Chapter 2

Selection of the best extractant

2.1 Problem statement and aim of the exercise

There has been a substantial increase in the number of papers where authors have screened plants for antimicrobial properties. It is clear that researchers use many different extractants, for example 80% ethanol (Vlietinck *et al.,* 1995), methanol (Taylor, *et al.,* 1995), petroleum ether, chloroform, ethanol and water (Salie *et al.,* 1996). After reviewing the re-awakening of pharmacognosy, Cordell (1993) concluded, "There is clearly substantial room for improvement in the extraction methodologies given that there are a variety of techniques that could be used to prepare extracts". Farnsworth (1996) stated that the biggest problem in drug development with plants is answering a very simple question: what kind of extract should we test?

There are many ways in which to extract the active components from plants.
It was decided in this study to evaluate a number of extractants with different polarities (and selectivity) to determine the extractant that would firstly give a high yield of extracted residue, and secondly extract the active components. If an extractant could be found that extracted mainly biologically active compounds, it would simplify the isolation

of these compounds to a large degree. A number of possible solvents, along with their polarities and grouping based on selectivity, are listed in Table 2.1.

Table 2.1 Different solvents with their distinctive polarities and selectivity groups (Snyder and Kirkland, 1979)

SOLVENT	POLARITY	GROUP
Hexane	0.1	I
Isopropylether	2.4	I
Diethyl ether	2.8	I
Ethylacetate	4.4	IV a
Tetrahydrofuran	4.0	III
Dichloromethane	3.1	V
Acetone	5.1	VI a
Methanol	5.1	II
Ethanol	4.3	II
Water	10.2	VIII

Following published investigation on several *Terminalia* species, it was decided to investigate the antibacterial activities of *Terminalia* species found in southern Africa. This would serve the purpose of scientifically verifying the use of extracts for their wound-healing properties by rural people, personal communication (traditional healer in Pretoria city central). An additional aspect is that proven antibacterial activity of these

selected plants would have clear advantages for rural people reliant on medicinal plants for cost-effective treatment of various bacterially induced afflictions. We decided to concentrate on leaves and not other plant parts for sustainable utilisation reasons. Initially only three species (based on their availability) representative of the three sections of *Terminalia* (Table 3; Carr, 1988), i.e. *T. sericea (*Section Psidioides), *T. phanerophlebia* (Section Platycarpae) and *T. prunoides* (Section Abbreviate) were selected for the evaluation of the best extractant.

2.2 Materials and Methods

2.2.1 Collection and processing of plant material

The study was performed on the leaf material of *T. sericea*, *T. phanerophlebia* and *T. prunoides*. Plant material was collected from the Lowveld National Botanical Garden in Nelspruit. The permanent label on the tree assisted in the identification. The collection origin and voucher specimens are deposited in the Lowveld National Botanical Garden Herbarium in Nelspruit.

2.2.2 Extraction of plant material

Leaves were dried in the shade at room temperature. Stems and thick veins were removed and the remainder of the leaf material ground to a fine powder using a Jankel and Kunkel Model A10 mill. Extraction was carried out on different samples (1g) with 10 different solvents (10µl) of varying polarities as described in Table 2.1. Extracts were dried in a cold air stream and the mass of residue resulting from extraction with each solvent for the three plant species was noted.

2.2.3 Analysis of extracts by thin layer chromatography

Thin layer chromatography (TLC) was used to separate the components of each extract. The efficacy of the eluant used to separate the components by TLC depends on the polarity of the components and the selectivity of the eluents (Kirkland, 1979). Table 2.2 depicts the various eluting solvent systems developed in the Program for Phytomedicine at the University of Pretoria, employed in TLC studies.

Table 2.2. Three different eluant systems used to analyze plant extracts

ELUANT	COMPOSITION	SEPARATION CHARACTERIZTICS
BEA	Benzene:ethanol:ammonia 9:1:0.1 (v/v/v)	Separates low polarity components
CEF	Chloroform:ethyl acetate:formic acid 10:8:2 (v/v/v)	Separates intermediate polarity components
EMW	Ethyl acetate:methanol:water 20:2.7:2 (v/v/v)	Separates high polarity components

TLC was an integral part of the process of identification and isolation of active substance(s) with antibacterial activity in the three *Terminalia* species. The dried extracts of the ten different solvents were reconstituted to a concentration of 10 mg/ml in acetone. Acetone was the solvent of choice owing to its wide extraction capacity and low toxicity towards the test organisms in the bioassay procedures (Eloff, 1998b).

Approximately 100 µg aliquots (5 µl of a 20 mg/ml solution) of each of the extracts were loaded in 1 cm bands on three 20 x 10cm F_{254} TLC plates (Merck, 0.25 mm thick) and each of these was developed with CEF, EMW or BEA (Table 2.2). The extracts were applied approximately 1 cm from the bottom of the plates with a micropipette and allowed to develop for 8 to 9 cm in a tank containing eluant. The atmosphere in the tank was saturated by placing filter paper wetted with the eluant against the walls of the tanks, which were then sealed with lids.

Once developed, the separated compounds were investigated under a Camac Universal TL-600 UV light at 360 nm and 254 nm and the fluorescing (360nm), or quenching(254nm) compounds marked. The chromatograms were then sprayed with vanillin spray reagent (0.59 g vanillin dissolved in 100 ml sulphuric acid: ethanol [4:1] according to Stahl, 1969) and *p*-anisaldehyde spray reagent (5 ml *p*-anisaldehyde dissolved in 90 ml ethanol and 5 ml concentrated sulphuric acid (Carr and Rogers, 1986). The plates were heated in an oven at 105 °C for several minutes until the coloured bands showed clearly on the plates. On each of the plates, from 1 to 12

different compounds could be seen appearing as different coloured bands across the length of the run.

2.2.4 Antibacterial assay

Minimum inhibitory concentration (MIC) values were determined by a microplate serial dilution method (Eloff, 1998c). The test organisms were the Gram-positive *Staphylococcus aureus* (ATCC 29213) and *Pseudomonas aeruginosa* (ATCC 25922), and the Gram-negative *Escherichia coli* (ATCC 27853) and *Enterococcus faecalis* (ATCC 29212). These are the reference strains recommended by the National Committee for Clinical Laboratory Standards (Villanova, Pennsylvania, USA). These bacteria are responsible for most nosocomial infections. The cultures were grown at 37°C in Mueller-Hinton broth (Merck), maintained at 4 °C, and were regularly subcultured using a 1% inoculum. Approximately every six months, fresh cultures were obtained from Dr. F. Huygens in the Department of Medical Microbiology of the University of Pretoria.

The MIC assay used subsequently as well is described here:
Dried plant extracts were reconstituted to 10 mg/ml in acetone. Four 96-well microtitre plates were labeled with sample codes in landscape mode. Using a Socorex multichannel pipette, the plant extracts (100 µl) were serially diluted 50% with water in 96-well microtitre plates. An aliquot (100 µl) of each test bacterial species was added to the plant extracts in the wells and mixed. MH broth cultures of preferably 24 hours, but not more than 10 days old, were used in the assay, following the recommendation of

Eloff (1998b). The inoculation of the wells as well as the subculturing of the bacteria was undertaken in a laminar flow cabinet in order to minimize contamination. The four microplates (one for each of four test organisms) were stacked upon each other, covered by an empty plate to serve as a lid and sealed in a plastic bag. The plates were then incubated at 37° C in an incubating oven.

After incubating overnight for approximately 18 hours, the plates were removed from the incubator and 40 µl of a 0.2 mg/ml solution of INT (ρ-iodonitrotetrazolium violet) were added to each well with a multichannel micropipette. The plates were incubated for a further half an hour at 37° C before the first evaluation of colour development was made. The coloured tetrazolium salt (INT) acts as an electron acceptor and is reduced to a colourless formazan product by biologically active organisms (Eloff, 1998c), so where bacterial growth is inhibited, the solution in the well will remain clear after incubation with INT. In this study, the wells with decreased colour development were regarded as showing growth inhibition, and were used to calculate the MIC values. The microplates were again evaluated after 60 minutes and 120 minutes to confirm the results. The microtitre plate containing *Enterococcus faecalis* took up to 8 hours of incubation to show bacterial growth (or the lack thereof), as reflected by colour development. The minimum inhibitory concentration (MIC) values of each plant extract for each bacterial species were calculated from the original concentration of the extracts.

2.3 Results and Discussion

2.3.1 Extraction of raw material

The mass that each solvent extracted from 500 mg leaf material is recorded in Table 2.3.

Table 2.3. The mass (mg) of residue extracted by each of the ten different extractants from 500 mg of dried leaves of *T. sericea*, *T. phanerophlebia* and *T. prunoides*

EXTRACTANT	MASS RESIDUE EXTRACTED (mg)			AVERAGE (mg)
	T. sericea	*T. phanerophlebia*	*T. prunoides*	
Hexane	8	8	9	8.3
Isopropylether	8	10	10	9.3
Ethylether	5	11	19	11.6
Ethyl acetate	8	16	20	14.6
Tetrahydrofuran	24	26	132	60.6
Dichloromethane	6	13	34	17.6
Acetone	11	14	97	40.6
Methanol	10	11	96	39
Ethanol	13	19	126	52.6
Water	4	6	5	5
AVERAGE	9.7	13.4	54.8	25.9

Tetrahydrofuran (60.6 mg), methanol (39 mg), acetone (40.6 mg) and ethanol (52.6 mg) extracted the most material in terms of mass from the three plants. These extractants were situated in the intermediate to polar region of solvents. The highest average mass was extracted from *T. prunoides*.

2.3.2 TLC analysis of extracts

The reason for using different extractants was to determine if any of the extractants would preferentially extract antibacterial compounds to facilitate subsequent isolates of these compounds. In this section the complexity of the extracts were evaluated by TLC. An extract with few compounds and high antibacterial activity would be a logical choice for isolating the antibacterial compounds. On the other hand, the higher the number of compounds extracted by different extractants, the better the chance that bioactive compounds will be extracted. Each of the extracts was analyzed by TLC using the three solvent systems. The average number of bands separated with the different extracts varied from 2.5 (water) to 7.2 (dichloromethane) (Table 2.4)

Dichloromethane, isopropylether and acetone extracted the highest number of compounds that reacted with the vanillin spray reagent on the TLC plates, based on the number of visible bands on the TLC. BEA as an eluant showed the best results. Because BEA separates non-polar compounds well, this indicates the character of the compounds present in *Terminalia* extracts. Most compounds had R_f values in the mid- to more polar range, correlating with the polarity of the solvents that extracted a higher mass of residue from the plants. (Table 2.4)

Table 2.4. Average number of compounds visible on chromatograms of 10 *Terminalia* leaf extracts using three different eluents (chromatograms treated with vanillin spray reagent)

EXTRACTANT	AVERAGE NUMBER OF COMPOUNDS			
	BEA (non-polar)	CEF (intermediate)	EMW (polar)	AVERAGE
Hexane	4.7	4.3	2.8	3.9
Isopropylether	8.4	7.8	5.0	7.0
Ethylether	7.4	6.0	5.0	6.1
Ethyl acetate	6.7	6.7	4.3	5.9
Tetrahydrofuran	5.2	6.5	3.0	4.9
Dichloromethane	8.4	8.2	5.0	7.2
Acetone	8.2	7.5	4.5	6.7
Methanol	7.7	6.7	5.0	6.4
Ethanol	6.5	6.2	3.8	5.5
Water	2.7	2.5	2.3	2.5

2.3.3 Antibacterial assay

The average MIC of all four test organisms were evaluated of all 10 extractants as was done by Kotze and Eloff (2001). The results were similar for acetone although the results shown in table 2.5 were the average for all four organisms, ranging from 0.58 for water to 1.14 mg/ml for acetone.

Table 2.5. Average MIC values in mg/ml of each of the extractants against the four test organisms

Extractant	Average MIC value mg/ml
Hexane	0.78
Isopropyl ether	0.74
Ethyl ether	1.08
Ethyl acetate	0.91
Tetrahydrofurane	0.69
Dichloromethane	1.10
Acetone	1.14
Methanol	0.70
Ethanol	0.89
Water	0.58

The average total activity values of the various extracts against the four test bacteria varied from 11 to 126 ml for the different extractants (Table 2.6). *T. phanerophlebia* had the highest total activity for the different extractants (75 ml) compared to 51 and 50 ml for *T. prunoides* and *T. sericea* respectively.

Table 2.6. Total activity values (ml/g) of each of the plant extracts evaluated against Ps= *Pseudomonas aeruginosa, Ec= Escherichia coli, En= Enterococcus faecalis* and St= *Staphylococcus aureus*

PLANT	*T. sericea*				*T. phanerophlebia*				*T. prunoides*				
Organism	***Ps***	***Ec***	***St***	***En***	***Ps***	***Ec***	***St***	***En***	***Ps***	***Ec***	***St***	***En***	**Ave/ Extr**
Extractant													
Hexane	200	15	44	44	5	5	5	42	200	15	44	44	56
Isopropyl alcohol	32	4	16	32	6	12	48	100	32	4	16	32	28
Ethyl ether	125	4	14	28	6	48	48	100	125	4	14	28	45
Ethyl acetate	80	47	5	19	8	64	267	123	80	47	5	19	64
Tetrahydrofuran	200	141	14	57	26	51	51	104	240	141	14	57	91
Dichloromethane	50	2	13	13	8	65	130	260	50	2	13	13	51
Acetone	52	2	6	26	8	33	280	280	52	2	6	26	64
Methanol	90	3	11	45	15	61	122	122	90	3	11	45	52
Ethanol	433	4	16	65	34	34	271	136	433	4	16	65	126
Water	11	11	11	21	7	7	7	7	11	11	11	21	11
Average TA	127	23	15	35	12	38	123	127	131	23	15	35	59
Average TA/Plant	50				75				51				

TA = total activity =(mass extracted in mg from 1g / MIC in mg ml^{-1})

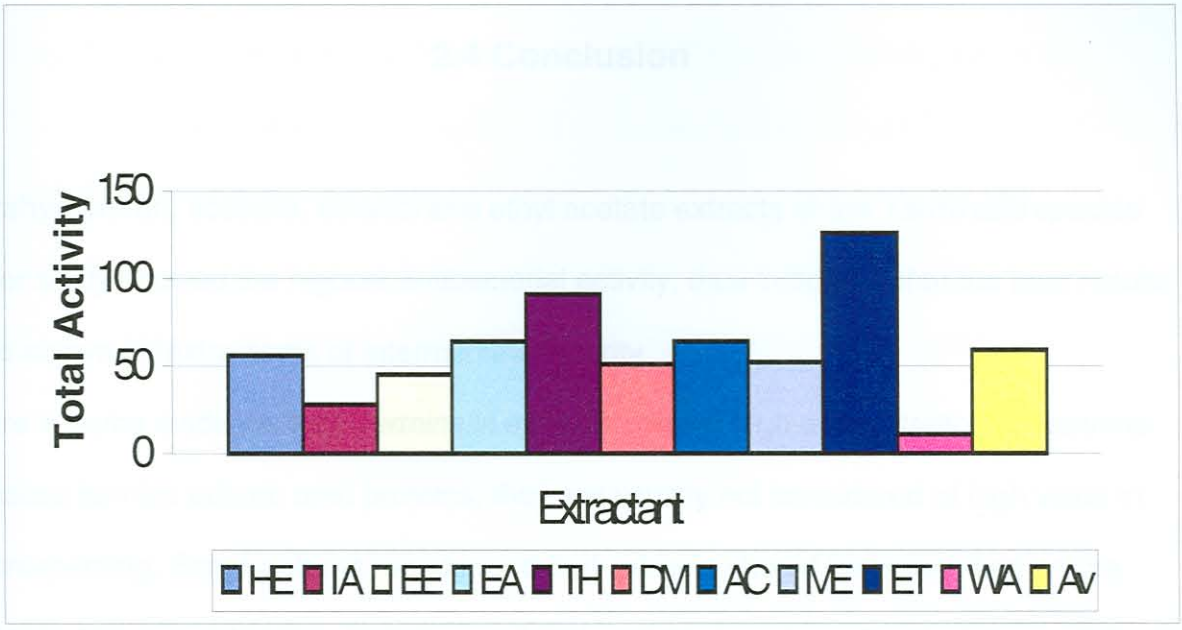

Fig.2.1. The average value of the total activity of each of the extractants evaluated against *Pseudomonas aeruginosa, Escherichia coli, Enterococcus faecalis* and *Staphylococcus aureus.* HE= Hexane, IA= Isopropyl alcohol, EE= Ethylether, EA= Ethyl acetate, TH=Tetrahydrofuran, DM=Dichloromethane, AC=Acetone, ME=Methanol, ET= Ethanol, WA= Water and Ave= Average

2.4 Conclusion

Tetrahydrofuran, acetone, ethanol and ethyl acetate extracts of the *Terminalia* species under study showed the highest antibacterial activity, thus indicating that the best results were shown by extractants of intermediate polarity.

There is some evidence that *Terminalia* extracts contain high concentrations of tannins. Because tannins adsorb onto proteins, they are usually not considered of high value in bioprospecting. Some authors recommend that extracts should be detannified before bioassay to remove tannins. Tannins are highly soluble in polar solvents. Intermediate polarity extracts had the highest antibacterial activity in this experiment, indicating that tannins are probably not responsible for the antibacterial activity in the leaves of the *Terminalia* species.

The average total activity of the three plants compared relatively well with each other with *T. phanerophlebia* showing the highest total activity against *S. aureus* (267 ml for ethyl acetate, 280 ml for acetone and 271 for ml ethanol) whereas the total activity values for *T. prunoides* (5-44 ml) and *T. sericea* (5-44ml) were not as promising against the same bacterial species.

In considering various solvents as extractants, tetrahydrofuran (60.6 mg) yielded the largest residue mass, followed by acetone (40.6 mg) and ethanol (52.6 mg). Ethyl ether and ethyl acetate extracts showed the best results concerning clear separation of components by TLC, while dichloromethane and acetone also provided good results. On

counting the number of compounds visible on the TLC chromatograms, both with vanillin- and anisaldehyde spray reagents, dichloromethane (Average 7.2) showed the best results, with acetone also producing good results. On determining the total activity, the ethanol extract (126 ml) had the highest total activity with tetrahydrofuran (Average 91 ml), acetone (64 ml) and ethyl acetate (64 ml) also displaying promising activity.

In conclusion, because of the qualities of reasonable extraction yield, clear separation and high number of components in TLC, and good total activity against various test bacteria, acetone was a good general extractant. A similar study was done by Kotze and Eloff (2001) on *Combretum microphyllum* and obtained results that compared well with the study done here with MIC values against *S. aureus* of 0.01 mg/ml with acetone as extractant. They found MIC values against *E. faecalis* (0.11 mg/ml), *P. aeruginosa* (0.23 mg/ml) and *E. coli* (0.03 mg/ml). This also supports the results of Eloff (1998a) who investigated different solvents with respect to the diversity of compounds extracted, the number of inhibitors extracted, the rate of extraction, the ease of removal of solvent and the potential biological hazard posed by the solvent. Consequently acetone was selected as the best extractant for the large-scale isolation of antibacterial compounds.