Chapter 3

Selection of the best *Terminalia* species for isolation of antibacterial compounds

3.1 Problem statement and aim of the exercise

Rogers and Verotta (1996) stated that of the 99 African species of *Combretum,* only 25 species have been subjected to any form of scientific study. The same situation holds for *Terminalia* spp. Of the 11 species occurring in South Africa, only *T.sericea* has been investigated superficially to date. In the previous chapter, it could be seen that there were large differences in specificity of the different extracts to the different bacterial species. *T. phanerophlebia*, for example, had the highest average total antibacterial activity but *T. prunoides* provided the highest yield in terms of mass extracted. It was therefore decided to expand the research to seven *Terminalia* species found in southern Africa. Each species would be subjected to an *in vitro* investigation for their potential antibacterial activity. From these results the best *Terminalia* species to use for isolating the antibacterial compounds would be determined.

3.2 Material and Methods

3.2.1 Collection and processing of plant material

Leaf material of *T. sericea, T. prunoides, T. phanerophlebia, T. gazenzis, T. sambesiaca, T. mollis* and *T. brachystema* was collected from the Lowveld Botanical Gardens in Nelspruit during autumn. Voucher specimens were deposited in the Garden's herbarium. The leaves were dried at room temperature and after the stems were removed, ground to a fine powder with a Jankel and Kunkel model 10A mill. The powder was stored in a closed glass container at room temperature in the dark to limit photo-oxidative changes.

3.2.2 Extraction and TLC analysis

One gram of each powdered plant was extracted in 10 ml of acetone based on the results presented in Chapter 2. The amount extracted was increased from the 500 mg used in the previous chapter because of the relatively low antibacterial activity and low quantity extracted in the previous experiment. TLC was performed on the extracts using CEF as eluant. The TLC plate was sprayed with vanillin after development. Gallic acid, which has been isolated from *Terminalia arjuna* and shown to have antibacterial activity (Sato *et al.,* 1997) was used as a standard.

3.2.3 Antibacterial assay

The antibacterial activity of the seven *Terminalia* species was evaluated using bioautography (Buege and Kline, 1972) against four test organisms, namely *S. aureus, E. faecalis, E. coli* and *P. aeruginosa.*

Plant extract components were separated using TLC as described in section 2.2.3. TLC plates were developed using the eluents CEF, EMW and BEA, one for each of the four test organisms. The fluorescent components on each chromatogram were visualised under UV light. Areas of fluorescence (360 nm) or fluorescent quenching (254 nm) were marked and the chromatogram was scanned for future reference.

The plates were left overnight in front of an air stream in a fume cabinet to completely remove residual solvent that might be lethal to the bacteria in the bioautography experiments. The plates were sprayed the next morning with 48-hour-old bacterial cultures. Approximately 20 ml of the culture was centrifuged at 3500 rpm for 10 minutes. The clear supernatant was decanted and discarded. The bacterial pellet was retained and, if it was smaller than 2 to 3 mm in diameter, the process was repeated to obtain a clearly visible pellet. This pellet was resuspended in 20 ml sterile MH broth using a vortex shaker. The resulting mixture was poured into a glass spray-tube that had previously been tested with clean water to verify the spray pattern. The marked TLC plates were sprayed until clearly wet with bacteria and then allowed to dry for 5 to 10 minutes to remove excess moisture. Each plate was then placed in a closed container on a raised surface with enough water below the TLC plate (but not touching it) to

provide 100% relative humidity. The container was placed in an incubating oven and left overnight at 37^{0}C. The next morning the plates were sprayed with a 2 mg/ml solution of INT (Sigma) in water and placed back in the incubating container in the oven. The plates were examined after 30, 60 and 120 minutes before being dried and scanned to provide a permanent record. Clear zones against a pink background indicated regions of bacterial growth inhibition, as the tetrazolium salt (INT) was reduced to a pink colour only where bacteria were not actively growing.

MIC values were also determined for each of the *Terminalia* species against each of the four test bacteria.

3.3 Results and Discussion

3.3.1 TLC analysis

The chromatogram in Fig. 3.1 indicates the similarities in compounds extracted from the seven different *Terminalia* species under investigation. The complexity of the plants can be seen from the number of components in each of the plant extracts. Similar compounds are visible in most of the extracts.

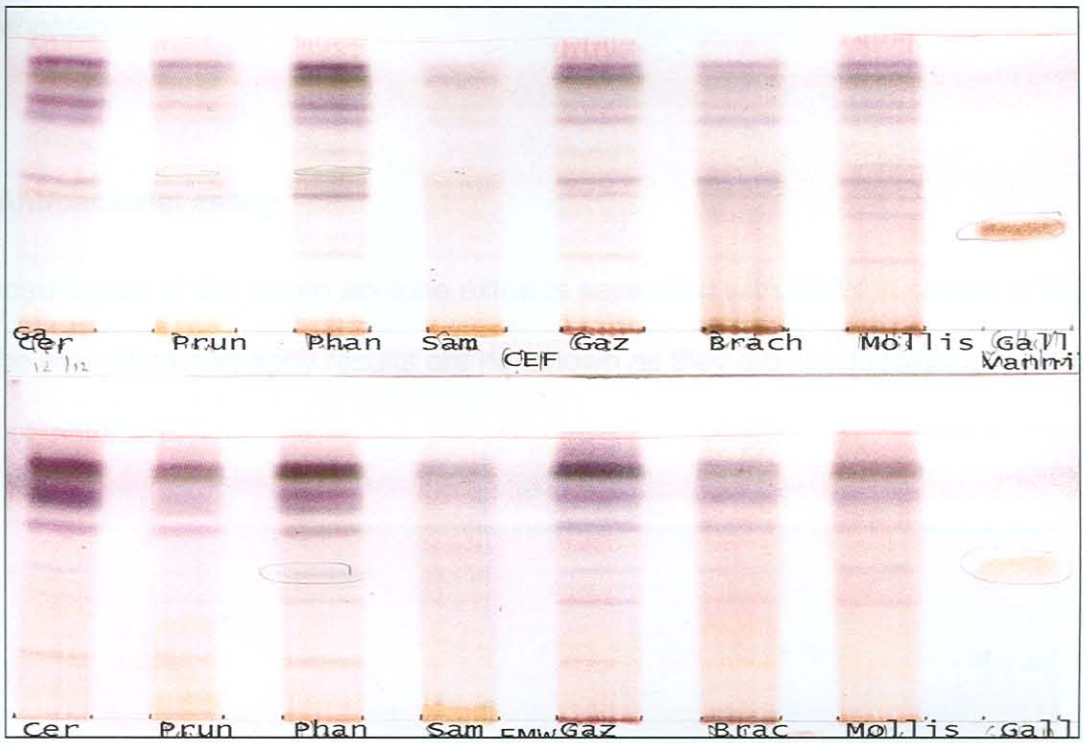

Fig. 3.1. Chromatogram of seven *Terminalia* species developed with CEF as eluant [top] and EMW [bottom] and sprayed with vanillin spray reagent where, Cer = *T. cerisea,* Prun = *T. prunoides,* Phan = *T. phanerophlebia,* Sam = *T. sambesiaca,* Gaz = *T. gazenzis,* Brac = *T. brachystema,* Mollis = *T. mollis,* Gal = Gallic acid.

T. sericea and *T. brachystema* are of the same Psidiodes section, *T. prunoides* belongs to the Abbreviate section and the rest are from the Platycarpae section. It does not appear that a differentiation on the basis of sections can be done using TLC as a technique other than the fact that *T. prunoides* has a slightly different chromatographic

profile from the rest. Gallic acid seems to be present in all the plants, most visibly in *T. phanerophlebia*.

3.3.2 Antibacterial assay

The bioautogram of the seven acetone extracts separated with EMW is shown in Figure 3.2. The other bioautography results are not shown as they did not display such clear zones of inhibition.

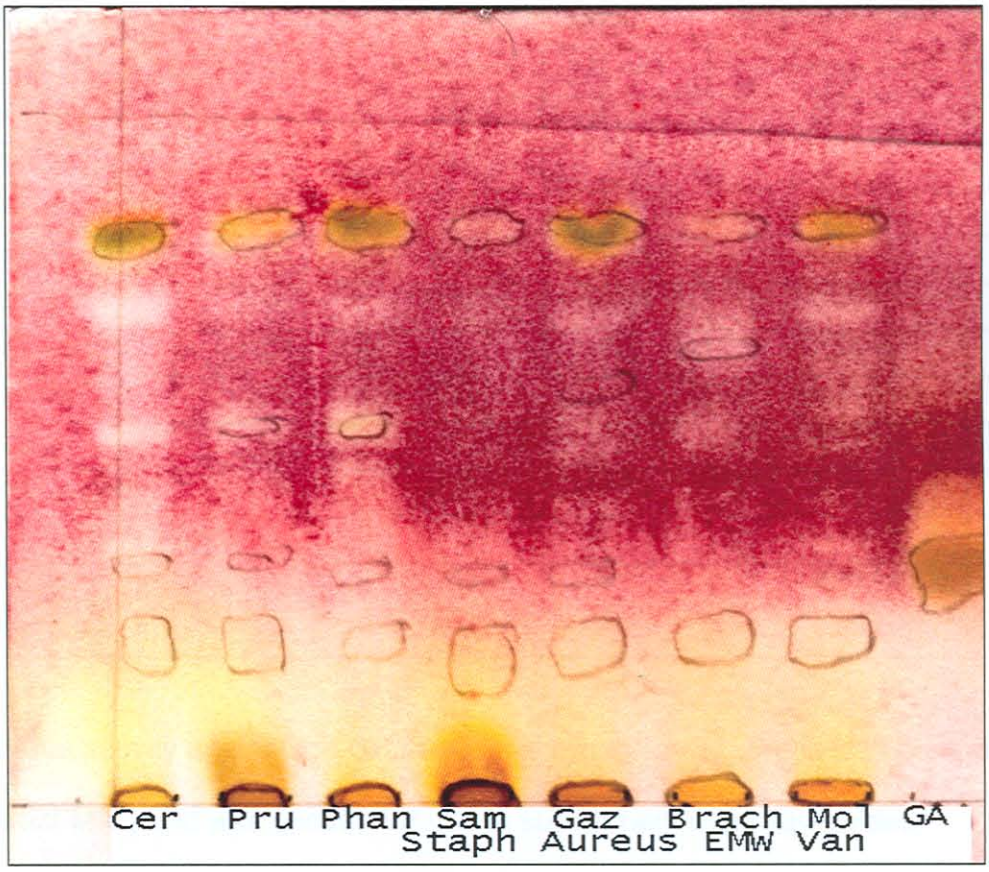

Fig. 3.2. Bioautogram of acetone extracts of seven *Terminalia* species separated with EMW and sprayed with *S. aureus*. Areas circled indicate fluorescent compounds.

MIC values for each plant extract against each bacterial species are shown in Table 3.1. The results are expanded further in Table 3.2 where the mass extracted, the acetone soluble fraction of the extract and the total antibacterial activity for each *Terminalia* species are recorded.

Table 3.1. MIC values (mg/ml) of the seven *Terminalia* species tested against *Pseudomonas aeruginosa, Escherichia coli, Enterococcus faecalis* and *Staphylococcus aureus*

EXTRACT	MIC (mg/ml)				
	S. aureus	*E. faecalis*	*E. coli*	*P. aeruginosa*	AVERAGE
T. sericea	0.16	0.63	0.31	0.16	0.32
T. prunoides	0.13	2.13	2.13	2.13	1.63
T. phanerophlebia	0.11	3.38	0.21	0.21	0.98
T. sambesiaca	0.20	3.25	0.20	0.41	1.02
T. gazenzis	0.06	0.25	0.25	0.25	0.2
T. brachystema	0.28	1.13	0.14	0.56	0.53
T. mollis	0.34	1.38	0.34	0.09	0.54
Average/organism	0.18	1.74	0.51	0.54	0.75
Gallic acid	0.31	2.50	0.31	0.31	0.86
Gentamycin*	0.16	1.00	0.16	0.63	0.49

The antibacterial activity against the Gram-positive organism *S. aureus* was generally the best (lowest MIC) and was chosen for use in subsequent bioassay-guided fractionation for the discovery of antibacterial compounds.

Table 3.2. Mass (mg) extracted with acetone from 500 g leaf material with the total mass soluble in acetone, the MIC values against four bacteria, and the total activity of the seven different *Terminalia* species

PLANT/COMPOUND	TOT EXT (mg)	TOT AC SOL (mg)	MIC (mg/ml)	TOTAL ACTIVITY
T. sericea	56	10	2.2	4.60
T. prunoides	99	34	4.7	7.20
T. phanerophlebia	53	27	1.4	19.80
T. sambesiaca	126	52	1.5	33.80
T. gazenzis	53	16	0.7	23.80
T. brachystema	89	36	1.4	19.30
T. mollis	63	22	1.1	20.00
Gallic acid			0.09	
Gentamycin			0.03	

Tot Extr = Total mass extracted in mg

Tot Ac sol = Total mass soluble in acetone (mg)

MIC = Average minimum inhibitory concentration

The total activity was determined by dividing the quantity (in mg) dissolved as acetone soluble by the MIC value. It became clear from this that redisolving in acetone was a problem. *T. sambesiaca* not only had the highest yield in terms of mass of compound extracted that was soluble in acetone but also had the highest total activity, while *T. gazenzis* had the lowest MIC of the test plant species.

3.4 Conclusion

In his study Eloff (1999b) acetone leaf extracts of *Terminalia sericea* had MIC values ranging from 0.1 – 6 mg/ml for the four test organisms compared to the average values of 0.16 to 0.63 mg/ml for *T. sericea*. The average values for the other species were 0.7 mg/ml (*T. gazenzis*) and 4.7 mg/*ml (T. prunoides*) in this study.

Eloff (1999b) evaluated the total activity and stability of 27 members of the Combretaceae. The total activity values obtained from the plants in this experiment were much lower than those obtained with previous work on other Combretaceae species (Eloff, 1999b) but were still worth investigating. The activity of the plants was tested against *S. aureus* because of the relative ease of performing bioautography with this organism.

In the bioautography experiments, *T. sericea* showed two distinctive areas of bacterial growth inhibition worthy of further investigation, the R_f values of 0.55 and 0.75 in a CEF/vanillin system, in addition to substantial antibacterial activity in the polar components (Fig 3.2). Gallic acid had a R_f value of 0.57 and appears not to be the compound that has the major antibacterial activity in this case.

There was substantial antibacterial activity in the polar fractions, which correlated with the results from Chapter 2 where ethanol, a relatively polar solvent extracts had the highest total antibacterial activity but the water extract had very low antibacterial activity. The average total activity of *T. sericea* against all four test organisms was 50 ml, compared to 51 ml for *T. prunoides* and 75 ml for *T. phanerophlebia*.