Chapter 3 Preliminary extraction studies

3.1 Introduction

The main aim of preliminary extraction studies was to identify the solvents that extracted the largest quantities of material while also extracting high antibacterial and antioxidant activity in their extracts. An important factor governing the choice of solvents used in an extraction is the type of phytochemical groups that are to be extracted (Houghton and Raman, 1998). Several researchers have used different solvents while extracting compounds from plants, for example 80% ethanol in water solution (Vlietinck *et al.*, 1995), ethanol-water (50:50,v/v), methanol (Taylor *et al.*, 1996), petroleum ether, chloroform, ethanol, methanol and water (Salie and Eagles, 1996).

Many scientists extract dried plant materials using solvents that are liquid at room temperature, however, other techniques such as steam distillation and the use of supercritical fluids or pressurised gas may be employed (Houghton and Raman, 1998).

Extraction can be direct, which involves bringing the plant material in contact with the solvent for a period of time; or serial which involves the use of many solvents of varying polarities successively on the same material. Soxhlet extraction can be very useful for the exhaustive extraction of material with a particular solvent, but this cannot be used for thermolabile compounds. The problem may be overcome by extracting under reduced pressure.

The Combretaceae is particularly rich in stilbenes, alkaloids and flavonoid compounds (Rogers, 1996). These compounds are intermediate polar compounds and as such would be extracted by intermediate polar solvents like diethyl ether, ethylacetate, acetone, ethanol and methanol. However, most antibacterial compounds isolated from the Combretaceae are non-polar (Kotze and Eloff, 2002), while most antioxidant compounds are polar (Re *et al.*, 1999) hence the need to extract with a wide range of solvent polarities.

Many solvents can be used to achieve the desired result, but the extract was intended for use in poultry, therefore it was necessary to select solvents that are non-toxic or otherwise would be easy to remove by evaporation. Preliminary extraction studies should be

regarded as a selection process and further studies were conducted using the solvents that presented the desired properties.

The secondary objective was to compare the biological activities of extracts from plant samples collected from two locations. Since it was necessary for us to have positive identification and a record of the plants we were using, we worked with plants growing in botanical gardens. Leaf material samples were collected from the Manie van der Schyff Botanical Garden at the University of Pretoria main campus (UP) and from the Nelspruit Lowveld National Botanical Garden (LNBG).

The antibacterial and antioxidant activities of the extracts from these two samples were determined using the MIC method and the DPPH assay on TLC respectively.

3.2 Extraction method

A direct extraction method was performed with 5 solvents of varying polarities; acetone, ethanol, ethylacetate, dichloromethane and hexane were used. These solvents were chosen based on their safety in application or their ease of evaporation.

One gram of samples were mixed with 10 ml each of the extractant in a shaker for 5 minutes and centrifuged at 3600 rpm for five minutes to separate the marc from the extract. The extracts were dried under a cold air fan, weighed and reconstituted in acetone to a concentration of 10 mg/ml

3.3 Results and discussion

Table 3:1: Amount in milligrams extracted in a single direct extraction of one gram of sample in 10 ml solvents, for the Nelspruit Lowveld National Botanical Garden (LNBG) and University of Pretoria main campus garden (UP) samples.

	Acetone	Ethanol	Ethylacetate	Dichloromethane	Hexane	Average
LNBG sample	82	88	87.5	81	38	75.3
UP sample	68.5	71	76.5	77.5	35.5	65.8

There was not much difference in quantities extracted between acetone, ethanol, ethylacetate and dichloromethane extracts of either sample. However, larger quantities of material were present in the extracts from the LNBG sample (75.3 mg/g) compared to the UP sample (65.8 mg/g).

Hexane extracted the lowest quantities (approximately half the quantity extracted by the other four solvents) while ethanol extracted the largest quantities (8.8%), followed by ethylacetate (8.75%), acetone (8.2%) and dichloromethane (8.1%) for the LNBG sample. Conversely for the UP sample the largest quantities were in the dichloromethane extract (7.75%), followed by ethylacetate (7.65%), ethanol (7.1%), acetone (6.85%) and hexane (3.55%). In general, acetone, ethanol, ethylacetate and dichloromethane all extracted comparable quantities for their respective samples.

3.4 Phytochemical analysis

3.4.1 Introduction

Thin layer chromatography (TLC) was used to investigate the composition of the various extracts. TLC can be used for qualitative as well as semi-quantitative analysis of crude extracts for identification of constituents (Houghton and Raman, 1998). Qualitative analysis is done by comparing the retardation factor (R_f value) on the TLC against a reference value of a standard.

R_f value = distance moved by analyte / distance moved by solvent front

For semi-quantitative analysis, the size and colour intensity of zones on TLC plates are related to the amount of substance present. The amount of substance initially applied to the plate must be an accurately measured volume of a solution of known concentration. The zone produced after development must exhibit a measurable function proportional to the amount of substance present. This relationship can be measured by visual inspection, examining the size and intensity of the sample zone in relation to those of standards applied in the same volume.

The detection methods used in TLC are determined largely by the nature of substances present in the mixture. Although some components of an extract may be coloured and thus easy to visualize if the stationary phase is white, the vast majority will have little or no colour and other methods have to be used to make them visible. The two most common methods are examination under UV-light and the use of spray reagents to produce fluorescent or, more commonly, coloured derivatives. This latter method usually requires subsequent heating of the plate (Houghton and Raman, 1998).

3.4.2 Method

Ten µl of each extract (100 µg) was loaded onto TLC (Merck, Kieselgel 60 F_{254}) plates and developed in three solvent systems as indicated in 2.3 above. The air-dried plates were then sprayed with vanillin-sulphuric acid reagent and heated in an oven at 105 °C for two minutes to enable visual detection of extract composition.

3.4.3 Results and discussion

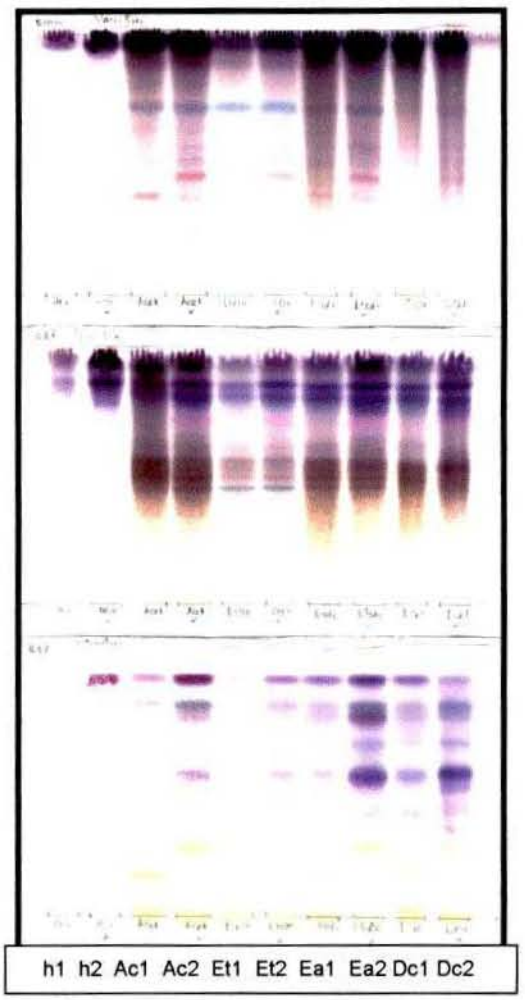

Figure 3:1:TLC profiles of the LNBG sample (1) and UP sample (2) developed side by side in EMW (top), CEF (centre), and BEA (bottom) solvent systems and sprayed with vanillin–sulphuric acid. **Lanes from left to right**: Hexane extracts (h), acetone extracts (Ac), ethanol extracts (Et), ethylacetate extracts (Ea) and dichloromethane extracts (Dc).

The chromatographic profiles of the LNBG and UP samples were generally similar in terms of numbers of compounds visible on the chromatograms, the size of bands and their colour intensities when the plates were sprayed with vanillin–sulphuric acid reagent [Figure 3.1], suggesting similar composition and occurrence of the compounds in approximately the same quantities in the two samples

3.5 Antioxidant screening

3.5.1 Introduction

Various assays have been used to test for antioxidant activity but the mostly widely used methods are those that involve generation of free radical species that are then neutralised by antioxidant compounds (Arnao *et al.*, 2001). In qualitative analysis of antioxidant activity, the 2, 2,diphenyl-1-picrylhydrazyl (DPPH) assay on TLC plates was used as a screen test for the radical scavenging ability of the compounds present in the different extracts.

DPPH is a purple coloured compound that does not dimerize and can hence be prepared in crystalline form. It is a stable free radical and following interaction with antioxidants, they either transfer electrons or hydrogen atoms to it thus neutralizing its free radical character (Naik *et al.*, 2003).

The DPPH method measures electron-donating activity of other compounds and hence provides an evaluation of antioxidant activity due to free radical scavenging. Any molecule that can donate an electron or hydrogen to it will react with DPPH, thus quenching its absorption, DPPH is therefore reduced from a purple compound to a light yellow compound by electrons from oxidant compounds. Reaction of DPPH with hydroxyl groups involves a homolytic substitution of one of the phenyl rings of DPPH. The concentration of DPPH at the end of a reaction will depend on the concentration and structure of the compound being scavenged (Naik *et al.*, 2003).

3.5.2 Method

One hundred µg of each extract was loaded on TLC (Merck, Kieselgel 60 F_{254}) plates and developed in three solvent systems described in 2.3. The dried plates were sprayed with 0.2% DPPH in methanol and the presence of antioxidant compounds was detected by yellow bands against a purple background.

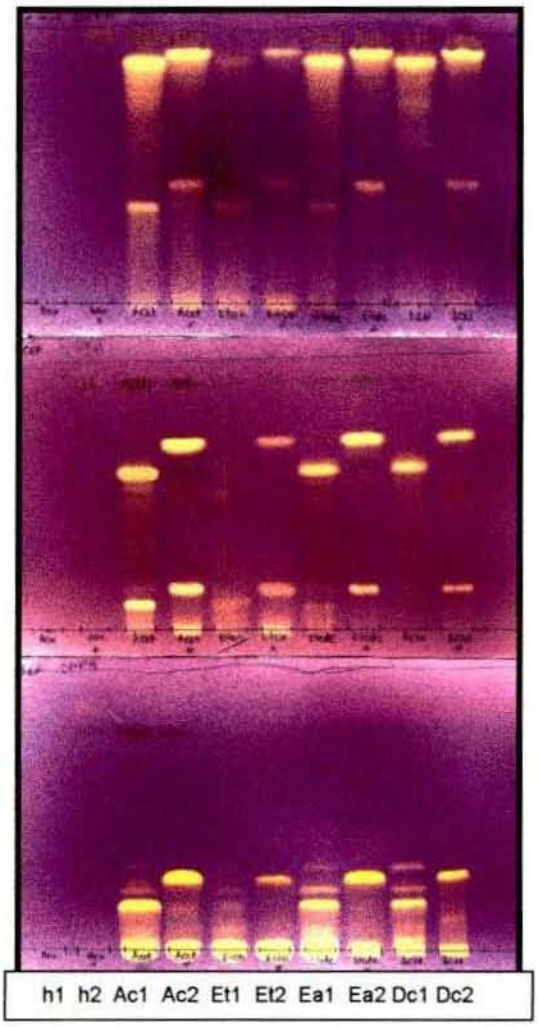

Figure 3:2: TLC profiles of the LNBG sample (1) and UP sample (2) developed side by side in EMW (top), CEF (centre), and BEA (bottom) solvent systems and sprayed with 0.2% DPPH in methanol. **Lanes from left to right**: Hexane extracts (h), acetone extracts (Ac), ethanol extracts (Et), ethylacetate extracts (Ea) and dichloromethane extracts (Dc).

TLC plates sprayed with 0.2% DPPH in methanol revealed the presence of two major antioxidant compounds that appeared immediately after spraying [Figure 3.2]. Though the antioxidant compounds had different R_f values for the different samples, the colour intensities and reaction speed with which they formed yellow bands of inhibition on TLC plates were similar suggesting similar radical scavenging characteristics. Hexane extracts did not possess antioxidant compounds.

Table 3:2: R_f values of the 2 major antioxidant compounds present in the acetone, ethanol, ethylacetate and dichloromethane extracts of *C. woodii* leaves when developed in EMW, CEF and BEA, for the UP and the LNBG samples.

	EMW		**CEF**		**BEA**	
	1st	2nd	1st	2nd	1st	2nd
U. P sample	0.90	0.40	0.69	0.15	0.25	0
LNBG sample	0.85	0.35	0.56	0.08	0.15	0
Difference	0.05	0.05	0.13	0.07	0.1	0

The antioxidant active compounds in the UP sample had higher R_f values compared to the LNBG sample by a consistent magnitude of 0.05 when developed in EMW solvent system [Table 3.2]. This means that the two samples have different antioxidant compounds as evidenced by their different R_f values, but the difference may be because the compounds in one sample occurred as derivatives e.g. glycoside or hydroxylated derivatives, in the other. The large difference in R_f values in either sample when developed in CEF meant that CEF is the best separation system for the antioxidant compounds in *C. woodii* leaves and also suggests that these compounds are of intermediate polarity. BEA (a non-polar) system was the least ideal system for separation of the antioxidant compounds of *C. woodii* extracts with only one of the two major compounds moving up on TLC.

3.6 **Bioautography**

Bioautography of the extracts from the two samples was performed to identify the antibacterial compounds present in them. Clear zones against a red background on the chromatogram sprayed with 2 mg/ml INT indicated growth inhibition.

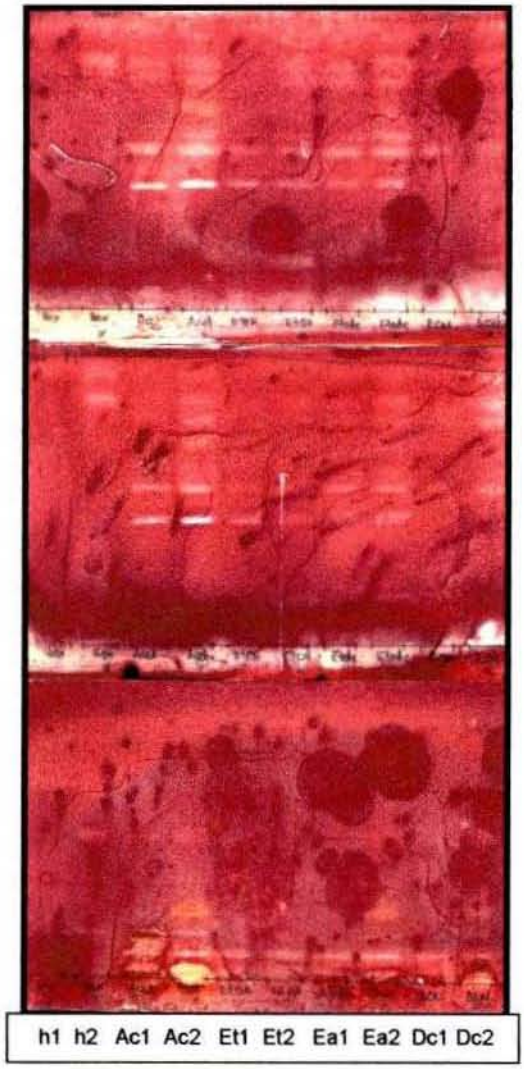

Figure 3:3 Bioautography profiles of the LNBG sample (1) and UP sample (2) developed side by side in EMW (top), CEF (centre), and BEA (bottom) solvent systems and sprayed with *S. aureus*. **Lanes from left to right**: Hexane extracts (h), acetone extracts (Ac), ethanol extracts (Et), ethylacetate extracts (Ea) and dichloromethane extracts (Dc).

Bioautography worked well with the two Gram-positive bacteria, *E. faecalis* and *S. aureus* [Figure 3.3] but not as well with the Gram-negative bacteria *E. coli* and *P. aeruginosa*. Antibacterial compounds were present in the acetone, ethanol and ethylacetate extracts (about 6 bands of inhibition on TLC developed in EMW solvent system), while the dichloromethane extracts showed fewer zones of inhibition and the hexane extracts had the lowest number of antibacterial compounds (one band of inhibition on TLC developed in EMW solvent system) [Figure 3.3].

The best separation of antibacterial compounds was obtained with EMW and CEF solvent systems suggesting the antibacterial compounds to be intermediate polar to polar compounds. It was necessary to completely remove formic acid from TLC plated developed in CEF solvent systems because it is quite toxic to bacteria, therefore plates developed in this solvent system were allowed to dry for two days before spraying with bacteria.

Bioautography technique has a drawback in that coloured compounds may mask the growth inhibition of the bacteria. Some of the more polar components have a green, yellow or brown colour and it is possible that some of these compounds may also inhibit the growth of one or more of the test organisms but their colour may mask the presence of clear zones of growth inhibition. This situation occurred with *Combretum erythrophyllum* extracts (Martini and Eloff, 1998).

3.7 Quantification of antibacterial activity of C. woodii leaf extracts

3.7.1 Introduction

Agar diffusion assays cannot be used to determine the antibacterial activities of plant extracts. The technique works well with a single compound (Hewitt and Vincent, 1989), but when examining extracts containing unknown compounds there are problems leading to false negative and false positive results (Eloff, 1998b).

An alternative technique is serial dilution of the extracts in a number of test tubes followed by addition of test organisms to determine the minimum inhibitory concentration (MIC) for the test organism using turbidity as an indicator of growth. This technique requires large quantities of extracts and is therefore not useful in bioassay-guided isolation of antimicrobial compounds (Eloff, 1998b).

Eloff (1998b) developed a microdilution technique that involves the use of 96-well microplates and tetrazonium salts to indicate bacterial growth. The method worked well with *S. aureus, E. faecalis, P. aeruginosa*, and *E. coli* and is useful in bioassay work because it requires small quantities of materials. This method was used in the quantification of the antibacterial activity of various *C. woodii* extracts.

3.7.2 Method

The minimum inhibitory concentration (MIC) values were determined using the microplate dilution method (Eloff, 1998b). ATCC strains of *S. aureus, E. faecalis, P. aeruginosa* and *E. coli* were used as test organisms and the method was performed as outlined in 2.5.

3.7.3 Results and discussion

Table 3:3: Amount in mg extracted from one gram samples and MIC values in mg/ml for *C. woodii* leaf extracts of hexane (H), dichloromethane, (Dc), ethylacetate (Ea), acetone (Ac) and ethanol (Et) for the LNBG (N) and UP (M) samples against ATCC strains of the four test bacteria.

	H(N)	H(M)	Ea(N)	Ea(M)	Dc(N)	Dc(M)	Ac(N)	Ac(M)	Et(N)	Et(M)
Quantity	38	35.5	87.5	76.5	81	77.5	82	68.5	88	71
					MIC in mg/ml					
E. coli	0.31	0.31	0.08	0.16	0.08	0.16	0.16	0.16	0.04	0.08
S. aureus	0.63	0.63	0.08	0.08	0.16	0.16	0.04	0.16	0.16	0.16
E. faecalis	0.63	0.63	0.04	0.08	0.08	0.08	0.04	0.08	0.08	0.16
P. aeruginosa	0.63	0.63	0.31	0.31	0.63	0.63	0.31	0.31	0.31	0.31
Average MIC	0.55	0.55	0.13	0.16	0.24	0.26	0.14	0.18	0.15	0.18

MIC values for the extracts tallied with the results from bioautography with the acetone, ethanol and ethylacetate extracts showing the highest bacterial growth inhibitory properties while the hexane extract showed the least antibacterial activity [Table 3.3]. MICs as low as 0.04 mg/ml were realized in the acetone and ethylacetate extracts of the LNBG sample against *S. aureus* and *E. faecalis*.

3.8 Reciprocal of MIC value

One way of expressing antibacterial activity on a positive scale is to express it as the reciprocal of MIC. The units of this measure are ml/mg and the value indicates the volume to which one mg of extract can be diluted in ml and still inhibit bacterial growth.

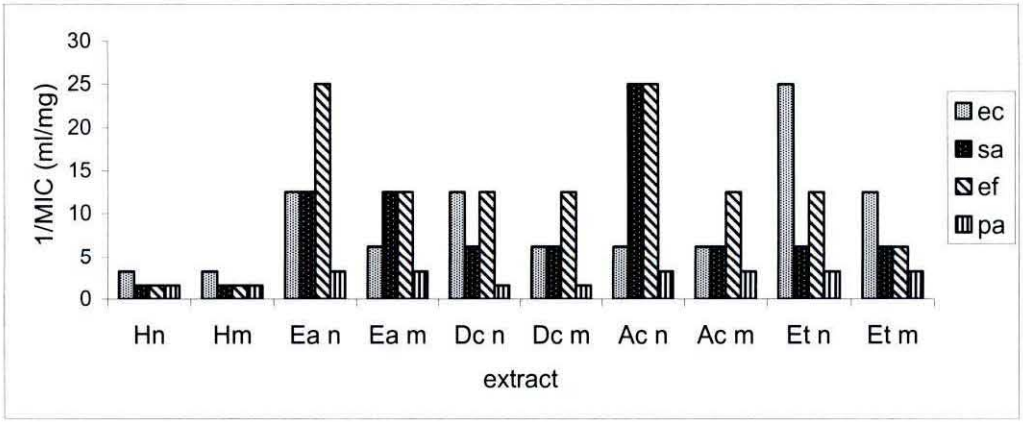

Figure 3:4: Reciprocal of the MIC values of the extracts against *E. coli* (ec), *S. aureus* (sa), *E. faecalis* (ef), and *P. aeruginosa* (pa) for hexane (H), dichloromethane, (Dc), ethylacetate (Ea), acetone (Ac) and ethanol (Et) extracts from the LNBG at Nelspruit (n) and UP main campus (m) samples.

The reciprocal of the MIC values showed the acetone extract from LNBG sample (Ac n) to possess the most potent antibacterial compounds against the four tested bacteria with the highest activity against *S. aureus* and *E. faecalis,* while hexane extracts showed the least potency. Except in the hexane extracts where antibacterial activity of the two samples was similar, greater activity was realized in the LNBG sample compared to the UP sample for all the other extracts [Figure 3.4].

3.9 Total activity

The reciprocal of the MIC value is related to the potency of antibacterial compounds present, but it does not take account of the quantity of material in the extract. Total activity is an arbitrary measure of the quantity of antibacterial compounds present in the extract and is calculated by dividing the mass in mg of the extract by the MIC in mg/ml, thus, the unit of this arbitrary measure is ml. Total activity therefore indicates the volume to which the bioactive compounds present in the extract can be diluted and still inhibit growth of bacteria (Eloff, 1999).

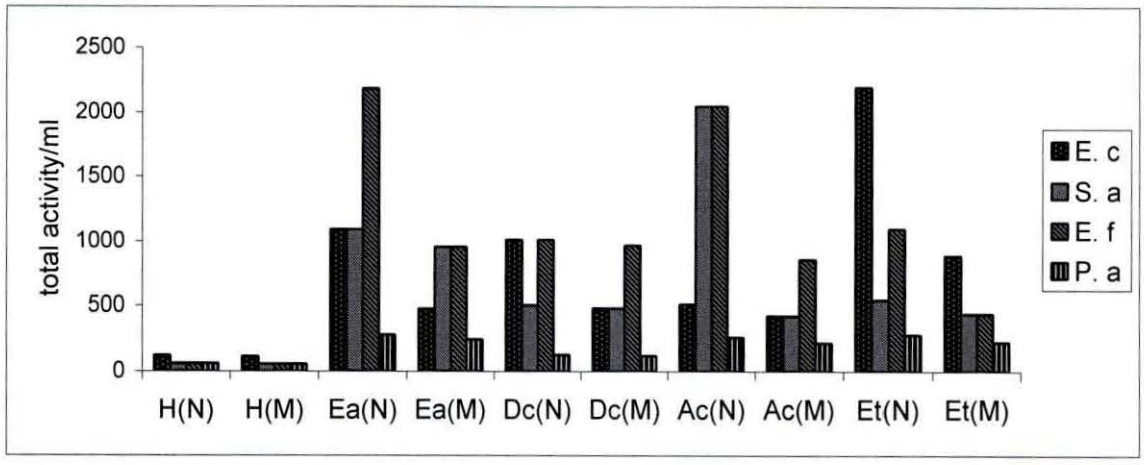

Figure 3:5 Total activity of the four test pathogens *E. coli* (E.c), *S. aureus* (S.a), *E. faecalis* (E.f), and *P. aeruginosa* (P.a) for extracts of hexane (H), dichloromethane, (Dc), ethylacetate (Ea), acetone (Ac) and ethanol (Et) extracts from the LNBG at Nelspruit (n) and UP main campus (m) samples.

Acetone, ethanol and ethylacetate extracts from the LNBG sample showed the best total activity values with the ethanol extract possessing the highest average total activity with a value of 2440 ml. In all the extracts the total activity for the LNBG sample was greater then the total activity of the UP sample and in some cases (for the acetone and ethanol extracts) was more than two fold higher.

The highest average total activity was found in intermediate polar solvents ethylacetate, ethanol and acetone, dichloromethane had lower total activity values compared to the other intermediate polarity solvents while the hexane extracts had the lowest total activity values [Figure 3.5]. This suggests that the antibacterial compounds present in *C. woodii* leaves are intermediate polar compounds and agrees with the observations made in bioautography [Figure 3.3].

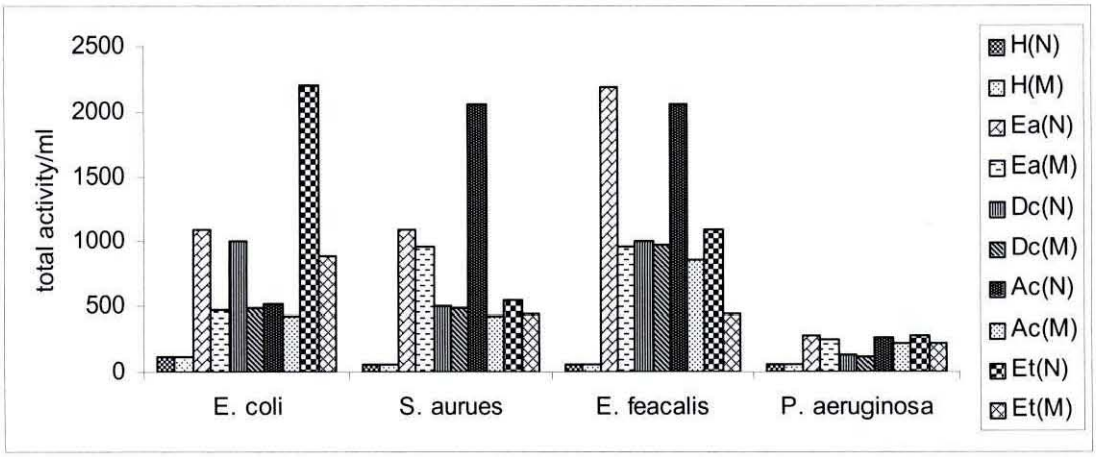

Figure 3:6 Total activity of *C. woodii* extracts of hexane (H), dichloromethane, (Dc), ethylacetate (EA), acetone (Ac) and ethanol (Et) for the LNBG (N) and UP (M) garden samples against four test pathogens.

3.10 General discussion and conclusion

Drying is the most common method of preservation and is achieved by leaving the material in warm dry air, however, the time delay between collecting and processing may affect extractability. Increases in exposure to light, oxygen, temperature and moisture can accelerate deterioration and also affect extractability (Houghton and Raman, 1998).

The method employed for extraction has the following advantages; it is quick, easy to use and compounds that are heat sensitive are extracted successfully from the leaf material. The time frame in which the procedure is performed limits the possible degradation of compounds due to prolonged exposure to solvent. The great limitation of this method is that a number of compounds may not be extracted due to the short time frame during which the samples are in contact with the solvents. However, for its use in these studies, the advantages of direct extraction far outweighed its limitations.

Preliminary extraction studies showed that for all five test solvents, larger quantities of material were present in extracts from the LNBG sample as compared to samples from UP [Table 3.1].

TLC plates developed in CEF solvent system showed the largest numbers of compounds and the best resolution compared to EMW and BEA [Figure 3.1]. This result suggests that the leaves of *C. woodii* have more intermediate polar compounds and tallies with the conclusion in *C. microphyllum* (Kotze and Eloff, 2002).

The fact that polar solvents like ethanol, ethylacetate, dichloromethane and acetone also extracted similar concentrations of non-polar compounds, as did hexane [Figure 3.1], suggested the presence of saponin compounds with polar and non-polar ends, which solubilize in either polar or non-polar solvents (Bruneton, 1995).

MIC and total activity values showed extracts from the LNBG sample to possess more potent antibacterial properties when compared to extracts from the UP sample [Table 3.2]. For these reasons, the LNBG sample was used for further work.

Data from TLC analysis of LNBG sample extracts revealed the presence of two major antioxidant compounds (one with R_f values of 0.85 and the other with an R_f value of 0.35 in EMW separation system) [Figure 3.2], while bioautography showed the presence of a number of antibacterial active compounds in the acetone, ethanol and ethylacetate extracts with R_f values ranging from 0.85 to 0.46 for the chromatogram developed in the EMW system [Figure 3.3]. These results meant that the antibacterial and antioxidant activity of *C. woodii* leaves occurred in compounds whose R_f values lie between 0.85 and 0.35 on TLC plates developed in EMW solvent system.

Hexane extracted 4% of the original material of *C. woodii* leaves [Table 3.1]. TLC plates sprayed with 0.2% DPPH in methanol showed that hexane extracts did not contain any antioxidant active compounds [Figure 3.2], while the bioautograms of the hexane extracts showed one zone of inhibition [Figure 3.3]. All the compounds present in the hexane extract were located near the front of the chromatogram of a TLC plate developed in EMW and sprayed with vanillin spray reagent and had R_f values greater than 0.84 [Figure 3.1]. Thus, hexane could be used to remove ~ 4% of material that is largely inactive from the leaves of *C. woodii* before subsequent extraction with selected solvents. This "washing" procedure would effectively enrich the extract by removing inactive components.

Gram-negative bacteria are generally more resistant to antibiotics and antimicrobials because their cell wall is thicker compared to Gram-positive bacteria.

The two Gram-positive organisms, *S. aureus* and *E. faecalis* as expected showed high sensitivity to the different extracts. The Gram-negative *E. coli* showed sensitivity that compared to that of the two Gram-positive bacteria while *P. aeruginosa* was less sensitive to the test extracts [Figure 3.6]. Because *E. coli* is an important poultry pathogen these results are promising. The quantity of antibacterial compounds present in one g of *C. woodii* leaves diluted to two litres would theoretically still inhibit the growth of *E. coli*, *S. aureus* and *E. faecalis*. The high total activity value for some of the extracts indicates their potential use as replacements for antibiotic feed additives.

Acetone and ethanol are preferred solvents in bioassay application for reasons of safety, cost and availability. Since both solvents extracted large quantities and retained high activity in their extracts, further work was carried out using acetone and ethanol as the main extractants.

Chapter 4 Enrichment procedures

4.1 Introduction

The primary objective of this project was to develop an extract containing high antibacterial and antioxidant activity. In enrichment procedures studies, the aim was to identify solvents that could be used individually, in mixtures or serially as pretreatment or final extractants in a simple extraction procedure.

Many enrichment procedures have been published during the last two decades. The first procedures were extremely time-consuming and error-prone comprising up to 35 partitioning steps or column chromatography steps and not validated (Lobstein-Guth *et al.*, 1983). The first validated method for sample clean-up procedures was published in 1991 (van Beek *et al.*, 1991). An aqueous leaf extract of *Gingko biloba* was enriched over a combination of a polyamide and an 18-carbon (C_{18}) column. The desired phenols remained on the polyamide column while remaining impurities were removed on the C_{18} column. Other researchers have made use of solvent-solvent partitioning steps by means of separatory funnels (Lang and Wai, 1999). The major disadvantage of this procedure is the limited solubility of some compounds in the extraction solvents used. Another technique for extract enrichment involves the use of silica gel in column chromatography.

Bioassay-guided fractionation on column separation simplifies extracts based on polarity of their components. Although the entire procedure may be lengthy, the sample clean up is robust and has been applied successfully by other researchers (van Beek and Taylor, 1996). The effective fractionation of relevant compounds also depends on the sample preparation prior to column chromatographic separation. Alternative extraction procedures to the conventional e.g. the use of supercritical fluids, may also be helpful. However, because of the commercial nature of this project, other factors like cost of setting up and running the separation, the time it will take and quantities that can be yielded have to be considered.

In this project we investigated low cost procedures that would yield an extract with high antioxidant and antibacterial activity.

4.2 Pathway 1

The first pathway was based on results from preliminary extraction studies. Leaf extracts of *C. woodii* contained significant amounts of nonpolar compounds that were largely inactive.

Hexane could be used to defat (remove ~ 4% of non-polar material that is largely inactive from the leaves of *C. woodii*) before extraction with acetone or ethanol for reasons cited in the discussion in Chapter 3. This defatting procedure would effectively enrich the extract by means of removing the inactive components.

4.3 Pathway 2

The second pathway involved the use of various mixtures of acetone in water and ethanol in water solvents to evaluate their application as pretreatment or final extractants. To avoid too many polar impurities, in almost all approaches water is an important constituent of the solvent initially used for the extraction of compounds (Fuzzati *et al.*, 2003). Normally an organic solvent like methanol or acetone is added to improve the rate of extraction because, while most polar compounds are water-soluble, some compounds are poorly soluble in 100% water at room temperature.

4.4 Hexane "wash" (defatting)

A single direct extraction with 10 ml of hexane was performed on one g sample of leaf material from LNBG as a pretreatment procedure before subsequent extraction with ethanol or acetone on the plant residue. The extract was dried at room temperature under a fan and reconstituted in acetone to a concentration of 10 mg/ml.

4.4.1 Results

The resultant hexane-pretreated extracts of acetone and ethanol were analysed together with their respective crude extracts and the hexane extracts to determine how much was removed in the pretreatment and what changes were realised in the pretreated extracts. The crude acetone and ethanol extracts were therefore reference extracts.

4.4.2 Phytochemical analysis

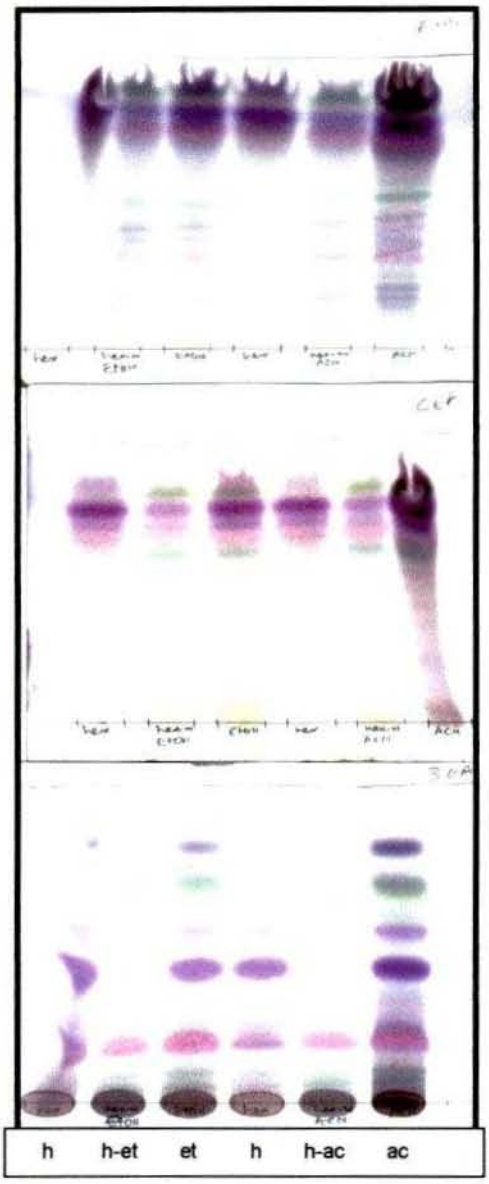

Figure 4:1 TLC profiles of the hexane "wash" extracts developed in EMW (top), CEF (centre), and BEA (bottom) solvent systems and sprayed with vanillin–sulphuric acid reagent. **Lanes from left to right**: Hexane "wash" extracts (h), hexane-pretreated ethanol extract (h-et) and its acetone equivalent (h-ac), crude ethanol extract (et) and crude acetone extract.

Hexane "washing" did not completely remove the inactive components lying at the top of the chromatogram developed in EMW, however, their concentration was reduced as evidenced by the reduction in colour intensity of these compounds in hexane pretreated extracts.

4.4.3 DPPH assay

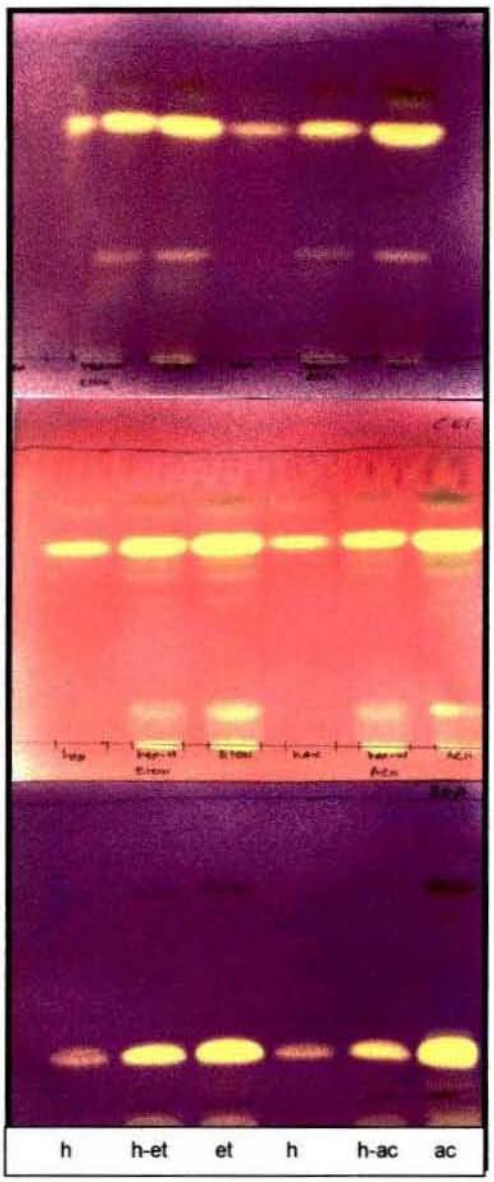

Figure 4:2 TLC profiles of the hexane wash extracts developed in EMW (top), CEF (centre), and BEA (bottom) solvent systems and sprayed with 0.2% DPPH in methanol. **Lanes from left to right**: Hexane "wash" extracts (h), hexane-pretreated ethanol extract (h-et) and its acetone equivalent (h-ac), crude ethanol extract (et) and crude acetone extract.

Contrary to earlier observations, the hexane extracts contained one of the two major antioxidant compounds. However, the compound was extracted in low concentrations as evidenced by the small size and lower intensity of the zone of inhibition. The deviation

from earlier observations could have been caused by a more rigorous extraction procedure in the latter.

4.4.4 MIC and total activity determination

Table 4:1: Amount extracted in mg from one gram, MIC values in mg/ml and total activity values (ml) of hexane "wash" (H), acetone (ac) and ethanol (et), and hexane-pretreated acetone (h-ac) and ethanol (h-et) extracts.

	H	Ac	h-ac	Et	h-et
Quantities	33	82	87	86	92
MIC values in mg/ml					
E. coli	0.63	0.16	0.08	0.08	0.08
S. aureus	0.63	0.16	0.08	0.16	0.08
E. faecalis	0.31	0.04	0.04	0.08	0.04
P. aeruginosa	0.63	0.31	0.16	0.31	0.31
Average MIC	0.55	0.17	0.08	0.16	0.13
Total activity values in ml					
E. coli	52	513	1088	1075	1150
S. aureus	52	513	1088	538	1150
E. faecalis	106	2050	2175	1075	2300
P. aeruginosa	52	265	544	277	297
Average	66	835	1495	741	1224

4.4.5 Discussion

Pretreatment with hexane resulted in an increase in the quantities of materials that were present in the subsequent extracts of acetone and ethanol compared to their crude extracts.

The MIC values of hexane-pretreated acetone and ethanol extracts were enhanced especially against *S. aureus* where MICs were improved by 100% (0.16 mg/ml to 0.08 mg/ml) [Table 4.1], while the changes in sensitivity of *E. coli* and *P. aeruginosa* to the hexane-pretreated extracts were marginal where they were realized. The magnitude of change in average MIC was larger for the acetone extracts (0.17 mg/ml to 0.08 mg/ml) while change in average MIC in the ethanol extracts was less significant (from 0.16 mg/ml to 0.13 mg/ml) [Table 4.1], average MIC values of the acetone extracts were improved to a larger extent compared to ethanol extracts.

The hexane-pretreated extracts showed a significant increase in total activity values compared to their crude extracts. Total activity is doubled against *S. aureus* for hexane-pretreated extracts (513 ml to 1088 ml in acetone extract and from 538 ml to 1150 ml ethanol extracts)[Table 4.1]. In all instances, the total activity of the pretreated extracts was higher when compared to their crude extracts, while there were slight differences in average total activity values between the pretreated acetone and ethanol extracts.

The slight difference in total activity values of hexane-pretreated acetone and ethanol extracts even as relatively large differences in average MIC values were realized can be explained since total activity takes into account both the MIC value and quantity extracted. The poorer MICs of the ethanol extracts were compensated by their larger extraction quantities relative to acetone.

TLC plates sprayed with 0.2% DPPH in methanol showed one of the two major antioxidant compounds to be present in the hexane "wash" extract [Figure 4.2], a result that deviates from earlier observations [Figure 3.2], this result could have arisen owing to increased exposure of solvent to leaf sample or a more vigorous shaking procedure compared to previous extractions

The overall observation from hexane "wash" studies was that pretreatment with hexane prior to extraction with acetone or ethanol not only increases the quantities present in the extracts, but also improves significantly on the antibacterial activity of these extracts.

4.5 Ethanol in water and acetone in water extracts

4.5.1 Introduction

Most biological assays are carried out in water therefore problems arise if the active compounds are only lipid soluble. An alternative is to use a water miscible general solvent like acetone or ethanol. Eloff (1998a) reported that acetone gave the best results in bioassays, due to its low toxicity to test organisms when compared to other solvents such as methanol, chloroform and dichloromethane. Acetone and to a lesser extent ethanol dissolve many hydrophilic and lipophilic components, are miscible with water, volatile and have low toxicity to bioassays.

Ethanol or acetone in water mixtures are safer solvents in extraction procedures and could result in products that may safely be incorporated in feed. They are also useful as extraction solvents because they are inexpensive and readily available. In preliminary extraction studies, ethanol and acetone as individual extractants had high antibacterial activity and high extraction yields. In work on extraction of proanthocyanidins from grape seed, Pekic *et al.*, (1998) concluded that the presence of water in solvent mixtures increased permeability of seed tissue enabling better extraction. Results on other Combretaceae indicate that 100% water does not extract antibacterial compounds (Kotze and Eloff, 2002), however, water extracts antioxidant compounds.

The rationale of using various ratios of solvent in water was to identify mixtures that would selectively extract the bioactive components or otherwise mixtures that would selectively extract inactive material, because different ratios of solvent in water have different polarities and strengths, they can be used to extract different components. Once identified, the suitable solvent mixtures would be used as final extractants or as pretreatment extractants depending on their applicability.

4.5.2 Methods

Various mixtures of acetone in water and ethanol in water were developed and tested as described in 2.2.2, phytochemical analysis was performed as outlined in 2.3 (vanillin spray) and 2.6.1 (DPPH assay).

4.5.3 Results

4.5.3.1 Phytochemical analysis of acetone in water extracts

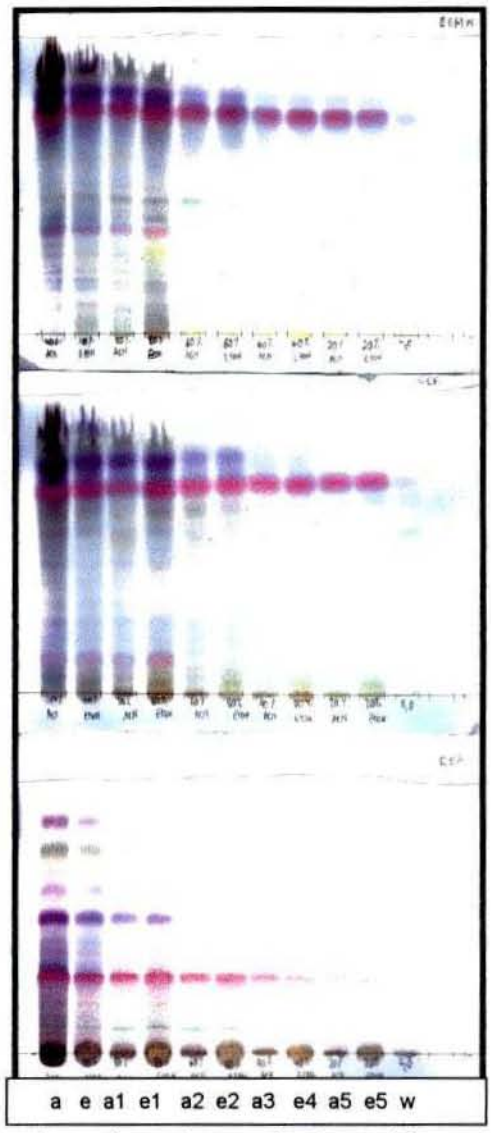

Figure 4:3 TLC profiles for acetone in water and ethanol in water extracts developed side by side in EMW (top), CEF (centre) and BEA (bottom) and sprayed with vanillin–sulphuric acid. **Lanes from left to right**: 100% acetone (a), 100% ethanol (e), 80% acetone in water (a1), 80% ethanol equivalent (e1) 60% acetone in water (a2) 60% ethanol (e2), 40% acetone in water (a3) 40% ethanol equivalent (e3), 20% acetone in water (a4) 20% ethanol equivalent (e4) and water (W) extracts.

As the water content of the solvent mixtures increased, the number of vanillin active compounds that were extracted decreased. All the solvents except water extracted a compound that gave a deep red in colour with vanillin spray and had an R_f value of 0.85 in EMW solvent system.

4.5.3.2 DPPH assay

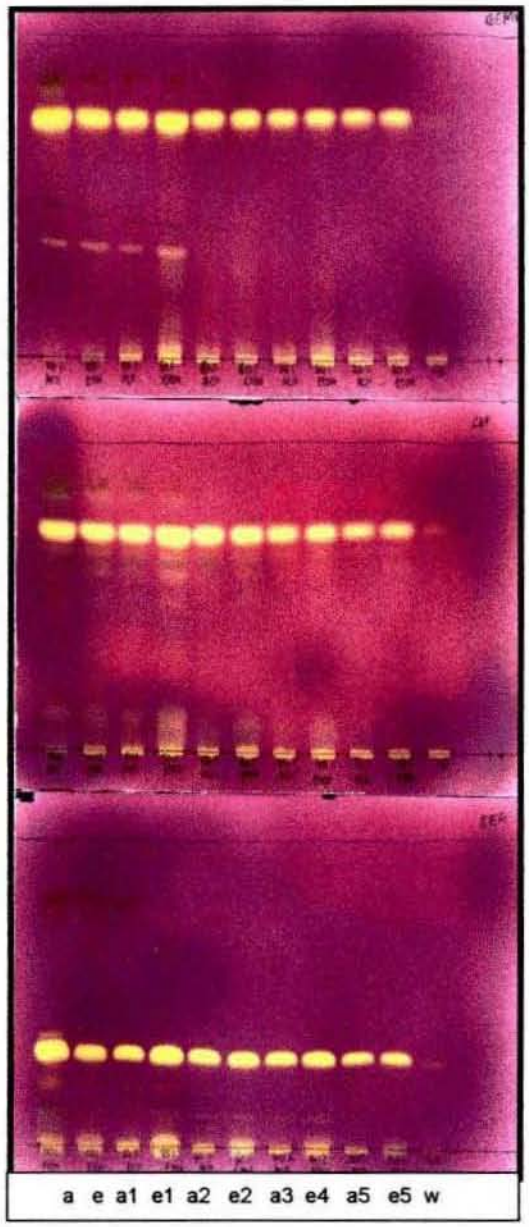

Figure 4:4 TLC profiles for acetone in water and ethanol in water extracts developed side by side in EMW (top), CEF (centre) and BEA (bottom) and sprayed with 0.2% DPPH in methanol. **Lanes from left to right**: 100% acetone (a), 100% ethanol (e), 80% acetone in water (a1), 80% ethanol equivalent (e1) 60% acetone in water (a2) 60% ethanol (e2), 40% acetone in water (a3) 40% ethanol equivalent (e3), 20% acetone in water (a4) 20% ethanol equivalent (e4) and water (W) extracts.

Only 100% acetone, 100% ethanol, 80% acetone and 80% ethanol in water mixtures retained the two major antioxidant compounds. The compound that gave a deep red colour

with vanillin spray reagent and had an R_f value of 0.85 in EMW solvent system was one of the major antioxidant compounds.

4.6 MIC determination of acetone in water and ethanol in water extracts.

The antibacterial activities of acetone, ethanol and acetone or ethanol in water and water extracts were quantified using the 96-well microtitre plate technique (Eloff, 1998b), as described in 2.5. Ten mg/ml solutions of the extracts were tested against *S. aureus*, *E. coli*, *E. faecalis* and *P. aeruginosa*.

4.6.1 MIC and total activity values of acetone in water extracts

Table 4:2: Amount in mg extracted from one g samples and MIC values in mg/ml of 100% acetone (Ace), 80% acetone in water (80% Ace), 60% acetone in water (60% Ace), 40% acetone in water (40% Ace), 20% acetone in water (20% Ace) and water extracts of *C. woodii* leaves.

	Ace	80% Ace	60% Ace	40% Ace	20% Ace	Water
Quantity	84	167	169	164	128	68
			MIC values (mg/ml)			
E. coli	0.16	0.16	0.16	0.31	0.63	1.25
S. aureus	0.16	0.16	0.31	0.31	0.63	2.5
E. faecalis	0.08	0.08	0.16	0.31	0.63	1.25
P. aeruginosa	0.63	0.31	0.63	0.63	0.63	2.5
Average MIC	0.26	0.18	0.32	0.39	0.63	1.88

Eighty percent acetone extracts had higher antibacterial potency compared to the acetone extracts with average MIC of 0.18 mg/ml compared to 0.26 mg/ml respectively. As the water content of the extracts increased from 60% acetone in water to water, the antibacterial activity of the extracts decreased and in all cases the average MICs were poorer than that of 100% acetone extract. The lowest antibacterial activity was present in the water extract.

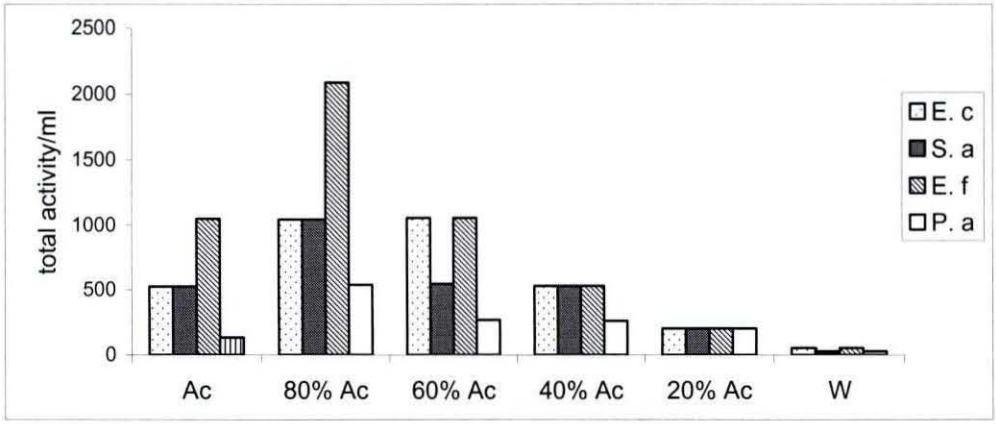

Figure 4:5 Total activity of *C. woodii* extracts of 100% acetone (Ac), 80% acetone in water (80% Ac), 60% acetone in water (60% Ac), 40% acetone in water (40% Ac), 20% acetone in water (20% Ac) and water (W) against *S. aureus* (S.a), *E. faecalis* (E.f), *E. coli* (E.c), and *P. aeruginosa* (P.a).

The highest total activity values were in 80% acetone in water extracts followed by 60% acetone in water and 100% acetone. Though 100% acetone extracts had the best MIC values, 80% and 60% acetone in water solvents extracted larger quantities hence the better total activity values.

4.6.2 **MIC and total activity values of ethanol in water extracts.**

Table 4:3: Amount in mg extracted from one gram and MIC values in mg/ml of 100% ethanol, 80% ethanol in water (80% Ethanol), 60% ethanol in water (60% Ethanol), 40% Ethanol in water (40% Ethanol), 20% Ethanol in water (20% Ethanol) and water extracts of *C. woodii* leaves.

	Ethanol	**80% Ethanol**	**60% Ethanol**	**40% Ethanol**	**20% Ethanol**	**Water**
Quantity	92	184	198	168	149	72
			MIC values in mg/ml			
E. coli	0.16	0.16	0.31	0.31	0.31	1.25
S. aureus	0.16	0.16	0.16	0.31	1.25	1.25
E. faecalis	0.08	0.04	0.16	0.31	0.63	1.25
P. aeruginosa	0.63	0.31	0.31	0.63	0.63	1.25
Average MIC	0.26	0.17	0.24	0.39	0.71	1.25

The average MIC values of the 80% ethanol in water and 60% ethanol in water extracts were better compared to the ethanol extracts. Eighty% and 60% ethanol in water extracts showed good activity against *P. aeruginosa* (MIC values of 0.31 mg/ml for both extracts).

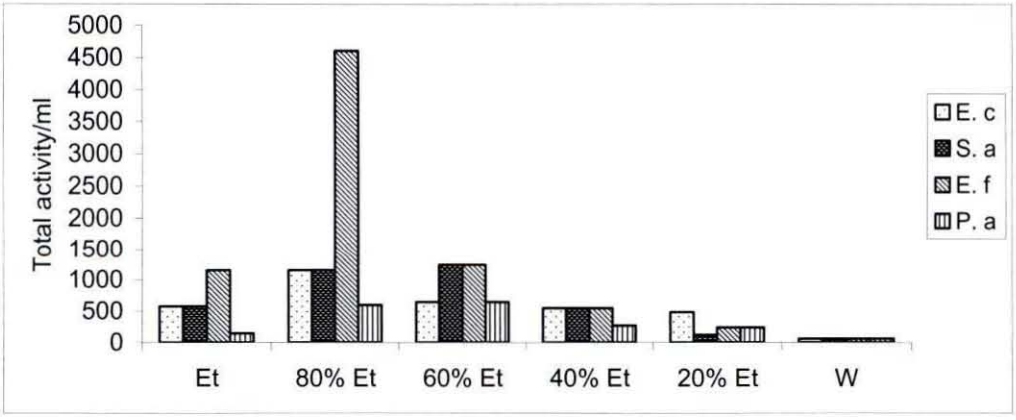

Figure 4:6 Total activity of *C. woodii* extracts of 100% ethanol (Et), 80% ethanol in water (80% Et), 60% ethanol in water (60% Et), 40% Ethanol in water (40% Et), 20% Ethanol in water (20% Et) and water (W) against *S. aureus* (S.a), *E. faecalis* (E.f), *E. coli* (E.c) and *P. aeruginosa* (P.a).

The highest total activity values were in 80% ethanol in water extracts followed by 60% ethanol in water and 100% acetone. As observed with acetone in water extracts, the antibacterial activity of the ethanol in water extracts decreased as the water content of the extractant increased after 80% ethanol in water.

4.7 Summary discussion and conclusions

Hexane is often used as a defatting solvent, removing oils and fatty acids from test samples. The subsequent increase in quantities extracted and improvement in activity values realized in hexane-pretreated extracts could be because; hexane had removed the fatty acid composition of the leaves resulting in rupturing of cellular membranes consequently exposing more compounds in the leaves of *C. woodii* to better extraction with acetone and ethanol.

Acetone and ethanol in water extracts had fewer vanillin-active compounds on TLC plates as the water content of the mixture increased [Figure 4.1] and the DPPH assay showed that only the acetone, ethanol and 80% acetone or ethanol in water extracts contained the two major antioxidant compounds while the other mixtures extracted only one (with R_f 0.85 in EMW solvent system) [Figure 4.2]. The water extract had a few vanillin active compounds and showed no antioxidant active compounds in the DPPH assay [Figures 4.1 and 4.2]. The absence of antioxidant compounds in the water extract was surprising as antioxidant compounds are usually considered to be water-soluble. Possibly the antioxidant compounds though water-soluble, may be present within cellular organelles whose membranes did not rupture in water.

Different ratios of acetone or ethanol in water extractants resulted in larger quantities of material being extracted compared to the individual extraction abilities of acetone, ethanol or water [Tables 4.2 and 4.3]. The quantity extracted gradually increased, reaching a maximum when 60% acetone or 60% ethanol in water solvents were used. This increase could be because the presence of water in the extraction solvents brought water-soluble compounds in contact with acetone or ethanol enabling better extraction.

Eighty percent acetone or ethanol in water extracts possessed the most potent antibacterial activity of the mixtures and had similar or better MIC values to their respective crude extracts [Tables 4.2 and 4.3]. Because they extracted larger quantities while retaining similar MIC values, 80% mixtures had higher total activity values. This result means that 80% acetone and 80% ethanol in water solutions are better solvents for extraction of the antibacterial compounds in the leaves of *C. woodii* compared to their

individual solvents. It is interesting that 70 % ethanol has been used frequently in the extraction of amino acids from plants (JN Eloff, personal communication)

Antibacterial activity of the extracts decreased as the water content of the mixtures increased with the water extracts having the lowest antibacterial activity against the test organisms. This result tallies with the observations in *Combretum microphyllum* (Kotze and Eloff, 2002). Twenty percent acetone or ethanol in water solvent mixtures extracted a large quantity of material (~ 15%), but their extracts had very little antibacterial and antioxidant activity. This meant that these solvent mixtures could be employed as pretreatment solvents in extract enrichment.

The low antibacterial activity of water extracts *in vitro* does not support its common use as the principal extraction solvent by traditional healers. The healing properties of their remedies could possibly be linked to antioxidant activity because antioxidants are usually water-soluble. Another possibility is that the extraction procedures employed during preparation of these remedies allows for more rigorous extractions e.g. boiling. Extraction procedures also often involve soaking the plant material in water over days, this might allow for fermentation thus changing the extractant from water to an alcoholic mixture that will better extract the antibacterial compounds.

Although water extracted c.7% of the material from the leaves of *C. woodii*, the extracts had no antioxidant activity in the DPPH assay and very little antibacterial activity. It was not considered for application as a pretreatment extractant because it would be difficult to remove traces of water remaining in the extract prior to extraction with acetone or ethanol, and also because water extracts did not retain many vanillin active compounds as seen on TLC plates sprayed with vanillin reagent. The 7% that was extracted by water could have been some salts and sugars.

In summary, these extraction studies provided a clean-up step using 20% acetone in water and 20% ethanol in water while 80% acetone or ethanol in water mixtures were identified as possible final extractants.