. celastroides* ssp. *orientale* and *C. celastroides* ssp. *celastroides* acetone extracts against *S. aureus* (SA), *E. faecalis* (EF), *E. coli* (EA) and *P. aeruginosa* (PA) .

Extracts	**No of bioactive compounds on bioautogram**				**MIC mg/ml)**			
Acetone extracts	**SA**	**EF**	**PA**	**EC**	**SA**	**EF**	**PA**	**EC**
C. imberbe	8	7	0	4	0.625	0.625	2.5	2.5
				Total activity (ml)	**416**	**416**	**<5.2**	**< 5.2**
C. padoides	7	6	0	2	0.625	0.625	2.5	1.25
				Total activity(ml)	**250**	**250**	**7.2**	**14.4**
C. celastroides ssp. *celastroides*	1	0	0	0	1.25	1.25	2.5	2.5
				Total activity (ml)	**28**	**28**	**<14**	**<14**
C. celastroides ssp. *orientale*	1	0	0	0	1.25	1.25	1.25	2.5
				Total activity (ml)	**28**	**28**	**28**	**< 14**

4.3.1.2 Bioautography of serial exhaustive extraction extracts

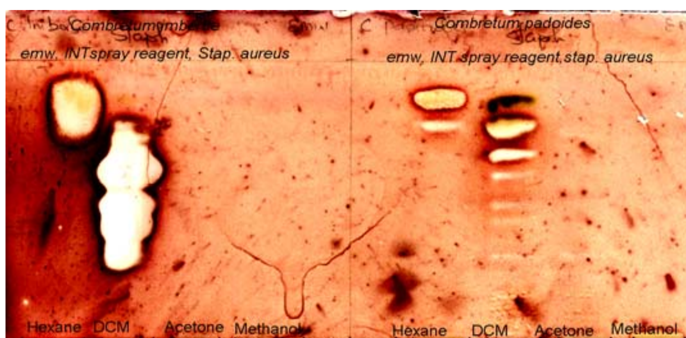

Figure 4-6: Bioautography of serial exhaustive extraction extracts on *S. aureus* sprayed with INT sprayed reagent. White areas indicate zones of inhibition.

In the serial exhaustive extraction screening, acetone and methanol did not extract any visible antibacterial compounds (**Fig. 4-6**). *S. aureus* was used as the indicator organism in the bioassay guided pathway as indicated in the bioautogram. The activities of other pathogenic organisms are indicated in **Table 4-1**. DCM extracted most of the active compounds in both *C. imberbe and C. padoides.* The active compounds extracted by DCM were of varying polarity. Hexane extracted fewer active compounds, most of which were present in the non-polar region of the bioautogram **(Fig. 4-7**) as could be expected.

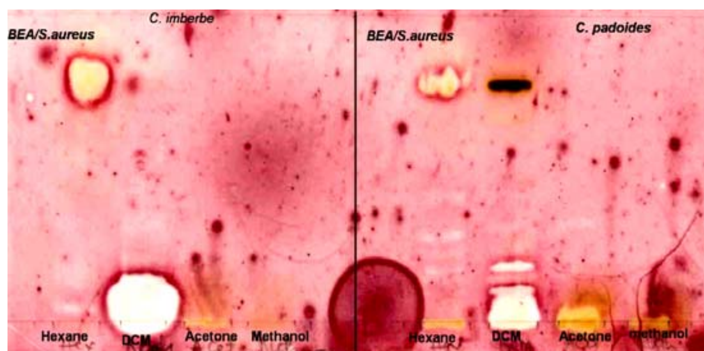

Figure 4-7: Bioautography (BEA solvent system) of serial exhaustive extraction extracts against *S. aureus* sprayed with INT spray reagent. White areas indicate zones of inhibition.

Most of the active compounds occurred in the DCM extracts of both *C. imberbe* and *C. padoides*. The hexane extracts of both plants species indicated just a few antibacterial compounds and no active

compound was seen from acetone and methanol extracts (**Fig. 4-6**)**.** Extracts of both *C. imberbe* and *C. padoides* had minimal activity against *E. coli* **(Fig. 4-8**). The activity profile appeared to be the same when chromatograms were developed with BEA but as expected from previous results all the active compounds were found at the base of the bioautogram **(Fig. 4-7**)

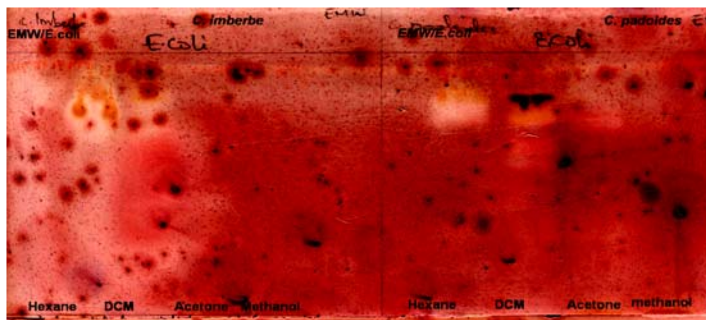

Figure 4-8: Bioautography of serial exhaustive extraction extracts against *E. coli,* using EMW solvent system and sprayed with INT spray reagent. White areas indicate zones of inhibition.

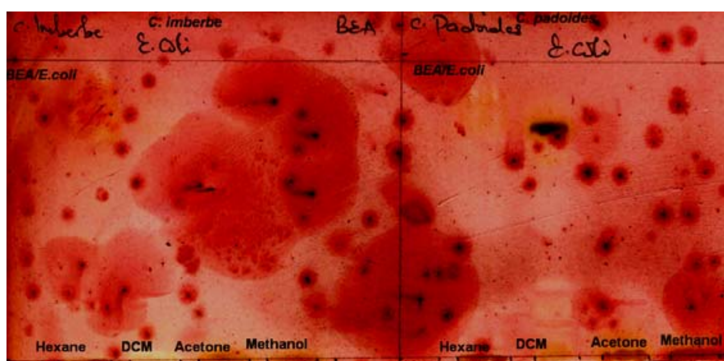

Figure 4--9: Bioautography of serial exhaustive extraction extracts against *E.coli*, using BEA solvent system and sprayed with INT spray reagent. White areas indicate zones of ihibition.

4.3.2 Minimum inhibitory concentration of extracts from serial exhaustive extraction

The hexane and DCM extracts of *C. imberbe* and *C. padoides* were the only extracts that indicated promising activity in the bioautogram results. The microtitre plate assay **(Fig. 4-10 and 4-11)** was then used to quantify the level of activity of these extracts by determining their minimum inhibitory concentration (MIC) values. In both species (*C. imberbe* and *C. padoides*), the DCM extract indicated a much lower MIC (an

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indication of good antibacterial activity) on both Gram-positive and Gram-negative pathogens than the hexane extracts. The MIC values obtained against the four pathogenic organisms are indicated in **(Table 4-2).** Five mg/ml of each extract was used and the total activities against bacterial are indicated **Table 4-2.**

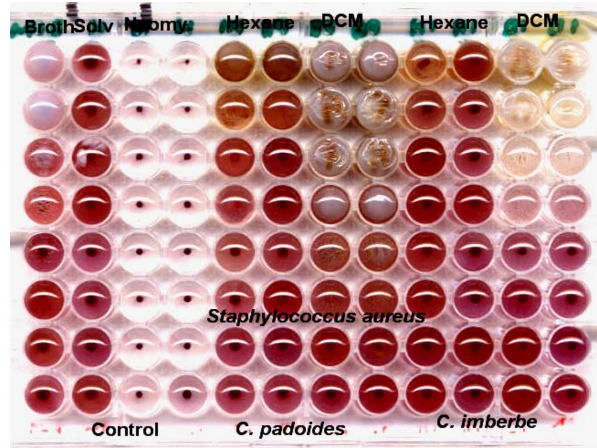

Figure 4-10: MIC of the hexane and DCM extracts of *C. imberbe* and *C. padoides* on *S. aureus* on microtitre plates. White wells indicate inhibition and purple wells indicate bacterial growth.

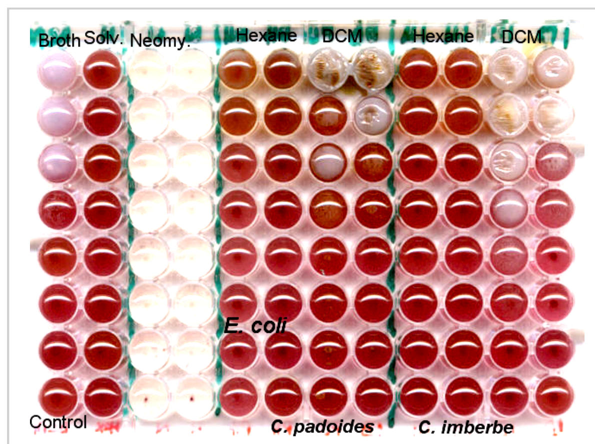

Figure 4-11: MIC of the hexane and DCM extracts of *C. imberbe* and *C. padoides* against *E. coli*

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4.2.3 Total activity of extracts

The total activity of the DCM extract was calculated for each organism (**Section 4.2.4**). The DCM extract of *C. imberbe* had a higher total activity (489.7 ml) than that of *C. padoides* (151 g/mg/ml) against *S. aureus* (**Table 4-2).**

Table 4-2: Bioautography, MIC and total activity (DCM extracts only) of serial exhaustive extracts (*S. aureus* (SA), *E. faecalis* (EF), *E. coli* (EC) and *P. aeruginosa* (PA)

	No of BAC on bioautogram				MIC (mg/ml)			
Extracts	**SA**	**EF**	**PC**	**EC**	**SA**	**EF**	**PA**	**EC**
***C. imberbe* extracts**								
Hexane	3	0	0	0	> 2.5	>2. 5	> 2.5	> 2.5
DCM	8	3	0	0	0.039	0.156	0.313	0.156
Acetone	0	0	0	0	nt	nt	nt	nt
Methanol	0	0	0	0	nt	nt	nt	nt
	Total activity DCM extract (ml)				**489.7**	**122.4**	**61**	**122.4**
***C. padoides* extracts**								
Hexane	3	0	3	1	>2. 5	>2. 5	> 2. 5	> 2. 5
DCM	8	0	3	1	0.078	0.019	0.078	0.313
Acetone	0	0	0	0	nt	nt	nt	Nt
Methanol	0	0	0	0	nt	nt	nt	nt
	Total activity DCM extract (ml)				**151**	**621**	**151**	**37.7**

(BAC) Biologically active compounds, (nt) Indicates that MIC was not carried on the corresponding extract because the extract did not exhibited activity in the bioautography.

Against *E. faecalis*, the DCM extract of C*. padoides* had a higher total activity (621 g/mg/ml) than that of *C. imberbe* (122.4 ml). It is obvious that the total activity for each extract depends on the organism in question. The higher the total activity, the better the activity of the whole plant extracts. There were also large differences between the total activity of the DCM extracts towards the different test organisms.

4.4 Discussion and conclusion

The lowest MIC indicating highest activity was recorded for the DCM extracts of *C.imberbe* and *C. padoides* against *S. aureus* (0.039 and 0.078 mg/ml, respectively and *E. faecalis* (0.156 and 0.019 mg/ml

respectively). The DCM extract of *C. padoides* also indicated very good activity against *P. aeruginosa*, with an MIC of 0.078 mg/ml. Comparing the activity of the two plant species according to total activity, the DCM extract of *C. padoides* recorded the highest total activity (621 ml) as compared to 122.4 ml for *C. imberbe* against *E. faecalis*. The DCM extract of *C. imberbe* had a higher (489.7 ml) total activity than the DCM extract of *C. padoides (*against *S. aureus)*, 151 ml/g. It is therefore evident that the activity of an extract varies with respect to different test organisms. The high activity and more antibacterial compounds present in the DCM extracts of *C. imberbe* and *C. padoides* provided the rational for further work on these extracts to be discussed in Chapter 4.

4.5 Summary

In this chapter, bioautogram and MIC values of *C. imberbe, C. padoides, C. celastroides* ssp. *orientale* and *C. celastroides* ssp. *celastroides* were determined for preliminary screening purposes. According to the results of the acetone extracts, *C. imberbe* (8 antibacterial compounds, MIC of 0.68 mg/ml) and *C. padoides* (7 antibacterial compounds, MIC of 0.625 mg/ml) were selected for further work. *C. celastroides* ssp. *orientale* and *C. celastroides* ssp. *celastroides* were eliminated due to their low number of antibacterial compounds (1 each) and high MIC (> 2.5 mg/ml) values as well as low total activity values (**Table 4-**T**1**). Bioautography and MIC were also carried out on the serial exhaustive extraction extracts of *C. imberbe* and *C. padoides* to find out which extractant extracted most of the antibacterial compounds.

Most of the active compounds were found in the DCM extract of each of the plant species. The DCM extracts of *C. imberbe* had about 8 antibacterial compounds with a MIC of 0.039 mg/ml against *S. aureus* as compared to 3 antibacterial compounds in the hexane extract (MIC > 2.5 mg/ml). The acetone and methanol extracts did not have any antibacterial compounds. DCM extracts of *C. padoides* had about 8 antibacterial compounds with an MIC of 0.078 mg/ml against *S. aureus* and 0.019 mg/ml *against E. coli* as compared to 3 antibacterial compounds in the hexane extract (MIC > 2.5 mg/ml). The acetone and methanol extracts did not have any antibacterial compounds. DCM extract of *C. imberbe* had a higher total activity against *S. aureus* than *C. padoides*. The DCM extract of *C. padoides* on the other hand indicated a higher total activity against *E. faecalis* than that of *C. imberbe.* Therefore the total activity depended on the organism used.