Chapter 4

A proposal for prioritising plant species to be investigated for treating diarrhoea in animals

4.1 Introduction

It was previously established in the current research that the extracts exhibited their best activity in the months of late summer and autumn (January until April) and also that there was no detectable intraspecies variation. The next step in the development of a commercially useful product for the treatment of diarrhoea was to investigate which plants had the overall best activity against pathogens and whether their activity was linked to their tannin content or not. A ranking system based on these results was developed to choose the most promising plant species for further in depth studies.

It is well known that infectious diseases account for a high proportion of the health problems in the Third World. Reports of antimicrobial activity of indigenous plants have been published from many regions (Desta, 1993). However, there is no record of an extensive comparison of such plants in southern Africa concerning their MIC values, TLC fingerprints and tannin content as has been performed in this study.

The plants for this study were chosen on their reported use as anti-diarrhoeal agents in ethnomedicine (Hutchings *et al.* 1996; Bossard, 1993; Bryant, 1966).

In the tree screening project conducted at our Phytomedicine Laboratory, the screening of leaves has proven successful to identify species with high antibacterial activity. Although different plant parts are used in ethnomedicine, it was decided to focus on the screening of leaf extracts as leaves are renewable resources. It is also possible that where activity in roots and barks exists, this may be reflected in the activity of leaf material. It has been proven that based on the minimum inhibitory concentration values, even though extracts of the inner bark of *Sclerocarrya birrea* were the most potent, the activity of the leaves and bark extracts did not differ (Eloff, 2001). McGaw *et al.* (2002) also found no statistically significant differences in antibacterial activity for certain bacteria between ethanol extracts of different parts of the species *Schotia brachypetala*.

The results of the preliminary screening will be added to the ever increasing database of the Phytomedicine Programme.

4.2 Materials and methods

4.2.1 Collection and preparation of plant material

Fifty-three plant species were chosen for the screening process; those species are listed in Table 4.1

Table 4.1: Selected plants for screening (Hutchings *et al.* 1996; Bossard, 1993; Bryant, 1966)

Plant species	Family
Acacia karroo Hayne	Fabaceae-Mimosoidae
Acacia sieberiana var. *woodii* DC	Fabaceae-Mimosoidae
Albizia adianthifolia (Schuhmach.) W.F. Wight	Fabaceae-Mimosoidae
Annona senegalensis Pers.	Annonaceae
Antidesma venosum E. Mey. ex Tul.	Euphorbiaceae
Berchemia zeyheri (Sond.) Grubov	Rhamnaceae
Bridelia micrantha (Hochst.) Baill.	Euphorbiaceae
Buddleja salviifolia Lam.	Loganiaceae
Capparis tomentosa Lam.	Capparaceae
Cassine aethiopica Thunb.	Celastraceae
Cassine transvaalensis (Burtt. Davy) Codd	Celastraceae
Cassinopsis ilicifolia (Hochst.) Kuntze	Icacinaceae
Cassinopsis tinifolia Harv.	Icacinaceae
Clausena anisata (Willd.) Hook. F. ex Benth.	Rutaceae
Clerodendrum myricoides (Hochst.) Vatke	Verbenaceae
Combretum molle R. Br. ex G. Don	Combretaceae
Combretum zeyheri Sond.	Combretaceae
Curtisia dentata (Burm. F.) C.A. Sm.	Cornaceae
Deinbollia oblongifolia (E. Mey. ex Arn.) Radlk.	Sapindaceae
Dichrostachys cinerea subsp. *africana* (L.) Wight et Arn.	Fabaceae-Mimosoidae
Dombeya rotundifolia (Hochst.) Planch.	Sterculiaceae
Ekebergia capensis Sparrman	Meliaceae
Elephantorrhiza elephantina (Burch.) Skeels	Fabaceae
Faidherbia albida (Del.) A. Chev.	Fabaceae-Mimosoidae
Faurea saligna Harvey	Proteaceae
Ficus sur Forssk.	Moraceae
Flueggea virosa (Roxb. Ex. Willd.) Pax & K. Hoffm.	Euphorbiaceae
Hippobromus pauciflorus (L. F.) Radlk.	Sapindaceae
Jatropha curcas L.	Euphorbiaceae
Kigelia africana/Kigelia pinnata (Jacq.) DC	Bignoniaceae
Lannea discolour (Sond.) Engl.	Anacardiaceae
Lippia javanica (Burm. f.) Spreng.	Verbenaceae
Lonchocarpus capassa Rolfe	Fabaceae
Olea eurpoea subsp. *africana* (Mill.) P.S. Green	Oleaceae
Oncoba spinosa Forssk.	Flacourtiaceae
Ozoroa obovata (Oliv.) R. & A. Fernandes	Anacardiaceae
Peltophorum africanum Sonder	Fabaceae-Caesalpinioideae
Pittosporum viridoflorum Sims	Pittosporaceae
Ricinus communis L.	Euphorbiaceae
Schotia brachypetala Sond.	Fabaceae
Sclerocarya birrea (A. Rich.) Hochst.	Anacardiaceae
Spirostachys africana Sond.	Euphorbiaceae
Strychnos spinosa Lam.	Loganiaceae
Syzigium cordatum Hochst. Ex Krauss	Myrtaceae
Tecomaria capensis (Thunb.) Spach	Bignoniaceae
Terminalia phanerophlebia Engl. & Diels	Combretaceae
Plant species	**Family**

Tetradenia riparia (Hochst.) Codd	Lamiaceae
Thespesia acutiloba (Bak. F.) Exell & Mendonca	Malvaceae
Trema orientalis (L.) Blume	Ulmaceae
Trichilia emetic Vahl	Meliaceae
Vangueria infausta subsp. *infausta* Burchell	Rubiaceae
Ximenia caffra Sond.	Olacaceae
Zizphus mucronata Willd.	Rhamnaceae

The leaf samples of the trees were collected at various sites and identified by qualified personnel (Pretoria Botanical Garden: Khangela Joseph Baloyi, Lowveld National Botanical Garden: Willem Froneman, Manie van der Schyff Botanical Garden University of Pretoria Main Campus: Lorraine Middleton) and by selecting labelled species in the Botanical Garden. The different sites from which plants were collected included: Lowveld National Botanical Garden (Nelspruit), Pretoria National Botanical Garden, the Main Campus of the University of Pretoria and the Campus of the Facultyof Veterinary Science, University of Pretoria (Onderstepoort).

The samples were dried indoors under constant air flow at the Phytomedicine Programme (University of Pretoria). After about a week of drying, the samples were ground to a fine powder with a Macsalab mill (Model 200 LAB). The powdered samples were stored in sealed glass containers in the dark until further use.

4.2.2 Extraction

The extracts were prepared as described in section 2.2.2.

4.2.3 TLC fingerprinting

The TLC plates were prepared as described in section 2.2.3, and again developed with the three solvent systems developed by the Phytomedicine Laboratory, University of Pretoria, namely BEA, CEF and EMW.

4.2.4 Bioautographic assays

The TLC plates were prepared as described in section 2.2.3 and dried for two days under a constant stream of air. Then they were sprayed with *E. coli* and *S. aureus* inoculated liquid cultures and incubated for 18 hours at 37°C and 100% humidity. Afterwards the plates were sprayed with a 2 mg/ml aqueous solution of INT and incubated for another hour for optimal colour development. Clear zones show bacterial growth inhibition due to antibacterial active compounds.

4.2.5 Total activity

Total activity was calculated as described in section 2.2.7.

4.2.6 Microdilution assay

MIC determinations for each plant extract against *E. coli* and *S. aureus* were conducted as described in section 2.2.6.

4.2.7 Tannin assays

Tannin contents for each extract were evaluated as described in section 2.2.6

4.3 Results and discussion

4.3.1 Quantity extracted

The extraction was performed twice to confirm the results obtained in the first extraction, and both extractions yielded similar results. Amounts in about the same range were extracted from all the species, from 0.68% to 6.2%, except for *Faurea saligna*, where much higher quantities of plant extract resulted. This species was subjected to a third round of extractions to exclude human error as the reason for the difference in extracted quantities. The extracted amount for *Faurea saligna* ranged from 10.5% to 15.1%. What was quite an interesting aspect when comparing the quantity of material extracted from *Combretum molle* (2.9%) and from *Combretum zeyheri* (3.5%) with the quantities of material that Eloff (1999b) extracted (9.8% and 6.6% respectively), was that the plants collected by Eloff in the Lowveld Botanical Garden seemed to yield higher quantities in extraction. This might be due to the different climate and soil conditions in the Mpumalanga area.

The quantities extracted are reported in Table 4.2, and Figure 4.1 supplies a visual comparison of the data.

Table 4.2: Percentage of plant material extracted with acetone, experiment repeated

Extract number	Plant species	1	2	Average
1	*Acacia karroo*	1.5%	1.3%	1.4%
2	*Acacia sieberiana* var. *woodii*	1.4%	1.3%	1.3%
3	*Albizia adianthifolia*	3.4%	2.0%	2.7%
4	*Annona senegalensis*	1.3%	1.4%	1.4%
5	*Antidesma venosum*	1.9%	1.6%	1.8%
6	*Berchemia zeyheri*	1.6%	0.8%	1.2%
7	*Bridelia micrantha*	2.9%	2.6%	2.7%
8	*Buddleja salviifolia*	3.9%	1.9%	2.9%
9	*Capparis tomentosa*	2.5%	1.5%	2.0%
10	*Cassine aethiopica*	3.3%	2.9%	3.1%
11	*Cassine transvaalensis*	8.1%	6.1%	7.1%
12	*Cassinopsis ilicifolia*	4.0%	2.2%	3.1%
13	*Cassinopsis tinifolia*	2.3%	2.2%	2.3%
14	*Clausena anisata*	4.4%	4.0%	4.2%
15	*Clerodendrum myricoides*	5.7%	1.5%	3.6%
16	*Combretum molle*	2.6%	3.2%	2.9%
17	*Combretum zeyheri*	3.5%	3.4%	3.5%
18	*Curtisia dentate*	7.2%	6.0%	6.6%
19	*Deinbollia oblongifolia*	2.7%	2.3%	2.5%
20	*Dichrostachys cinerea* subsp. *africana*	1.6%	1.0%	1.3%
21	*Dombeya rotundifolia*	1.7%	1.1%	1.4%
22	*Ekebergia capensis*	2.3%	1.8%	2.1%
23	*Elephantorrhiza elephantina*	2.8%	2.9%	2.9%
24	*Faidherbia albida*	2.8%	2.1%	2.4%
25	*Faurea saligna*	15%	12.3%	13.7%
26	*Ficus sur*	2.2%	1.5%	1.9%
27	*Flueggea virosa*	2.0%	0.7%	1.4%
28	*Hippobromus pauciflorus*	1.6%	1.3%	1.4%
29	*Jatropha curcas*	1.9%	3.6%	2.7%
30	*Kigelia africana*	1.4%	1.2%	1.3%
31	*Lannea discolour*	2.1%	2.0%	2.0%
32	*Lippia javanica*	1.9%	2.0%	1.9%
33	*Lonchocarpus capassa*	1.5%	1.3%	1.4%
34	*Olea europea* subsp. *africana*	7.0%	4.4%	5.7%
35	*Oncoba spinosa*	3.7%	3.6%	3.7%
36	*Ozoroa obovata*	4.3%	3.6%	3.9%
37	*Peltophorum africanum*	1.0%	1.2%	1.1%
38	*Pittosporum viridoflorum*	2.2%	2.4%	2.3%
39	*Ricinus communis*	2.8%	1.8%	2.3%
40	*Schotia brachypetala*	1.4%	1.0%	1.2%
41	*Sclerocarrya birrea*	2.3%	5.0%	3.7%
42	*Spirostachys Africana*	3.8%	2.9%	3.3%
43	*Strychnos spinosa*	2.6%	1.8%	2.2%
44	*Syzigium cordatum*	3.5%	3.7%	3.6%
45	*Tecomaria capensis*	2.7%	3.2%	2.9%
46	*Terminalia phanerophlebia*	3.0%	1.4%	2.2%
47	*Tetradenia riparia*	2.0%	1.1%	1.6%
48	*Thespesia acutiloba*	4.0%	3.8%	3.9%
49	*Trema orientalis*	3.5%	4.6%	4.0%
50	*Trichilia emetica*	0.9%	0.7%	0.8%
51	*Vangueria infausta* subsp. *infausta*	1.1%	1.2%	1.2%
52	*Ximenia caffra*	3.8%	4.0%	3.9%
53	*Ziziphus mucronata*	1.0%	1.5%	1.2%

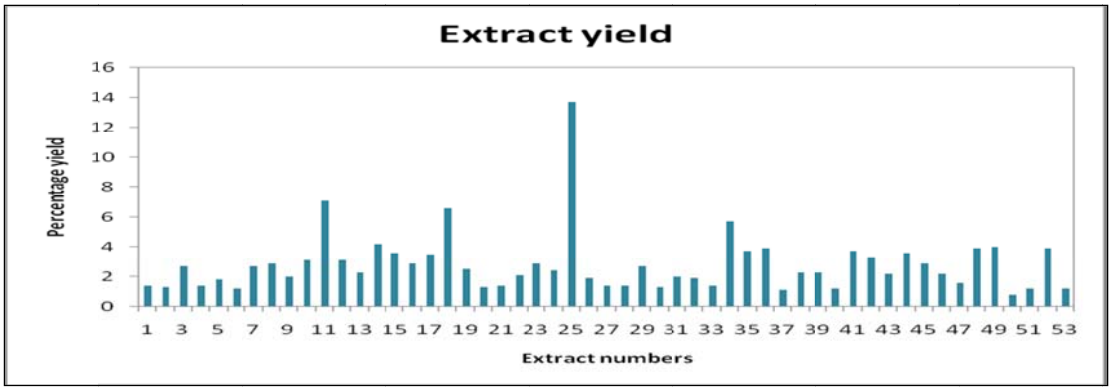


Figure 4.1: Average percentage extracted from the 53 selected plant species

4.3.2 Microdilution and tannin assays

The microdilution assays gave varying results regarding the antibacterial activity of the different plants. All the determined values are given in Tables 4.3 and 4.4. Some of the plant extracts had bactericidal activity whereas others extracts had bacteriostatic activity. The plants with the bactericidal effects are preferable, since they appear to be directly killing the bacteria, but there is always a risk that this effect means that the extract could be toxic, so it is wise not to disregard the plants with bacteriostatic effects. Furthermore, it will be interesting to investigate plants further that had good antibacterial activity against *E. coli*, but not against *S. aureus*, because if they were equally active against both, they may contain a general metabolic toxin that could also be toxic to mammals. Another important factor in choosing the most promising plant species is how high the extract yield is and of course, plants with a low tannin content (to favour the selection of plants that work with a different mechanism for the antibacterial activity of the extract) should be chosen. The extracts with the best overall average antibacterial activity against the strains of *E. coli* as well as *S. aureus* and also best total activity for both bacterial strains were: *Combretum zeyheri* (*E. coli*: MIC = 0.03 mg/ml, TA = 1077 ml/g, bactericidal; *S. aureus*: MIC = 0.04 mg/ml, TA = 829 ml/g, bactericidal), *Deinbollia oblongifolia* (*E. coli*: MIC = 0.09 mg/ml, TA = 309 ml/g, bacteriostatic; *S. aureus*: MIC = 0.12 mg/ml, TA = 234 ml/g, bactericidal), *Spirostachys africana* (*E. coli*: MIC = 0.12 mg/ml, TA = 317 ml/g, bactericidal; *S. aureus*: MIC = 0.09 mg/ml, TA = 438 ml/g, bactericidal), *Syzigium cordatum* (*E. coli*: MIC = 0.06 mg/ml; TA = 609 ml/g, bactericidal; *S. aureus*: MIC = 0.05 mg/ml, TA = 656 ml/g, bacteriostatic) and *Terminalia phanerophlebia* (*E. coli*: MIC = 0.08 mg/ml, TA = 374 ml/g, bactericidal; *S. aureus*: MIC = 0.03 mg/ml, TA = 900 ml/g, bactericidal). However, since they had such good activity against both the Gram-negative and the Gram-positive bacteria, these extracts could very likely prove to be toxic to animals.

The extract of *Faurea saligna* had a very good total activity (TA = 1022 ml/g) due to the higher extraction percentage in this plant (see 4.3.1). The MIC value against *E. coli* however was only 0.15 mg/ml.

Table 4.3: MIC values after 60 and 120 min against *E. coli.* Each value represents the average of 9 determinations

Plant species	Yield (mg)	Average activity60 (mg/ml) (± SD)	Total activity60 (ml/g)	Average activity120 (mg/ml) (± SD)	Total activity 120 (ml/g)
Acacia karroo	15	0.13 ± 0.04	115	0.13 ± 0.04	115
Acacia sieberiana var. *woodii*	14	0.13 ± 0.04	108	0.13 ± 0.04	108
Albizia adianthifolia	34	0.13 ± 0.09	255	0.14 ± 0.04	239
Annona senegalensis	13	0.12 ± 0.05	105	0.15 ± 0.08	88
Antidesma venosum	19	0.09 ± 0.00	222	0.15 ± 0.11	130
Berchemia zeyheri	16	0.08 ± 0.04	200	0.13 ± 0.04	120
Bridelia micrantha	29	0.13 ± 0.16	218	0.23 ± 0.12	124
Buddleja salviifolia	39	0.52 ± 0.10	75	0.52 ± 0.16	75
Capparis tomentosa	25	0.17 ± 0.08	148	0.20 ± 0.07	123
Cassine aethiopica	33	0.24 ± 0.07	136	0.35 ± 0.22	94
Cassine transvaalensis	81	0.19 ± 0.07	419	0.19 ± 0.07	419
Cassinopsis ilicifolia	40	0.26 ± 0.28	154	0.47 ± 0.24	85
Cassinopsis tinifolia	23	0.31 ± 0.23	75	0.31 ± 0.28	74
Clausena anisata	44	0.37 ± 0.05	118	0.45 ± 0.26	97
Clerodendrum myricoides	57	0.29 ± 0.06	194	0.31 ± 0.00	184
Combretum molle	16	0.18 ± 0.05	300	0.23 ± 0.08	244
Combretum zeyheri	35	0.03 ± 0.08	1077	0.03 ± 0.01	1077
Curtisia dentate	72	0.24 ± 0.06	296	0.45 ± 0.28	161
Deinbollia oblongifolia	27	0.09 ± 0.12	309	0.17 ± 0.13	158
Dichrostachys cinerea subsp.*africana*	16	0.19 ± 0.11	84	0.19 ± 0.12	84
Dombeya rotundifolia	17	0.20 ± 0.08	85	0.20 ± 0.11	85
Ekebergia capensis	23	0.21 ± 0.19	110	0.21 ± 0.08	110
Elephantorrhiza elephantina	28	0.26 ± 0.23	106	0.26 ± 0.19	106
Faidherbia albida	28	0.30 ± 0.08	92	0.30 ± 0.23	92
Faurea saligna	151	0.15 ± 0.26	1022	0.15 ± 0.08	1022
Ficus sur	22	0.26 ± 0.04	85	0.30 ± 0.25	73
Flueggea virosa	20	0.13 ± 0.12	154	0.13 ± 0.04	154
Hippobromus pauciflorus	16	0.11 ± 1.17	144	0.14 ± 0.13	117
Jatropha curcas	19	0.95 ± 0.12	20	0.96 ± 1.16	20
Kigelia africana	14	0.15 ± 0.06	92	0.29 ± 0.26	49
Lannea discolour	21	0.12 ± 0.11	183	0.12 ± 0.06	183
Lippia javanica	19	0.12 ± 0.24	156	0.13 ± 0.10	149
Lonchocarpus capassa	15	0.34 ± 0.00	44	0.33 ± 0.26	46
Olea europea subsp. *africana*	70	0.31 ± 0.16	226	0.42 ± 0.16	168
Oncoba spinosa	37	0.15 ± 0.07	69	0.26 ± 0.08	55
Ozoroa obovata	43	0.11 ± 0.06	403	0.11 ± 0.04	403
Peltophorum africanum	10	0.10 ± 0.13	105	0.10 ± 0.06	105
Pittosporum viridoflorum	22	0.22 ± 0.11	101	0.22 ± 0.13	101
Ricinus communis	28	0.22 ± 0.13	129	0.22 ± 0.11	129
Schotia brachypetala	14	0.20 ± 0.14	69	0.32 ± 0.25	43
Sclerocarrya birrea	23	0.22 ± 0.12	105	0.43 ± 0.30	53
Spirostachys africana	38	0.12 ± 0.09	317	0.13 ± 0.11	300
Strychnos spinosa	26	0.19 ± 0.03	135	0.19 ± 0.09	135
Syzigium cordatum	35	0.06 ± 0.26	609	0.06 ± 0.03	609
Tecomaria capensis	27	0.34 ± 0.06	79	0.36 ± 0.23	74

Plant species	Yield (mg)	Average activity60 (mg/ml) (± SD)	Total activity60 (ml/g)	Average activity120 (mg/ml) (± SD)	Total activity 120 (ml/g)
Terminalia phanerophlebia	30	0.08 ± 0.05	375	0.08 ± 0.06	375
Tetradenia riparia	20	0.09 ± 0.12	214	0.09 ± 0.05	214
Thespesia acutiloba	40	0.15 ± 0.21	267	0.15 ± 0.11	262
Trema orientalis	35	0.37 ± 0.26	95	0.37 ± 0.21	95
Trichilia emetica	9	0.34 ± 0.12	26	0.37 ± 0.22	24
Vangueria infausta subsp. *infausta*	11	0.21 ± 0.15	52	0.21 ± 0.12	52
Ximenia caffra	38	0.21 ± 0.12	178	0.22 ± 0.14	173
Ziziphus mucronata	10	0.15 ± 0.04	66	0.21 ± 0.10	47

Table 4.4: MIC values after 60 and 120 min against *S. aureus*. Each value represents the average of 9 determinations

Plant species	Yield (mg)	Average activity60 (mg/ml) (± SD)	Total activity 60 (ml/g)	Average activity 120 (mg/ml) (± SD)	Total activity120 (ml/g)
Acacia karroo	15	0.14 ± 0.04	107	0.14 ± 0.04	107
Acacia sieberiana var. *woodii*	14	0.10 ± 0.04	140	0.13 ± 0.04	108
Albizia adianthifolia	34	0.04 ± 0.03	765	0.04 ± 0.03	765
Annona senegalensis	13	0.07 ± 0.02	195	0.08 ± 0.00	163
Antidesma venosum	19	0.08 ± 0.00	238	0.07 ± 0.02	285
Berchemia zeyheri	16	0.04 ± 0.03	400	0.04 ± 0.03	400
Bridelia micrantha	29	0.05 ± 0.03	621	0.05 ± 0.03	621
Buddleja salviifolia	39	0.12 ± 0.15	334	0.12 ± 0.15	334
Capparis tomentosa	25	0.26 ± 0.07	96	0.28 ± 0.07	90
Cassine aethiopica	33	0.33 ± 0.18	100	0.47 ± 0.24	70
Cassine transvaalensis	81	0.18 ± 0.10	442	0.32 ± 0.24	256
Cassinopsis ilicifolia	40	0.19 ± 0.09	208	0.31 ± 0.00	129
Cassinopsis tinifolia	23	0.23 ± 0.12	99	0.26 ± 0.07	88
Clausena anisata	44	0.42 ± 0.16	106	0.42 ± 0.16	106
Clerodendrum myricoides	57	0.17 ± 0.11	327	0.33 ± 0.18	172
Combretum molle	16	0.21 ± 0.08	147	0.21 ± 0.07	141
Combretum zeyheri	35	0.04 ± 0.03	829	0.05 ± 0.02	656
Curtisia dentate	72	0.31 ± 0.00	232	0.31 ± 0.00	232
Deinbollia oblongifolia	27	0.12 ± 0.07	234	0.08 ± 0.05	338
Dichrostachys cinerea subsp. *africana*	16	0.15 ± 0.03	106	0.16 ± 0.00	100
Dombeya rotundifolia	17	0.15 ± 0.07	113	0.15 ± 0.07	113
Ekebergia capensis	23	0.16 ± 0.00	144	0.19 ± 0.07	119
Elephantorrhiza elephantina	28	0.15 ± 0.12	193	0.11 ± 0.07	260
Faidherbia albida	28	0.12 ± 0.06	233	0.13 ± 0.05	217
Faurea saligna	151	0.23 ± 0.08	666	0.26 ± 0.07	581
Ficus sur	22	0.26 ± 0.07	85	0.26 ± 0.07	85
Flueggea virosa	20	0.04 ± 0.01	450	0.05 ± 0.02	391
Hippobromus pauciflorus	16	0.06 ± 0.03	267	0.06 ± 0.02	248
Jatropha curcas	19	0.26 ± 0.07	73	0.35 ± 0.11	55
Kigelia Africana	14	0.40 ± 0.16	35	0.63 ± 0.00	22
Lannea discolour	21	0.22 ± 0.09	96	0.31 ± 0.00	68
Lippia javanica	19	0.18 ± 0.08	103	0.31 ± 0.00	61
Lonchocarpus capassa	15	0.23 ± 0.12	64	0.26 ± 0.07	58
Olea europea subsp. *africana*	70	0.11 ± 0.04	656	0.31 ± 0.00	226
Oncoba spinosa	37	1.67 ± 0.63	358	1.67 ± 0.63	358
Ozoroa obovata	43	0.19 ± 0.20	224	0.23 ± 0.17	189
Peltophorum africanum	10	0.11 ± 0.04	91	0.14 ± 0.04	71
Pittosporum viridoflorum	22	0.26 ± 0.07	85	0.26 ± 0.07	85
Ricinus communis	28	0.13 ± 0.04	210	0.16 ± 0.00	175
Schotia brachypetala	14	0.07 ± 0.02	210	0.07 ± 0.02	210

Plant species	Yield (mg)	Average activity60 (mg/ml) (± SD)	Total activity60 (ml/g)	Average activity120 (mg/ml) (± SD)	Total activity 120 (ml/g)
Spirostachys africana	38	0.09 ± 0.06	438	0.09 ± 0.06	438
Strychnos spinosa	26	0.11 ± 0.07	229	0.11 ± 0.07	229
Syzigium cordatum	35	0.05 ± 0.02	656	0.08 ± 0.00	438
Tecomaria capensis	27	0.52 ± 0.16	52	0.52 ± 0.16	52
Terminalia phanerophlebia	30	0.03 ± 0.01	900	0.03 ± 0.01	964
Tetradenia riparia	20	0.08 ± 0.06	237	0.13 ± 0.13	149
Thespesia acutiloba	40	0.16 ± 0.00	250	0.18 ± 0.05	226
Trema orientalis	35	0.22 ± 0.14	159	0.22 ± 0.14	159
Trichilia emetica	9	0.31 ± 0.00	29	0.43 ± 0.17	21
Vangueria infausta subsp. *infausta*	11	0.23 ± 0.12	47	0.23 ± 0.12	47
Ximenia caffra	38	0.13 ± 0.04	285	0.13 ± 0.04	285
Ziziphus mucronata	10	0.31 ± 0.00	32	0.31 ± 0.00	32

The tannin assays provided an indication of the tannin content of the different extracts. Returning to the five plant extracts with the best overall activity, only the extracts of *Deinbollia oblongifolia* and *Spirostachys africana* did not contain any detectable tannins.

Combretum zeyheri, *Syzigium cordatum* and *Terminalia phanerophlebia* all contained tannins in varying concentrations, with *Terminalia phanerophlebia* exhibiting a small ring of complexed protein, whereas the values for *Combretum zeyheri* and *Syzigium cordatum* were rather large with 4.33 and 4 times gallic acid equivalent.

The values obtained from the tannin assays are shown in Table 4.5.

Table 4.5: Tannin contents for the 53 selected plant species

Plant species	Average equivalent gallic acid	SD
Acacia karroo	1.67	1.00
Acacia sieberiana var. *woodii*	0.00	0.00
Albizia adianthifolia	0.00	0.00
Annona senegalensis	0.00	0.00
Antidesma venosum	0.00	0.00
Berchemia zeyheri	0.00	0.00
Bridelia micrantha	0.00	0.00
Buddleja salviifolia	0.00	0.00
Capparis tomentosa	0.67	0.58
Cassine aethiopica	0.00	0.00
Cassine transvaalensis	7.33	0.58
Cassinopsis ilicifolia	3.00	1.73
Cassinopsis tinifolia	1.33	1.15
Clausena anisata	4.00	0.00
Clerodendrum myricoides	0.00	0.00
Combretum molle	11.33	0.58
Combretum zeyheri	4.33	1.53
Curtisia dentate	3.33	1.53
Deinbollia oblongifolia	0.00	0.00
Dichrostachys cinerea subsp. *africana*	0.33	0.58
Dombeya rotundifolia	0.00	0.00
Ekebergia capensis	0.00	0.00
Elephantorrhiza elephantina	0.00	0.00
Faidherbia albida	1.33	1.15
Faurea saligna	1.67	1.00
Ficus sur	2.67	1.15
Flueggea virosa	0.00	0.00
Hippobromus pauciflorus	8.00	1.15
Jatropha curcas	1.00	0.00
Kigelia Africana	0.00	0.00
Lannea discolour	4.00	0.00
Lippia javanica	0.00	0.00
Lonchocarpus capassa	0.00	0.00
Olea europea subsp. *africana*	0.00	0.00
Oncoba spinosa	1.33	1.15
Ozoroa obovata	3.33	1.53
Peltophorum africanum	6.00	1.73
Pittosporum viridoflorum	0.00	0.00
Ricinus communis	3.00	1.73
Schotia brachypetala	4.67	1.00
Sclerocarrya birrea	11.33	0.58
Spirostachys africana	0.00	0.00
Strychnos spinosa	0.00	0.00
Syzigium cordatum	4.00	0.00
Tecomaria capensis	5.67	2.08
Terminalia phanerophlebia	0.67	0.58
Tetradenia riparia	0.00	0.00
Thespesia acutiloba	0.33	0.58
Trema orientalis	5.67	0.58
Trichilia emetica	0.33	0.58
Vangueria infausta subsp. *infausta*	4.08	1.04
Ximenia caffra	4.00	0.00
Ziziphus mucronata	0.67	0.58

When the activity against the bacterial pathogens was compared to the tannin content, there was no obvious correlation between those two values. See Figures 4.2 and 4.3 for a visual comparison.

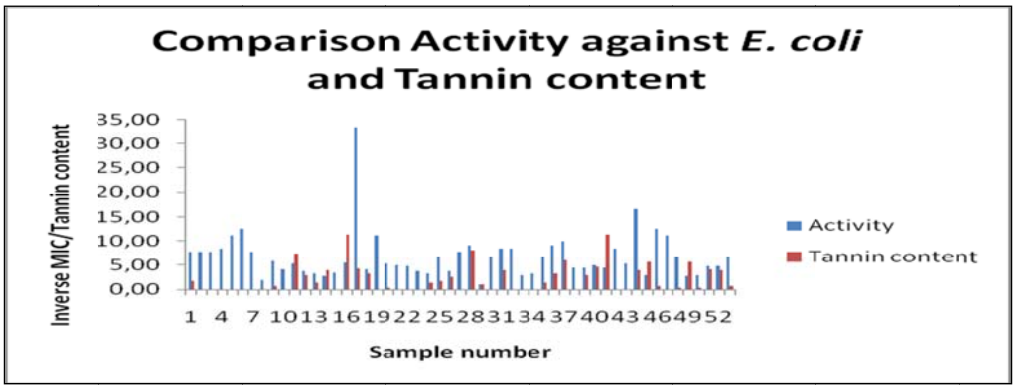


Fig. 4.2: Comparison of the activity of the 53 different plant samples against *E. coli* and their tannin content

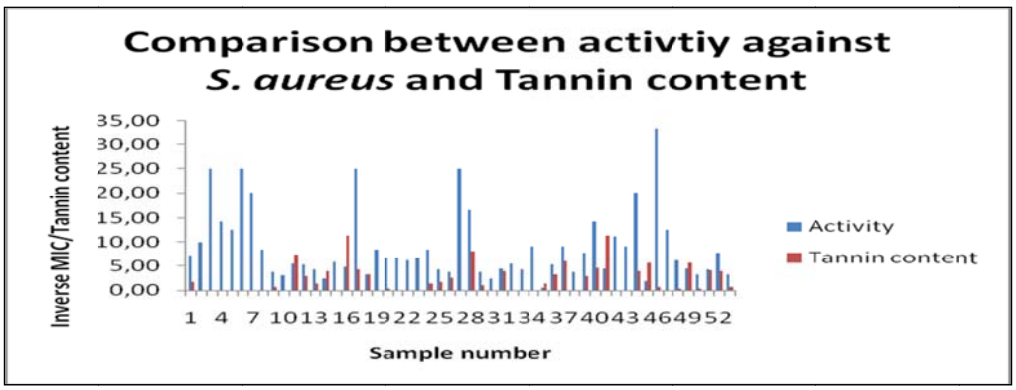


Fig. 4.3: Comparison of the activity of the 53 different plant samples against *S. aureus* and their tannin content

4.3.3 Parameters for the selection of the plant species for further investigation

The values obtained in the previous sections helped to select which plant extracts should be used for further investigations. Naturally extracts should have a high activity against *E. coli*, but not necessarily against *S. aureus*. If an extract has strong activity against *E.coli* and *S. aureus* it may contain a general metabolic toxin that could be toxic to other organisms as well. Furthermore the plant extract should have low tannin content since tannins in feed taste bitter and also complex with proteins and would therefore not benefit a prophylactic feed additive. The last point that should be taken into consideration for the selection of the plant species is the extract yield, which should be relatively high.

Therefore those values were tabulated in order of importance based on an assigned percentage value. Low activity against *E. coli*, being the most important point for our study objective, was allocated the

percentage of 60%, the low tannin content 25% (values of 0 were allocated the value of 0.003, resulting from the limit of detection), low activity against *S. aureus* 10% and a high extraction yield was allocated 5%. The sum of these values was calculated by using the following formula:

(0.60/MIC *E. coli*) + (0.25/gallic acid equivalent) + (0.10*MIC *S. aureus*) + (0.05*extract yield) = Rank

(For the plants that did not contain any tannins, the value of the limit of detection in our tannin assay was used, i.e. 0.003).

The values calculated for the different plant species tested (calculated from unrounded values) varied from about 2 to 107 (Table 4.6).

Table 4.6: Ranking of the extracts based on the formula developed

Plant species	MIC *E. coli* (mg/ml)	Tannin content	MIC *S. aureus* (mg/ml)	Yield(mg/g)	Value obtained with formula
Tetradenia riparia	0.09	0.003	0.13	20.00	90.78
Spirostachys africana	0.13	0.003	0.09	38.00	89.98
Albizia adianthifolia	0.14	0.003	0.04	34.00	89.26
Lippia javanica	0.13	0.003	0.31	19.00	89.03
Flueggea virosa	0.13	0.003	0.05	20.00	88.95
Acacia sieberiana var. *woodii*	0.13	0.003	0.13	14.00	88.66
Berchemia zeyheri	0.13	0.003	0.04	16.00	88.64
Antidesma venosum	0.15	0.003	0.07	19.00	88.41
Olea europaea subs. *africana*	0.42	0.003	0.31	70.00	88.30
Deinbollia oblongifolia	0.17	0.003	0.08	27.00	88.19
Clerodendrum myricoides	0.31	0.003	0.33	57.00	88.15
Annona senegalensis	0.15	0.003	0.08	13.00	88.07
Strychnos spinosa	0.19	0.003	0.11	26.00	87.77
Ekebergia capensis	0.21	0.003	0.19	23.00	87.36
Bridelia micrantha	0.23	0.003	0.05	29.00	87.36
Pittosporum viridiflorum	0.22	0.003	0.26	22.00	87.20
Dombeya rotundifolia	0.20	0.003	0.15	17.00	87.20
Elephanthorrhiza elephantina	0.26	0.003	0.11	28.00	87.02
Cassine aethiopica	0.35	0.003	0.47	33.00	86.74
Buddleja salviifolia	0.52	0.003	0.12	39.00	86.44
Kigelia africana	0.29	0.003	0.63	14.00	86.20
Lonchocarpus capassa	0.33	0.003	0.26	15.00	85.95
Combretum zeyheri	0.03	4.333	0.05	35.00	20.27
Syzigium cordatum	0.06	4.000	0.08	35.00	12.26
Faurea saligna	0.15	1.667	0.26	151.00	11.79
Terminalia phanerophlebia	0.08	0.667	0.03	30.00	9.38
Ozoroa obovata	0.11	3.333	0.23	43.00	7.87
Cassine transvaalensis	0.19	7.333	0.32	81.00	7.22
Combretum molle	0.11	11.333	0.18	26.00	6.97
Peltophorum africanum	0.10	6.000	0.14	10.00	6.87
Thespesia acutiloba	0.15	0.333	0.18	40.00	6.70
Lannea discolour	0.12	4.000	0.31	21.00	6.36
Acacia karroo	0.13	1.667	0.14	15.00	5.53
Hippobromus pauciflorus	0.14	8.000	0.06	16.00	5.23
Curtisia dentate	0.45	3.333	0.31	72.00	5.05
Dichrostachys cinerea subsp. *africana*	0.19	0.333	0.16	16.00	4.71
Ximenia caffra	0.22	4.000	0.13	38.00	4.70
Capparis tomentosa	0.20	0.667	0.28	25.00	4.61
Ricinus communis	0.22	3.000	0.16	28.00	4.27
Ziziphus mucronata	0.21	0.667	0.31	10.00	3.72
Clausena anisata	0.45	4.000	0.42	44.00	3.63
Faidherbia albida	0.30	1.333	0.13	28.00	3.58
Vangueria infausta subsp. *infausta*	0.21	4.083	0.23	11.00	3.47
Trema orientalis	0.37	5.667	0.22	35.00	3.45
Cassinopsis ilicifolia	0.47	3.000	0.31	40.00	3.38
Cassinopsis tinifolia	0.31	1.333	0.26	23.00	3.30
Ficus sur	0.30	2.667	0.26	22.00	3.20
Tecomaria capensis	0.36	5.667	0.52	27.00	3.10
Oncoba spinosa	0.66	1.333	0.42	36.30	2.95
Trichilia emetica	0.37	0.333	0.43	9.00	2.85
Schotia brachypetala	0.32	4.667	0.07	14.00	2.62
Sclerocarya birrea	0.43	11.333	0.33	23.00	2.59
Jatropha curcas	0.96	1.000	0.35	19.00	1.86

The effect of the tannin content within the calculation can be clearly seen in the rather big difference in the values of *Combretum zeyheri* (with a value of 20.27) and *Lonchocarpus capassa* (with a value of 85.95). The values of all the species containing tannins are much lower than those of the species without detectable tannins in this research.

Based on this list, 5 plants were selected based on their ranking values, their availability and on the extent of research that has been done on them already (plants with little research done on them being preferable). Those five plants were: *Acacia sieberiana* var. *woodii* (MIC *E. coli* = 0.13 mg/ml, MIC *S. aureus* = 0.13 mg/ml, Yield = 14 mg, Tannin content = 0 Gallic acid equivalent), *Albizia adianthifolia* (MIC *E. coli* = 0.14 mg/ml, MIC *S. aureus* = 0.04 mg/ml, Yield = 34 mg, Tannin content = 0 Gallic acid equivalent), *Deinbollia oblongifolia* (MIC *E. coli* = 0.17 mg/ml, MIC *S. aureus* = 0.08 mg/ml, Yield = 27 mg, Tannin content = 0 Gallic acid equivalent), *Spirostachys africana* (MIC *E. coli* = 0.13 mg/ml, MIC *S. aureus* = 0.09 mg/ml, Yield = 38 mg, Tannin content = 0 Gallic acid equivalent) and *Tetradenia riparia* (MIC *E. coli* = 0.09 mg/ml, MIC *S. aureus* = 0.13 mg/ml, Yield = 20 mg, Tannin content = 0 Gallic acid equivalent). Even though the activity of *Deinbollia oblongifolia* against *E. coli* was not that high after two hours of incubation with INT, we chose to further investigate it, because the activity had much better values after one hour of incubation with INT against *E. coli* (MIC = 0.09 mg/ml) and might therefore prove interesting if tested *in vivo.* A literature search also revealed that not much work had been done on this species so far. This might also provide the possibility to isolate novel compounds from this plant.

4.3.4 Bioautographic assays and TLC fingerprinting

Bioautographic assays on all of the 53 selected plant species were done, but only results of the 5 chosen plants (*Acacia sieberiana* var.woodii, *Albizia adianthifolia*, *Deinbollia oblongifolia*, *Spirostachys africana* and *Tetradenia riparia*) are discussed at this point. The number of compounds active against *E. coli* and *S. aureus* as representatives for a Gram-negative and a Gram-positive species were determined. White bands on the assays represent the compound(s) in the plant extracts responsible for inhibiting bacterial growth. *S. aureus* was included to help find selectivity of the different extracts toward *E. coli*, since we wanted to exclude general metabolic toxins. With the three different solvent systems used, some information on the character, such as polarity, of the antibacterial compounds could also be obtained.

No compounds present in any extracts had significant inhibition bands when separated by the non-polar BEA solvent system. Only *Acacia sieberiana* var. *woodii*, *Deinbollia oblongifolia* and *Tetradenia riparia* had active compounds against both bacterial strains, but those inhibition bands were positioned at the bottom of the plate. The compounds responsible for the activity in the extracts appeared to be relatively

polar, since they did not show any significant inhibition bands or only bands right on the bottom of the plate when developed with this BEA solvent, as this solvent system separates non-polar compounds well.

The bioautograms obtained with the CEF solvent system had significant bands in all the samples. All the bioautographies obtained when run with the EMW solvent system had active bands near the top of the plate. One faint inhibition band against *E. coli* developed when an *Acacia sieberiana* var. *woodii* extract was developed with the CEF solvent. Against *S. aureus* one faint and one pronounced inhibition zone developed after this plant extract was developed with the CEF solvent.

Three faint inhibition bands against *E. coli* could be seen when *Albizia adianthifolia* was run with the CEF solvent. It also had two faint inhibition bands against *S. aureus* when run with this solvent.

Deinbollia oblongifolia had various active bands against *S. aureus* and *E. coli* when developed with the CEF solvent, but there were also some other inhibition bands present that were only selectively active against *E. coli*.

Spirostachys africana, developed in the CEF solvent, had one inhibition band that was selectively active against *E. coli*.

Tetradenia riparia developed several inhibition bands against both bacteria after being run with the CEF solvent. It seems however that this plant extract possesses more active compounds against *E. coli*.

Even though TLCs of all the plant samples were done, only the results of the five chosen species (See section 4.3.2) are shown here to be able to visualize and compare the active compounds.

EMW and CEF were the best solvents for separating the compounds with high and intermediate polarity in the extracts. The BEA solvent system did not separate many plant compounds as they were usually situated on the bottom of the plates, except for the case of *Albizia adianthifolia* and *Tetradenia riparia*. This suggested that the polarity of the compounds in the other extracts was too high to be moved along the TLC plate by the non-polar solvent, showing that the most of the compounds possessed intermediate or high polarity.

The compounds present in the extract of *Deinbollia oblongifolia* were separated well with both the EMW and the CEF solvent systems indicating that the most of the plant's compounds possess intermediate as well as high polarity.

The compounds present in the extract of *Albizia adianthifolia* and *Tetradenia riparia* were separated well with each of the three solvent systems, suggesting that the plant possesses compounds of high, intermediate and low polarity.

The EMW and CEF solvent systems separated the compounds of the *Acacia sieberiana* var. *woodii* and *Spirostachys africana* extract very well indicating that all these plants possess compounds of intermediate and high polarity.

Scans of the chromatograms and bioautographic assay plates are presented in Figures 4.4 to 4.48.

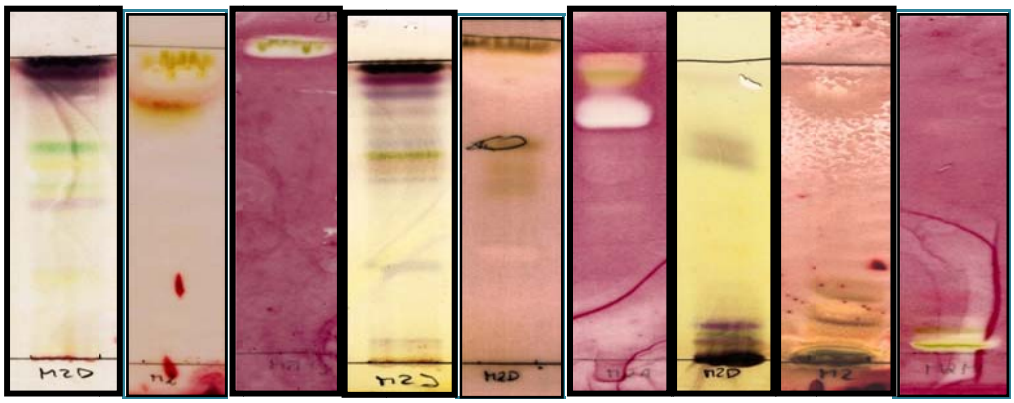


Fig. 4.4 Fig. 4.5 Fig. 4.6 Fig. 4.7 Fig. 4.8 Fig. 4.9 Fig. 4.10Fig. 4.11 Fig. 4.12

Fig. 4.4: *Acacia sieberiana* var. *woodii* EMW; Fig. 4.5: *Acacia sieberiana* var. *woodii* EMW against *E. coli*; Fig. 4.6: *Acacia sieberiana* var. *woodii* EMW against *S. aureus*; Fig. 4.7: *Acacia sieberiana* var. *woodii* CEF; Fig. 4.8: *Acacia sieberiana* var. *woodii* CEF against *E. coli*; Fig. 4.9: *Acacia sieberiana* var. *woodii* CEF against *S. aureus*; Fig. 4.10: *Acacia sieberiana* var. *woodii* BEA; Fig. 4.11: *Acacia sieberiana* var. *woodii* BEA against *E. coli*; Fig. 4.12: *Acacia sieberiana* var. *woodii* BEA against *S. aureus*

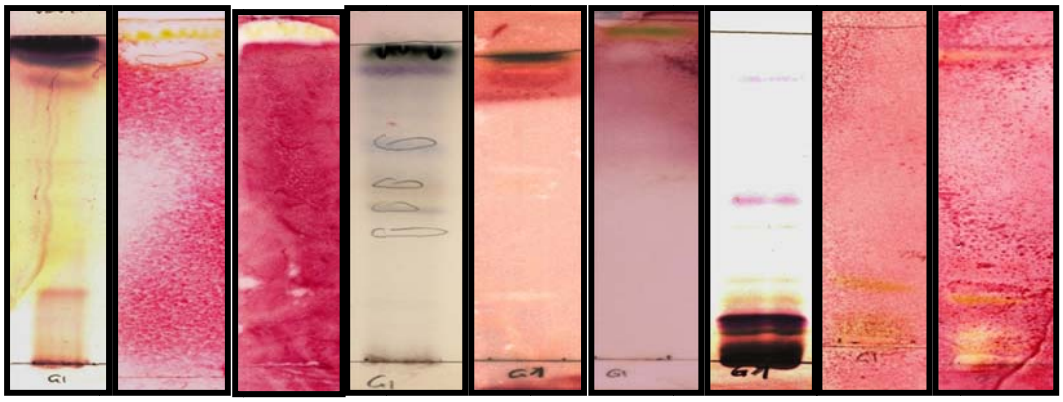


Fig. 4.13 Fig. 4.14 Fig. 4.15 Fig. 4.16 Fig. 4.17 Fig. 4.18 Fig. 4.19 Fig. 4.20 Fig. 4.21

Fig. 4.13: *Albizia adianthifolia* EMW; Fig. 4.14: *Albizia adianthifolia* EMW against *E. coli*; Fig. 4.15: *Albizia adianthifolia* against EMW *S. aureus*; Fig. 4.16: *Albizia adianthifolia* CEF; Fig. 4.17: *Albizia adianthifolia* CEF against *E. coli*; Fig. 4.18: *Albizia adianthifolia* CEF against *S. aureus*; Fig. 4.19: *Albizia adianthifolia* BEA; Fig. 4.20: *Albizia adianthifolia* BEA against *E. coli*; Fig. 4.21: *Albizia adianthifolia* BEA against *S. aureus*

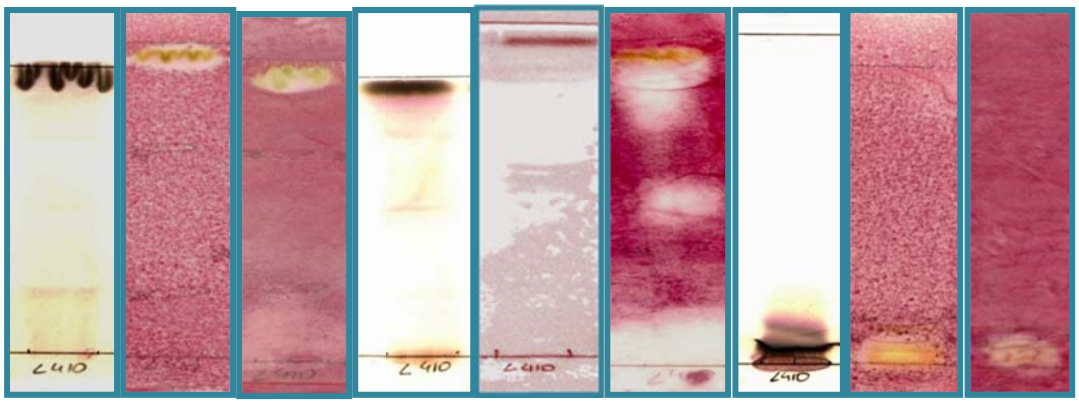


Fig. 4.22 Fig. 4.23 Fig. 4.24 Fig. 4.25 Fig. 4.26 Fig. 4.27 Fig. 4.28 Fig. 4.29 Fig. 4.30

Fig. 4.22: *Deinbollia oblongifolia* EMW; Fig. 4.23: *Deinbollia oblongifolia* EMW against *E. coli*; Fig. 4.24: *Deinbollia oblongifolia* EMW against *S. aureus*; Fig. 4.25: *Deinbollia oblongifolia* CEF; Fig. 4.26:*Deinbollia oblongifolia* CEF against *E. coli*; Fig. 4.27: *Deinbollia oblongifolia* CEF against *S. aureus*; Fig. 4.28: *Deinbollia oblongifolia* BEA; Fig. 4.29: *Deinbollia oblongifolia* BEA against *E. coli*; Fig. 4.30: *Deinbollia oblongifolia* BEA against *S. aureus*

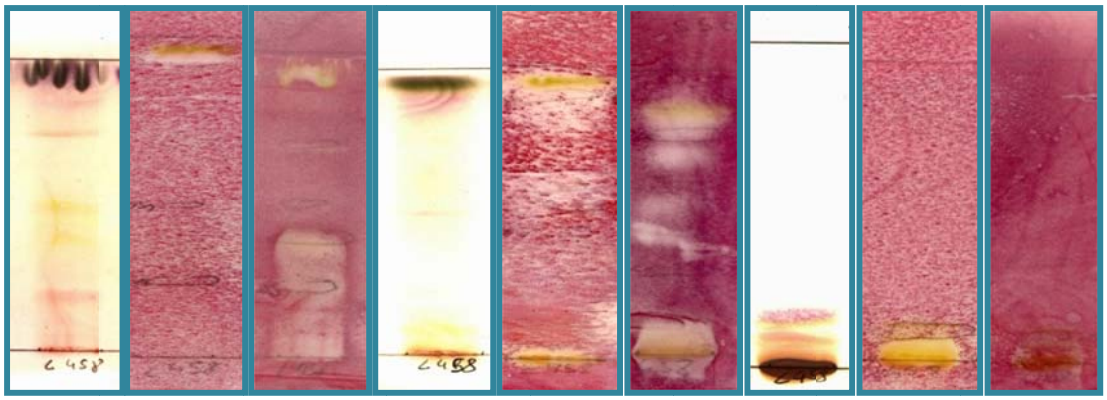


Fig. 4.31 Fig. 4.32 Fig. 4.33 Fig. 4.34 Fig. 4.35 Fig. 4.36 Fig. 4.37 Fig. 4.38 Fig. 4.39

Fig. 4.31: *Spirostachys africana* EMW; Fig. 4.32: *Spirostachys africana* EMW against *E. coli*; Fig. 4.33: *Spirostachys africana* EMW against *S. aureus*; Fig. 4.34: *Spirostachys africana* CEF; Fig. 4.35: *Spirostachys africana* CEF against *E. coli*; Fig. 4.36: *Spirostachys africana* CEF against *S. aureus*; Fig. 4.37: *Spirostachys africana* BEA; Fig. 4.38: *Spirostachys africana* BEA against *E. coli*; Fig. 4.39: *Spirostachys africana* BEA against *S. aureus*

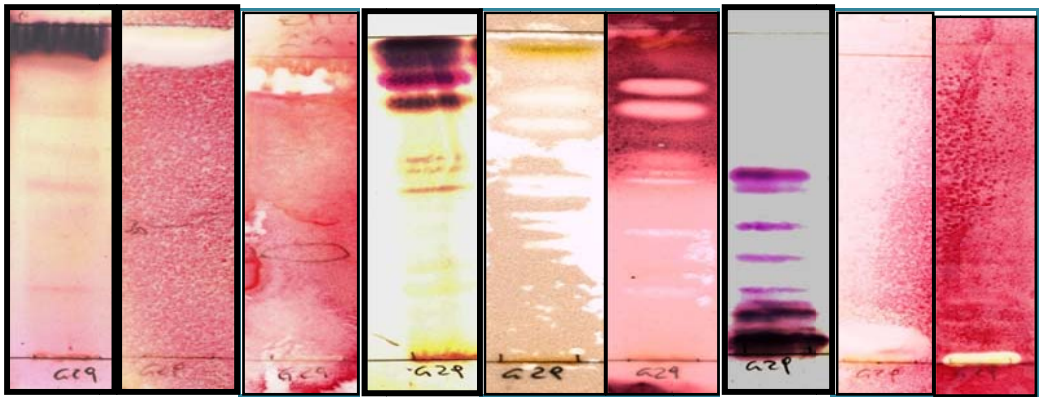


Fig. 4.40 Fig. 4.41 Fig. 4.42 Fig. 4.43 Fig. 4.44 Fig. 4.45 Fig. 4.46 Fig. 4.47 Fig. 4.48

Fig. 4.40: *Tetradenia riparia* EMW; Fig. 4.41: *Tetradenia riparia* EMW against *E. coli*; Fig. 4.42: *Tetradenia riparia* EMW against *S. aureus*; Fig. 4.43: *Tetradenia riparia* CEF; Fig. 4.44: *Tetradenia riparia* CEF against *E. coli*; Fig. 4.45: Tetradenia ripariaCEF against *S. aureus*; Fig. 4.46: *Tetradenia riparia* BEA; Fig. 4.47: *Tetradenia riparia* BEA against *E. coli*; Fig. 4.48: *Tetradenia riparia* BEA against *S. aureus*

4.4 Conclusions

This study compared 53 different plants, all extracted with acetone, in terms of the antibacterial activity of these plants with claimed medicinal value. The tannin determination assists in determining whether the antibacterial activity of the plant extracts is directly related to high tannin content or not. These results will help students of the Phytomedicine Programme in the future to choose plants with good activity but low tannin content for *in vivo* -and other further studies, which is important since tannins are

known to hinder the absorption of proteins and alkaloids, which might accelerate drug metabolism and result in blood levels of activities too low for a therapeutic effect (Williamson, 2001).

The five plants showing the overall best results regarding selectivity and activity in the antibacterial assays as well as a low tannin content and a high extract yield were *Acacia sieberiana* var. *woodii*, *Albizia adianthifolia*, *Deinbollia oblongifolia*, *Spirostachys africana* and *Tetradenia riparia*.

These five plants were selected based on the aspects discussed as well as on availability, on their bioautographic results and on the amount of work that has been done already on the plants as it is preferable to investigate plant species, on which little research has been done. The next step will be to determine the cytotoxicity *in vitro* of extracts of the five plant species. Based on these results, one of the five plants will be chosen for further analysis (i.e. animal experiments, isolation of active compounds and structure elucidation).

Chapter 5

Cytotoxicity of the five chosen extracts

5.1 Introduction

After selecting the five plant species *Acacia sieberiana* var. *woodii*, *Albizia adianthifolia*, *Deinbollia oblongifolia*, *Spirostachys africana* and *Tetradenia riparia* with the help of the ranking system established in chapter 4, the next step was to determine their *in vitro* cytotoxicity in order to make the decision regarding extracts to work on further.

In vitro cytotoxicity assays are useful for screening purposes to define cellular toxicity. This is considered primarily as the potential of an extract to induce cell death, in different cell types (Eisenbrand *et al.*, 2002). In order to produce a commercially useable product, the first step following bioactivity testing is to evaluate the toxicity of the plant extracts. Cell toxicity may be evaluated using the MTT (3 (4, 5-dimethyl thiazol-2yl)-2, 5-diphenyltetrazolium bromide) assay in which mitochondrial enzymes in viable but not dead cells cleave the yellow tetrazolium salt (MTT) to a blue formazan product (Mosmann, 1983). This assay is widely used as a preliminary screen to quantify cell proliferation, cytotoxicity and sensitivity (Mosmann, 1983; Fokkema *et al.*, 2002; Itamochi *et al.*, 2002). The MTT reduction is determined by the use of a microplate reader at a wavelength of 570 nm. The results are presented as a percentage of the value of the control cells. The concentration leading to a 50% death (LC_{50}) is calculated from concentration-response curves after linear regression analysis. Other cytotoxicity assays are available, for example the sulphorhodamine B assay and the neutral red assay which measure other aspects of cellular toxicity. The principle of the sulphorhodamine assay is based on the ability of the protein dye sulforhodamine B to bind electrostatically and pH dependently on protein basic amino acid residues of trichloroacetic acid-fixed cells (Houghton *et al.*, 2007). Under mild acidic conditions it binds to cells, and under mild basic conditions it can be extracted from cells and solubilised for measurement. Its sensitivity is comparable with that of several fluorescence assays (Voigt, 2005). The neutral red assay is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes (Mensah *et al.*, 2001; Repetto *et al.*, 2008). Since our laboratory at the Phytomedicine Programme has established a standard procedure for the MTT assay, this assay was used for the determination of the cell toxicity of the five extracts.

5.2 Materials and Methods

To determine the toxicity of the extracts, the MTT assay developed by Mosmann (1983) was used on African Green monkey kidney (Vero) cells (obtained from the Department of Veterinary Tropical Diseases of the Veterinary Faculty of the University of Pretoria) in a slightly modified version.

5.2.1 Cell culture

Minimum Essential Medium (MEM, Sigma) supplemented with 5% foetal calf serum (Highveld Biological), glutamine (Sigma) and gentamicin (Virbac) was used to culture the Vero cells. Cells were subcultured in 75 cm^2 culture flasks (Greiner) every week using trypsin-versene (Sigma) to remove cells from the culture flask.

5.2.2 Counting of cells and preparation of plates

The cells were counted using a Neubauer haemocytometer. Trypan blue (0.4%) in an equal volume was added to the cell aliquot to determine the number of viable cells. Those cells able to actively exclude the trypan blue appeared clear, while dead cells or those with a damaged cell membrane appeared blue.

In preparing cell suspensions for plating, cells were constituted to 10 000 cells/ml and 200 µl (2 500 cells per well) were pipetted into each well of a 96-well microtitre plate, except for columns 1 and 12 which contained medium alone to minimize evaporation from other wells. The plates were incubated overnight at 37°C in a 5% CO_2 incubator to allow the cells to attach.

5.2.3 MTT assay working method

Stock solutions of the plant extracts were prepared by reconstitution to a concentration of 100 mg/ml in acetone. Appropriate dilutions of each extract were prepared in growth medium and pipetted onto the cell monolayers after removing the original MEM from the wells. The viable cell growth after 120 h incubation (5 days in a 5% CO_2 incubator) with plant extracts was determined using the tetrazolium-based colorimetric assay (MTT assay) described by Mosmann (1983) with slight modifications. In this assay, the extract-containing MEM was removed and the cells were washed with 150 µl PBS. The PBS was removed and 200 µl fresh MEM was added to the wells. An aliquot of 30 µl MTT (stock solution of 5 mg/ml in PBS) was added to each well and the plates were further incubated for 4 h. After incubation, the MEM was removed from each well carefully, without disturbing the MTT crystals in the wells. The MTT formazan crystals were dissolved by adding 50 µl DMSO to each well. The plate was shaken gently until the solution was dissolved (or shaken in the microplate reader). The amount of MTT reduction was recorded by immediately measuring the absorbance at 570 nm (reference wavelength of

630 nm) in a Versamax microplate reader (Molecular Devices). The wells in column 1, which previously contained medium and MTT but no cells were used to blank the plate reader. The results were described as a percentage of the control cells. Berberine chloride (Sigma) was used as a positive control, and acetone served as the negative control. Tests were carried out in quadruplicate and each experiment was repeated three times. The assay was repeated two to three times to ensure reproducible results.

5.3 Results and Discussion

5.3.1 Determination of the LC_{50} values of the five different plant extracts

After determining in which range the cytotoxicity of the extracts lay, a second round of MTT assays was performed to determine a more accurate LC_{50} by testing 6 different concentrations within the range. The assay was repeated two to three times. The diagrams depicting these results can be seen in Figures 5.2 to 5.7.

Albizia adianthifolia was tested in the concentrations of 0.1 mg/ml, 0.075 mg/ml, 0.05 mg/ml, 0.025 mg/ml 0.01 mg/ml and 0.0075 mg/ml. From these results a response curve was drawn and the trend line was determined. With the trend line it was possible to determine the values for the equation $y = kx + d$. With this formula we determined that the LC_{50} was 0.068 mg/ml (standard deviation: 0.004).

Acacia sieberiana var. *woodii* was tested in the concentrations of 0.1 mg/ml, 0.075 mg/ml, 0.05 mg/ml, 0.025 mg/ml, 0.01 mg/ml and 0.0075 mg/ml. The response curve was drawn and the trend line determined. The resulting LC_{50} was 0.026 mg/ml (standard deviation: 0.005).

When doing the preliminary screening it was noted that the LC_{50} of *Deinbollia oblongifolia* lay somewhere between 1.0 mg/ml and 0.1 mg/ml, but when the second round of screening was done in the concentrations of 1.0 mg/ml, 0.75 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.1 mg/ml and 0.075 mg/ml it became obvious that the LC_{50} was closer to 0.075 mg/ml and so the assay was redone at six lower concentrations (0.1 mg/ml, 0.075 mg/ml, 0.05 mg/ml, 0.025 mg/ml, 0.01 mg/ml and 0.0075 mg/ml). The value of the LC_{50} was 0.078 mg/ml (standard deviation: 0.00).

Spirostachys africana was tested in the concentrations of 0.1 mg/ml, 0.075 mg/ml, 0.05 mg/ml, 0.025 mg/ml, 0.01 mg/ml and 0.0075 mg/ml. The response curve was drawn and the trend line determined. The resulting LC_{50} was 0.025 mg/ml (standard deviation: 0.005 mg/ml).

Tetradenia riparia was tested in the concentrations of 0.1 mg/ml, 0.075 mg/ml, 0.05 mg/ml, 0.025 mg/ml, 0.01 mg/ml and 0.0075 mg/ml. The response curve was drawn and the trend line determined. The resulting LC_{50} was 0.028 mg/ml (standard deviation: 0.005 mg/ml).

Figure 5.1 gives an example of how the linear regression curve was plotted to determine the LC_{50} in case of one of the *Tetradenia riparia* samples.

The selectivity index (SI) of all five species was determined using the following formula:

$SI = LC_{50}$ (mg/ml) / MIC (mg/ml)

Table 5.1 lists all the LC_{50} values and the values for the SI obtained.

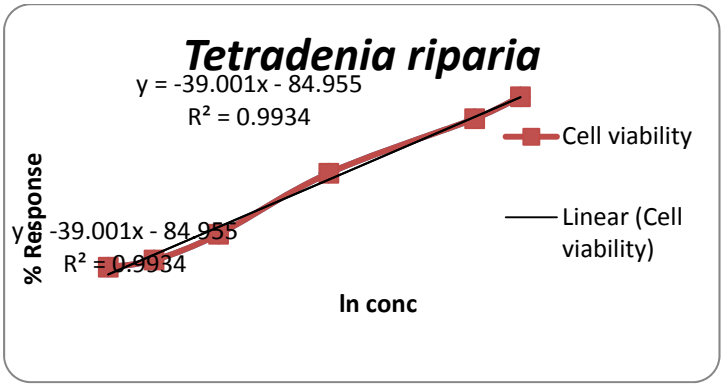


Fig. 5.1: Linear regression curve and equation for one of the *Tetradenia riparia* samples

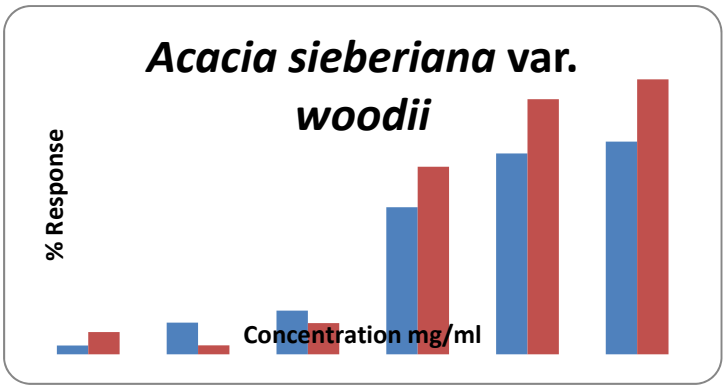


Fig. 5.2: Toxicity of *Acacia sieberiana* var. *woodii* extract

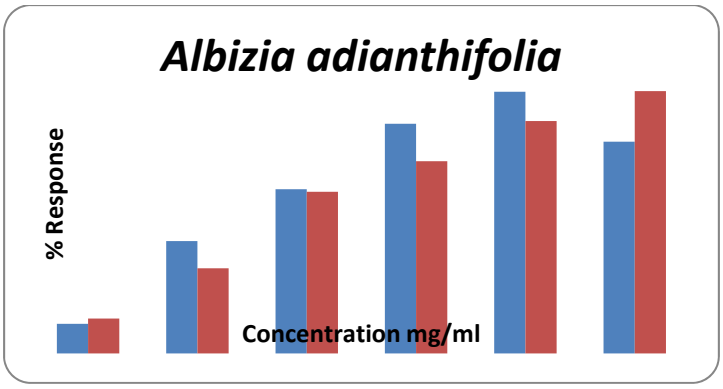


Fig. 5.3: Toxicity of *Albizia adianthifolia* acetone extract

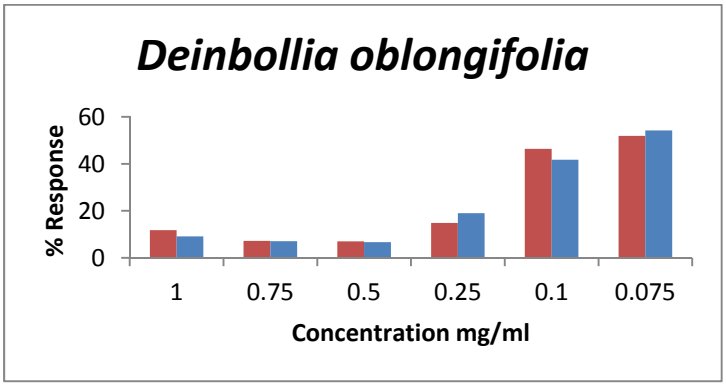


Fig. 5.4: Toxicity of *Deinbollia oblongifolia* extract1st experiment

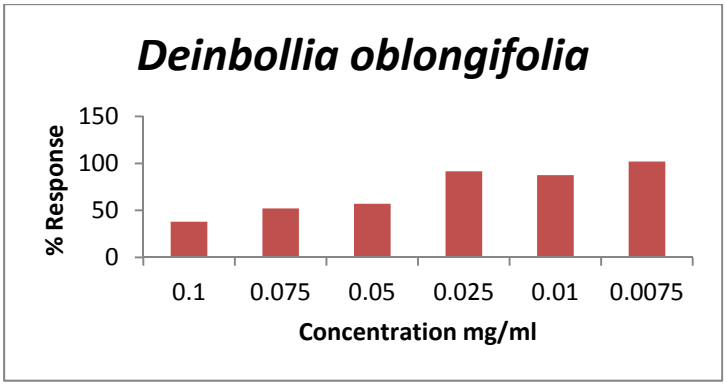


Fig. 5.5: Toxicity of *Deinbollia oblongifolia*extract

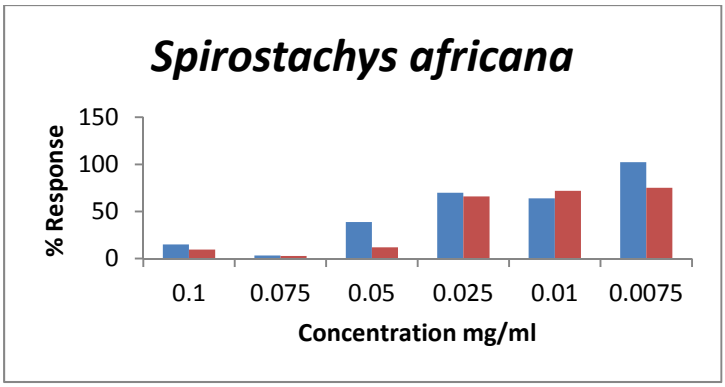


Fig. 5.6: Toxicity of *Spirostachys africana* extract

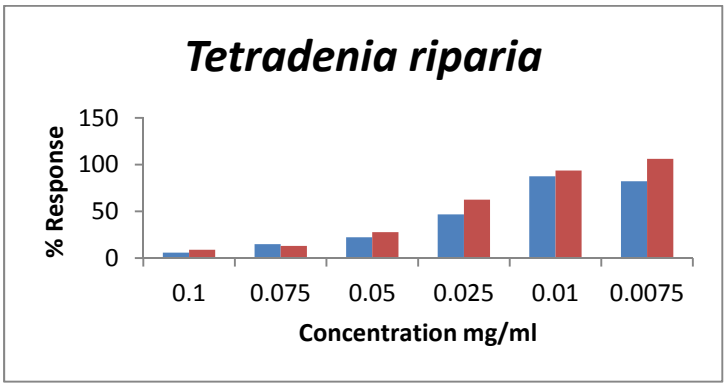


Fig. 5.7: Toxicity of *Tetradenia riparia* extract

Table 5.1: LC_{50} and SI values of the different species

Species	LC_{50} (mg/ml) (± SD)	SI
Acacia sieberiana var. *woodii*	0.026 ± 0.005	0.20
Albizia adianthifolia	0.068 ± 0.004	0.49
Deinbollia oblongifolia	0.078 ± 0.00	0.46
Spirostachys africana	0.025 ± 0.005	0.19
Tetradenia riparia	0.028 ± 0.005	0.31

5.4 Conclusion

Results from the MTT assays demonstrated that the least toxic extract was derived from *Deinbollia oblongifolia*. As a second plant on which to conduct further studies, *Spirostachys africana* was selected even though the extract was relatively toxic with an LC_{50} value of 0.025 mg/ml. The next step was to potentize these two extracts by increasing their activity while attempting to minimize toxicity.

Chapter 6

Potentization of *Deinbollia oblongifolia* and *Spirostachys africana* extracts

6.1 Introduction

After the previous study regarding cell toxicity, *Deinbollia oblongifolia* and *Spirostachys africana* were chosen to be potentized by solvent-solvent fractionation. These two plants were relatively active against *E. coli* (*Deinbollia oblongifolia* MIC = 0.17 mg/ml and *Spirostachys africana* MIC = 0.13 mg/ml) and based on the tannin assays performed earlier had a low concentration of tannins. The evaluation of their cytotoxicity resulted in relatively low cytotoxicity values (LC_{50}). The method of potentization allows us to enhance the activity as well as possibly reduce the toxicity of the plant extracts by splitting the crude extract into fractions which may separate active and toxic compounds. During this fractionation process inactive compounds might be concentrated in some of the fractions, leaving other fractions with the more active compounds and therefore enhancing their activity by reducing the dilution on a mass basis of the inactive compounds. The five resulting fractions in the method used (Suffness and Douros, 1979) each have different chemical properties due to the solvent-solvent fractionation process where solvents of increasing in polarity are used, i.e. hexane, chloroform, 35% water in methanol, n-butanol and water.

This process might allow for some fractions to be less toxic than others due to the fact that the toxic compounds might be present in a fraction with a different polarity from that of the active non-toxic compounds. It has already been proven that through solvent-solvent fractionation, the toxicity of certain extracts can be lowered due to the removal of toxic compounds (Liu et al, 2008). Unfortunately it is often the case that the compound(s) responsible for antibacterial activity are the same ones causing cytotoxicity.

6.2 Materials and Methods

For the potentization of the extracts the solvent-solvent fractionation method described by Suffness and Douros (1979) and modified by Eloff (1998) was used. With this method five different fractions were obtained, which were then evaluated for their antibacterial properties.

6.2.1 Mass extraction

Leaves of the two tree species, *Deinbollia oblongifolia* and *Spirostachys africana*,were collected at the SANBI Pretoria National Botanical Garden in the months of November and December 2009. The trees were identified by Joseph Khangela Baloyi, an employee of the Botanical Garden (Voucher specimens are being lodged at the herbarium). The leaves were dried in a dark room for one week and then ground to a fine powder using a Macsalab mill (Model 200 LAB). The resulting powder was stored in sealed glass containers.

Five hundred g of the ground leaves of *Spirostachys africana* and 300 g of the ground leaves of *Deinbollia oblongifolia* were exhaustively extracted with acetone in a 1:10 ratio (i.e. 5 l and 3 l of acetone respectively) in a 10 l glass container and shaken for one hour on a Labotec shaker model 20.2. This process was repeated three times with fresh aliquots of acetone on the same plant material. The extract was then left to settle and was filtered through cotton wool and then through Whatman No1 filter paper using a Buchner funnel. The extract was concentrated to a minimum volume using a Büchi rotavapor R-114 (Labotec) at 45°C. The remaining extracts were transferred to pre-weighed glass jars and left to completely dry under a constant flow of air.

6.2.2 Solvent-solvent fractionation

The *Spirostachys africana* and the *Deinbollia oblongifolia* extracts were fractionated using the solvent-solvent fractionation method developed by Suffness and Douros (1979) in a slightly modified version. The technique and schematic procedure of the solvent-solvent fractionation procedure is provided in Figure 6.1.

The method developed by Suffness and Douros (1979) was modified by leaving out one of the fractionation steps, namely the carbon tetrachloride step due to potential health problems with using large volumes of this solvent. There were therefore four fractionation steps in the solvent-solvent fractionation process. Each step of the fractionation was carried out once, and the steps are described below.

Step 1: Chloroform-water fractionation step: The dried extract was mixed with equal volumes of chloroform and water (100 ml each) and was transferred to a separating funnel. The liquid was mixed well by careful rotation of the funnel. After partition was achieved, the bottom phase which contained the chloroform was placed into a pre-weighed büchi (round-bottomed) flask.

Step 2: Water and n-butanol fractions: To the 100 ml of the water fraction 100 ml of n-butanol was added to yield a n-butanol and a water fraction after separation in a separating funnel. These fractions were placed in pre-weighed glass jars and then left to dry under a constant air flow.

Step 3: Hexane fraction: The chloroform fraction was reduced to a minimum volume in a rotavapor and left to dry under a constant flow of air. The dried fraction was dissolved in equal volumes of hexane and 10% water in methanol (each 100 ml) and placed into a separating funnel. Since no partition was achieved that way, a further 600 ml of the 10% water in methanol solvent was added to achieve partition into a hexane and a 10 % water in methanol fraction. The hexane fraction was collected into pre-weighed glass jars and left to dry under a constant flow of air.

Step 4: Chloroform and 35% water in methanol fraction: The 10% water in methanol fraction resulting from the hexane extraction was diluted to 35% water in methanol. An equal volume of chloroform was added and the mixture was extracted in the separating funnel and the resulting phases collected in pre-weighed glass jars. They were then dried under a constant flow of air.

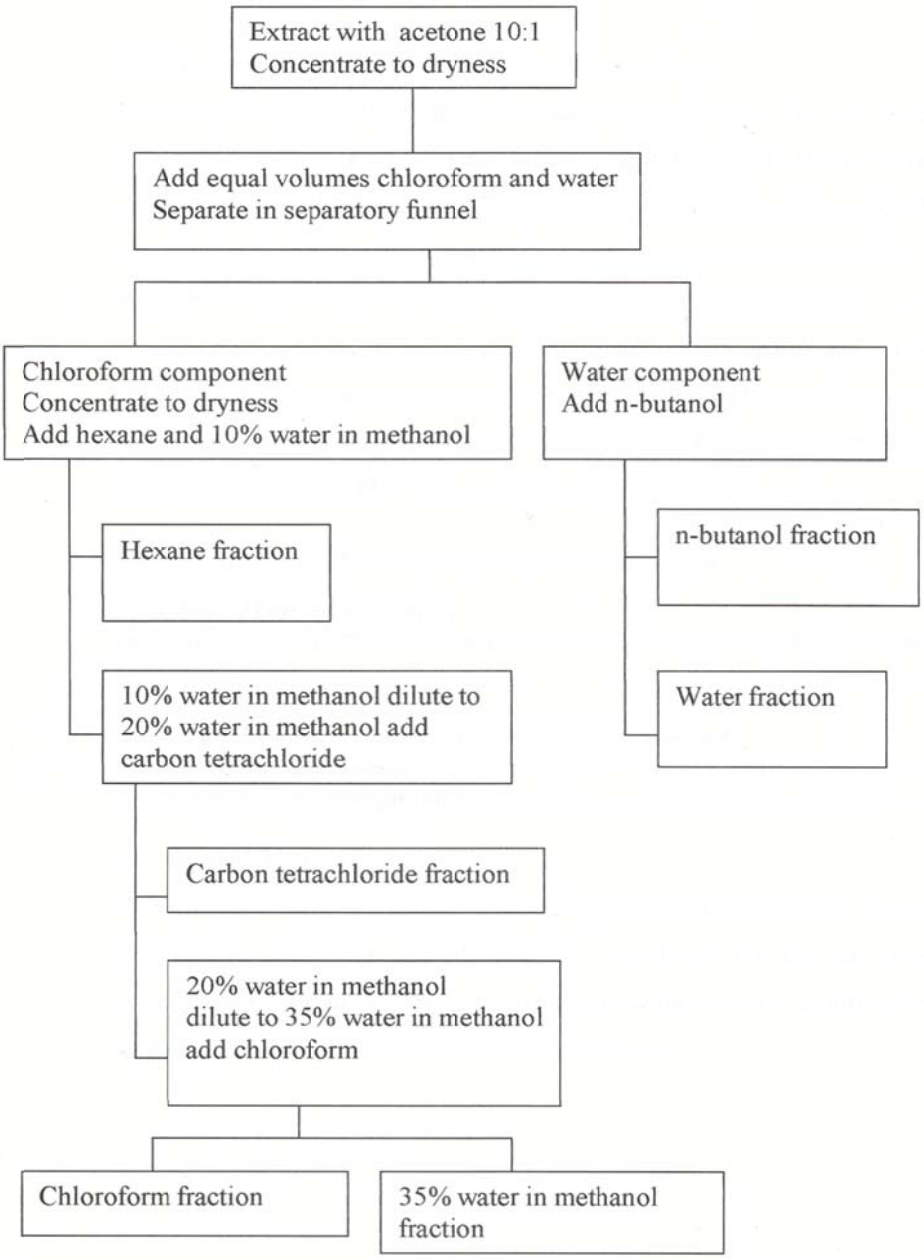


Fig 6.1: Representation of solvent-solvent fractionation procedure (Suffness and Douros, 1979)

6.2.3 TLC fingerprinting and bioautographic assays

The dried fractions were dissolved in acetone to a concentration of 10 mg/ml and then loaded (100 µg) onto two sets of three TLC plates and run in each of the three solvent systems developed in our lab (EMW, CEF, BEA). One set was sprayed with vanillin-sulphuric acid reagent to serve as a reference chromatogram.

The second set of TLC plates was used for bioautography using *E. coli* as test organism as described in section 2.2.5.

6.2.4 Minimum inhibitory concentration determination

The minimum inhibitory concentrations of the resulting fractions were determined by employing the 2-fold serial dilution assay described in section 2.2.6. This assay was done three times in triplicate to ensure reproducibility.

6.2.5 Cytotoxicity assays

6.2.5.1 Cell culture

The same method as described in section 5.2.1 was used to prepare the cell culture.

6.2.5.2 Counting of cells and preparation of plates

The same method as described in section 5.2.2 was used to count the cells and prepare the plates.

6.2.5.3 MTT-assay working method

The same method as described in section 5.2.3 was used.

6.3 Results and Discussion

6.3.1 Mass extraction

The resulting dried extract from 300 g *Deinbollia oblongifolia* weighed 11.18 g and the resulting dried extract from 500 g *Spirostachys africana* weighed 22.51 g. This means the extract yield for *Deinbollia oblongifolia* was 3.7% and for *Spirostachys africana* with acetone was 4.5%.

6.3.2 Solvent-solvent fractionation

The chloroform fraction resulting from the first step by mixing the dried extract with chloroform and water and separating the two phases had a dry weight of 16.2 g for *Spirostachys africana* and 10.38 g for *Deinbollia oblongifolia*.

The resulting fraction masses and their percentage in relation to the total extraction yield for *Deinbollia oblongifolia* are shown in Table 6.1.

Table 6.1: Fraction masses and percentages of *Deinbollia oblongifolia*

Fraction	Mass (g)	Percentage of total extraction yield
Water	1.32 g	0.44 %
n-Butanol	0.27 g	0.09 %
Hexane	8.02 g	2.67 %
Chloroform	1.11 g	0.37 %
35% water in methanol	0.53 g	0.18 %

These resulting masses total 11.25 g, which means only 0.05 g were lost during the separation process.

The resulting fraction masses and their percentage in relation to the total extraction yield for *Spirostachys africana* are shown in Table 6.2.

Table 6.2: Fraction masses and percentages of *Spirostachys africana*

Fraction	Mass (g)	Percentage of total extraction yield
Water	3.49 g	0.70%
n-Butanol	3.59 g	0.72%
Hexane	8.00 g	1.6%
Chloroform	3.69 g	0.74%
35% water in methanol	0.55 g	0.11%

These resulting masses total 19.32 g, which means that 3.19 g were lost during the separation process.

6.3.3 Minimum inhibitory concentration

The minimum inhibitory concentrations against *E. coli* of the two potentized extracts were determined by the MIC assay described in section 2.2.6.

The resulting MIC values for the fractions of each plant are shown in Tables 6.3 and 6.4.

Table 6.3: MIC values of the fractions of *Deinbollia oblongifolia* against *E. coli*

Fraction	Average MIC against *E. coli* after 60 min (mg/ml) (± SD)	Average MIC against *E. coli* after 120 min (mg/ml) (± SD)
Water	1.38 ± 0.92	1.38 ± 0.92
n-Butanol	0.75 ± 0.39	0.75 ± 0.39
Hexane	0.18 ± 0.10	0.30 ± 0.22
Chloroform	0.07 ± 0.02	0.08 ± 0.00
35% water in methanol	0.12 ± 0.04	0.20 ± 0.11

Table 6.4: MIC values of the fractions of *Spirostachys africana* against *E. coli*

Fraction	Average MIC against *E. coli* after 60 min (mg/ml) (± SD)	Average MIC against *E. coli* after 120 min (mg/ml) (± SD)
Water	0.50 ± 0.15	0.82 ± 0.33
n-Butanol	0.18 ± 0.10	0.25 ± 0.07
Hexane	0.20 ± 0.07	0.25 ± 0.07
Chloroform	0.05 ± 0.02	0.08 ± 0.06
35% water in methanol	0.18 ± 0.10	0.40 ± 0.15

The chloroform fractions of both extracts exhibited the best activity against *E. coli.* In the case of *Deinbollia oblongifolia* the chloroform fraction had a much better activity (MIC = 0.08 mg/m) than the crude extract (MIC = 0.17 mg/ml). With *Spirostachys africana* the chloroform fraction also had a better activity (MIC = 0.08 mg/ml) than the crude extract (MIC = 0.13 mg/ml).

6.3.4 TLC fingerprinting and bioautographic assays

The TLC plates gave a reference to the compounds in the plants and helped to identify the active compounds when the fractions were subjected to bioautography. The most active antibacterial compounds were detected in the chloroform fractions of both extracts. The compounds that were active in *Deinbollia oblongifolia* seemed to inhibit the bacteria to a stronger degree than the ones from *Spirostachys africana*.

The chromatograms and bioautographic assays can be seen in Figures 6.2 to 6.7

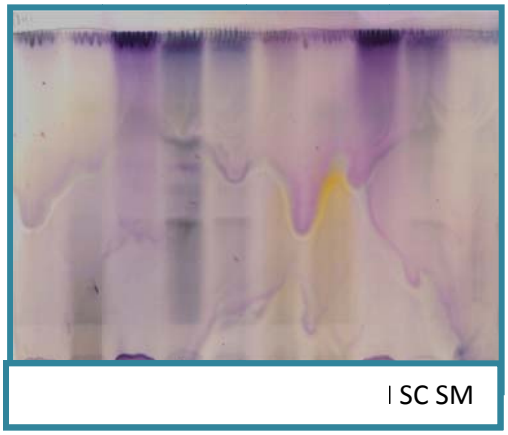


Fig. 6.2: Chromatograms EMW

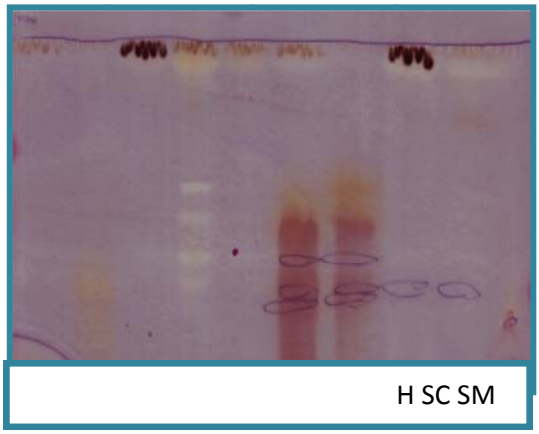


Fig. 6.3: Bioautography against *E. coli* EMW

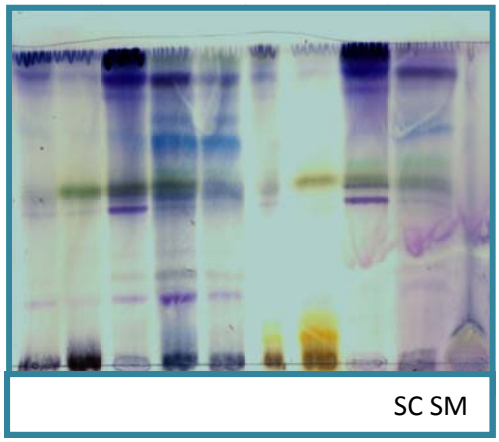


Fig. 6.4: Chromatograms CEF

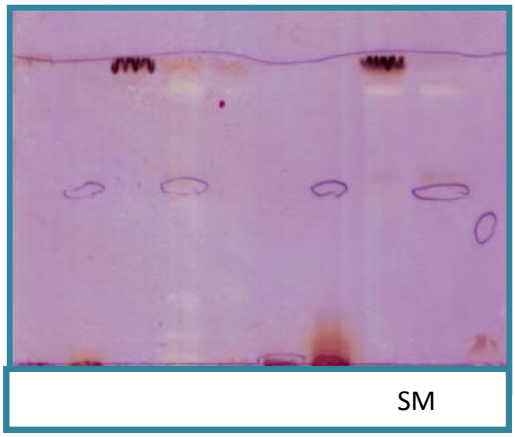


Fig. 6.5: Bioautography against *E. coli* CEF

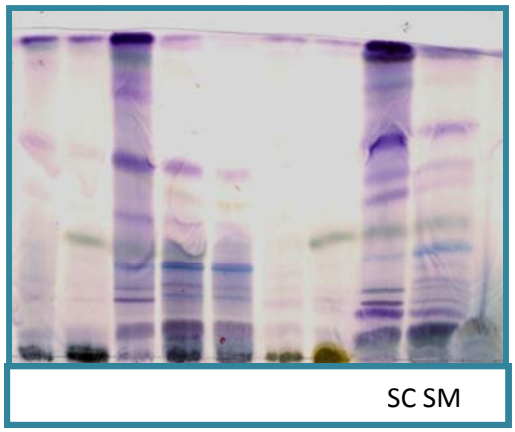


Fig. 6.6: Chromatograms BEA

Fig. 6.7: Bioautography against *E. coli* BEA

6.3.5 Cytotoxicity assays

The chloroform fraction of *Deinbollia oblongifolia* was tested in the range of 1 mg/ml to 0.1 mg/ml in the concentrations of 1 mg/ml, 0.75 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.1 mg/ml and 0.075 mg/ml. The resulting LC_{50} value was 0.188 mg/ml (standard deviation: 0.017).

The chloroform fraction of *Spirostachys africana* was tested in the range of 0.1 mg/ml and 0.01 mg/ ml in the concentrations of 0.1 mg/ml, 0.075 mg/ml, 0.05 mg/ml, 0.025 mg/ml, 0.01 mg/ml and 0.0075 mg/ml. The resulting LC_{50} value was.0.062 mg/ml (standard deviation: 0.02).

The assay was repeated twice to ensure reproducibility. The results are also shown in Figures 6.8 and 6.9.

The selectivity index (SI) was again determined and it was 2.35 for the chloroform fraction of *Deinbollia oblongifolia* and 0.78 for the chloroform fraction of *Spirostachys africana*.

The results for the values for the LC_{50} and the SI are shown in table 6.5.

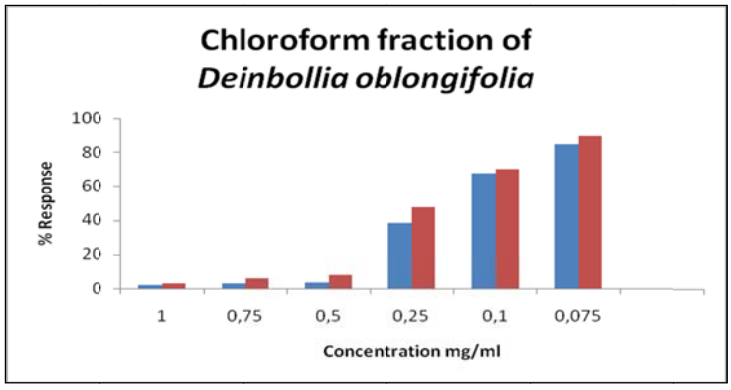


Fig. 6.8: Toxicity of the chloroform fraction of *Deinbollia oblongifolia*

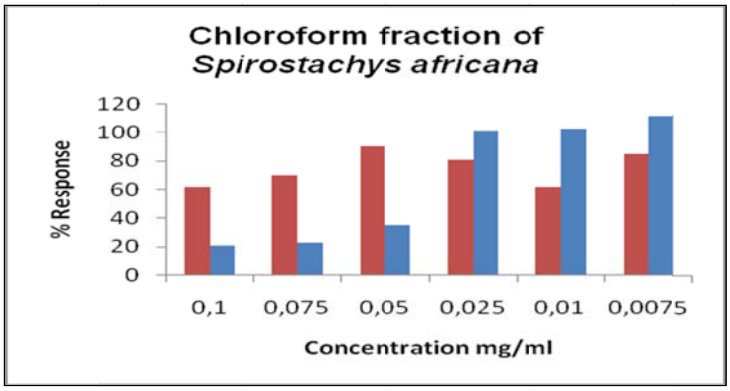


Fig. 6.9: Toxicity of the chloroform fraction of *Spirostachys africana*

Table 6.5: LC_{50} and SI of the chloroform fractions

Extract	LC_{50} (mg/ml) (± SD)	SI
Chloroform fraction of *Deinbollia oblongifolia*	0.188 mg/ml ± 0.017	2.35
Chloroform fraction of *Spirostachys Africana*	0.062 ± 0.02	0.78

6.4 Conclusion

The potentization of the two crude extracts of *Deinbollia oblongifolia* and *Spirostachys africana* showed that the intermediate polarity compounds in the chloroform fraction of both extracts had the highest activity. The next step was to determine the cytotoxicity of these chloroform fractions to determine which one of them is least toxic before testing the *in vivo* efficacy.

The chloroform fraction of *Deinbollia oblongifolia* was less toxic than that prepared from *Spirostachys africana*. Therefore further work was done on the chloroform fraction of *D. oblongifolia*. Even though the LC_{50} value of 0.188 mg/ml represented a rather high cellular toxicity, it is possible that the toxicity could be lower in a biological system since it may be metabolised and changed *in vivo* before it affects the animal. Since a lot of medications are excreted through the kidneys and therefore affect them, and most laboratories have available Vero kidney cells for cytotoxicity assays, the kidney cells were chosen for the cytotoxicity evaluation.

The values for the selectivity Index (2.35 for the potentized extract of *Deinbollia oblongifolia* compared to a value of 0.45 for the crude extract and 0.78 for the potentized extract of *Spirostachys*

*africana*compared to the lower value of 0.19 for the crude extract) show that a potentization of both extracts was indeed achieved.

The next step in this project was to isolate the active compounds from the extract and then to investigate the acute and subacute toxicity of the chloroform fraction as well as of the crude extract in a live rat model. The crude extract was not as active *in vitro* against *E. coli* as the chloroform fractions, but many substances are metabolically modified in a biological system and it is possible that the crude extract could exhibit a higher activity in an animal than in the laboratory tests, which would be ideal since it is economically more feasible to work with the crude extract.

Chapter 7

Isolation of active compounds from *Deinbollia oblongifolia*

7.1 Introduction

To discover new bioactive compounds from plants, bioassay-guided fractionation is often employed. Novel isolated bioactive compounds could lead to the development of new drugs if they are sufficiently active or non-toxic, and possibly the chemical structure of promising targets can be modified to achieve this objective. There is one major drawback to this process in that it frequently happens that known metabolites are isolated. However, new activities for known compounds are sometimes discovered, so useful information is still obtained.

The compounds in plant extracts are commonly isolated using column chromatography. In principle, plant constituents are distributed between the solid phase (for example silica gel or Sephadex) and the mobile phase, which comprises an eluting solvent. In silica gel the separation of compounds in an extract from each other is based on a number of factors including the polarity of compounds, hence compounds are eluted separately from the column with solvent systems of differing polarity. Silica gels constitute polar ends which interact strongly with polar compounds and they are eluted later from the column. In Sephadex gel filtration the separation of constituents in an extract depends on the size of the molecules. Constituents with a small molecular weight interact strongly with the matrix of the gel and tend to move slowly through the gel and are eluted later while the large molecular weight constituents are eluted early because they move fast through the column.

Deinbollia oblongifolia (Sapindaceae) or Dune soap-berry tree is a small tree or shrub up to 3.5 m in height, occurring in coastal open woodland, dune bush and forest, along riverine fringes and in bush clumps. Its distribution is mainly in KwaZulu Natal. To the knowledge of the author, no active compounds have been isolated from this species so far.

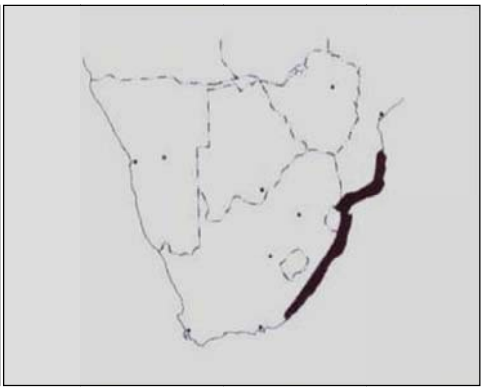


Fig. 7.1: *Deinbollia oblongifolia* **Fig. 7.2: Distribution of** *Deinbollia oblongifolia*

Materials and methods

7.2.1 Preparation of the bulk extract

Leaves of the tree species, *Deinbollia oblongifolia* were collected at the SANBI Pretoria National Botanical Garden in the months of November and December 2009. The trees were identified by Joseph Khangela Baloyi, an employee of the Botanical Garden. The leaves were dried in a dark room for one week and then ground to a fine powder using a Macsalab mill (Model 200 LAB). The resulting powder was stored in sealed glass containers.

The ground leaves (300 g) of *Deinbollia oblongifolia* were exhaustively extracted with the extracting solvent acetone in a 1:10 ratio (e.g. 3 l of acetone respectively) in a 10 l glass container and shaken for one hour on a Labotec shaker model 20.2. This process was repeated three times with fresh aliquots of acetone on the same plant material. The extract was then left to settle and was filtered through cotton wool and then through Whatman No1 filter paper using a Buchner funnel. The extract was concentrated to a minimum volume using a Büchi rotavapor R-114 (Labotec) at 45°C. The remaining extract was transferred to pre-weighed glass jars and left to completely dry under a constant flow of air. The mass of the resulting extract was 11.18 g.

7.2.2 Solvent fractionation

Since the chloroform fraction was the most active, the isolation of the active compounds was done using the chloroform fraction obtained by the solvent fractionation that was done as described in section 7.3.2. The weight of the resulting chloroform fraction was 3.69 g.

7.2.3 Column chromatography

A glass column of diameter 5 cm was used to separate the constituents of 3.69 g of the chloroform fraction of the *D. oblongifolia* extract. Silica gel 60 (400 g) (Merck) was mixed with hexane to form a slurry and this was then packed into the glass column up to a height of 37 cm. The chloroform fraction of the acetone extract (4 g) was dissolved in a small volume of acetone, mixed with about 25 g of silica gel 60 (Merck) and left to completely dry under a constant flow of air. The dried extract and silica powder combination was then delicately placed on top of the packed column. Initially the column was eluted with 200ml of 100% hexane and subsequently the polarity of the eluting solvent was sequentially increased by replacing hexane with ethyl acetate in an increasing percentage (80ml of 5%, 320ml of 10%, 120ml of 15%, 120 ml of 20% and 60 ml of 50%). The rest of the column was eluted with 100% methanol (200ml). The fractions were labelled 1 to 38. Fractions of approximately 20 ml each were collected in test tubes. In Table 7.1 the fractions are listed with their corresponding eluting solvent.

Table 7.1: Fractions with their corresponding solvent systems

Fraction	Solvent-system
1-2	100% hexane
3-6	95% hexane, 5% ethyl acetate
7-22	90% hexane, 10% ethyl acetate
23-28	85% hexane, 15% ethyl acetate
29-34	80% hexane, 20% ethyl acetate
35-37	50% hexane, 50% ethyl acetate
38 (200ml collected in a 250 ml flask)	100% methanol

7.2.4 Thin layer chromatography

The fractions were left to dry in front of a fan until the volume was decreased to concentrate the compounds. Then an aliquot of every third fraction was loaded onto 10 x 20 cm TLC plates. Two plates each were developed in a 10% ethyl acetate and 90% hexane solvent system. One set of TLC plates was sprayed with vanillin-sulphuric acid spray reagent and served as reference chromatograms. The second set of plates was sprayed with *E. coli* and incubated for 18 hours and then sprayed with INT to visualize the compounds that are active against *E. coli*. Fractions with similar R_f values and antibacterial activity were combined and placed in pre-weighed glass vials. They were then left to dry under a constant flow of air.

7.2.5 Purification of column fractions

From the TLC plates in section 8.2.4 any fraction(s) containing impurities were washed by adding 2 ml of hexane and shaking the glass vial to try and dissolve any impurities soluble in hexane. The dissolved fraction was transferred into a preweighed glass vial and both fractions were dried under a constant flow of air. This was done to obtain pure compounds, but the active compound also dissolved in the hexane and therefore this washing step did not prove effective.

7.2.6 Preparative thin layer chromatography

The active fraction was further separated by preparative TLC on silica gel plates (Merck Silica gel 60 F_{254}, 0.25 mm thick) using EMW. The active fraction was scraped off the TLC plates and eluted from the silica with ethanol. The active compound in ethanol was filtered through Millipore filters (0.45 µm and 0.22 µm) to remove the silica. To ensure complete removal of the silica particles, the filtrate was passed through a Silica 60 (Merck) column prepared in a Pasteur pipette. The Silica 60 (Merck) was loaded to a height of 30 mm, and the pipette had an internal diameter of 5 mm. The purity of the isolated compound was confirmed by TLC using various solvent systems.

7.2.7 Minimum inhibitory concentration

The minimum inhibitory concentration of the pure compound against *E. coli* was determined using the method described in section 2.2.6.This assay was carried out three times in triplicate.

7.2.8 Cytotoxicity of the isolated compound

The toxicity of the pure compound was tested against Vero kidney cells following the method described in section 5.2 with the only difference being the range of concentrations in which the compound was tested. The compound was dissolved in extracting solvent, i.e. acetone, to produce a 20 mg/ml solution which was used for testing in the assays. The compound was screened at a range of concentrations to determine the LC_{50}.

7.2.9 Identification of purified active compound

7.2.9.1 Nuclear Magnetic Resonance Spectroscopy

Nuclear Magnetic Resonance (NMR) Spectroscopy (^{1}H NMR and ^{13}C NMR) was carried out to confirm the structure of the compound using a Varian Inova 500 MHz spectrometer, based at the CSIR, Pretoria.

7.3 Results and Discussion

7.3.1 Thin layer chromatography and bioautography of the fractions resulting from column chromatography:

Every fraction was loaded onto two sets of TLC plates that were run in the 10% ethyl acetate and 90% hexane solvent system. One plate was sprayed with the vanillin-sulphuric acid spray reagent and the other plate was sprayed and incubated with *E. coli* for 18 hours and then sprayed with INT to visualize the bacterial inhibition bands. The fractions that showed the relevant compound are shown below in Figures 7.1 and 7.2.

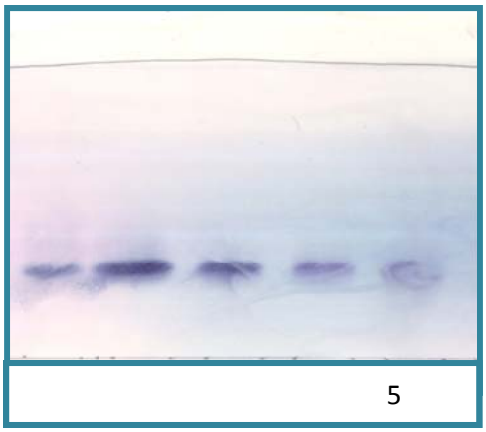


Fig. 7.3: Chromatograms 10% EA / 10%H

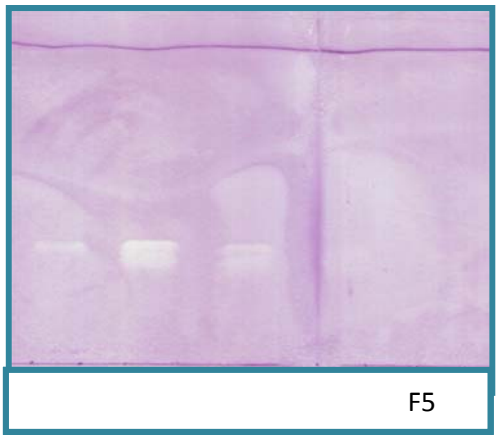


Fig. 7.4: Bioautography 10% EA / 90% H

The fractions 1 to 4 were combined and dried under a constant flow of air. They were spotted onto three sets of TLC plates together with the chloroform fraction for reference and run in the three solvent systems (EMW, CEF and BEA) and in the 10% ethyl acetate and 90% hexane solvent system. They were then sprayed with vanillin sulphuric acid spray reagent to show possible impurities. The combined fractions were also spotted onto another TLC plate and after being developed in the EMW solvent system, the plate was incubated with *E. coli* to show the activity. Figure 7.3 shows the respective plates.

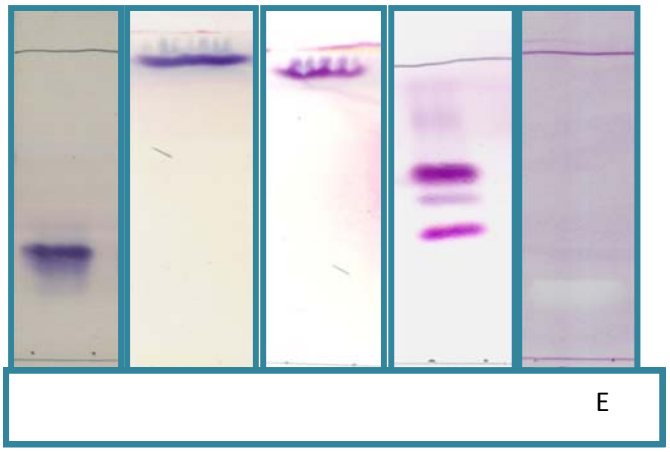


Fig. 7.5: A: Chromatogram combined fractions 10% EA / 90% H; B: Chromatogram combined fractions EMW; C: Chromatogram combined fractions CEF; D: Chromatogram combined fractions BEA; E: Bioautography combined fractions 10% EA / 90% H

The fractions proved to still contain impurities. These became especially obvious in the plate run with the BEA solvent system. The next step was to try and isolate the combined fractions further with preparative thin layer chromatography.

7.3.2 Preparative thin layer chromatography

The combined fractions were diluted with acetone to a concentration of 20 mg/ml and then loaded onto four TLC plates and run in the EMW solvent system. One small spot of the combined fractions was run alone at the edge of the plate to serve as a reference for the preparative TLC and was sprayed with vanillin-sulphuric acid reagent after development of the plate.

After preparative TLC was performed we were left with a pure compound. The compound was loaded onto three sets of two TLC plates that were developed in the EMW, CEF and BEA solvent systems. One plate developed in each solvent system was sprayed with the vanillin-sulphuric acid spray reagent to show the purity of the compound. The second plate developed in each of the solvent system was incubated with *E. coli* to show the compound's activity. The respective TLC and bioautographic plates can be seen in the Figures 7.4 to 7.5 below.

After the pure compound was dried, the total yield was between 10 and 20 mg.

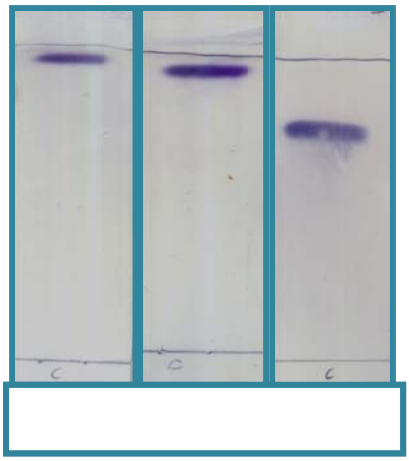


Fig. 7.6: Chromatograms of compound

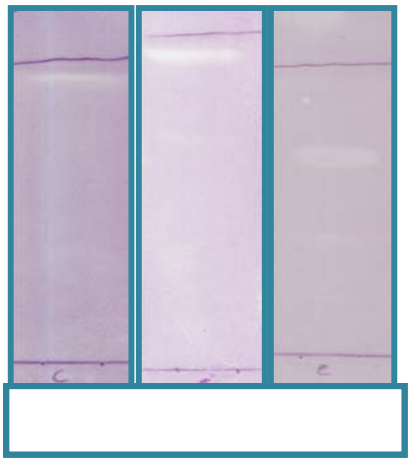


Fig. 7.7: Bioautography of compound against *E. coli*

7.3.3 Minimum inhibitory concentration

When determining the minimum inhibitory concentration of the purified compound against *E. coli* (ATCC 25922), it became apparent from the MIC value of 0.74 mg/ml after 60 and 120 minutes, that the antibacterial activity decreased by quite a lot in comparison with the potentized and even the crude extract. The total activity value was 0.07 ml/g, so it is also from the economic point of view clear that it would not be sensible to produce the pure compound as a treatment for diarrhoea.

7.3.4 Cytotoxicity of the isolated compound:

The cytotoxicity of the pure compound was tested using the MTT assay on Vero kidney cells. The range of concentrations tested lay between 10 and 200 µg/ml.

The LC_{50} for the pure compound, determined from the linear regression curve, was 42 µg/ml. The diagram depicting the cytotoxicity results is shown in Figure 7.6.

Based on the LC_{50} the selectivity index (SI) was determined to be 0.06.

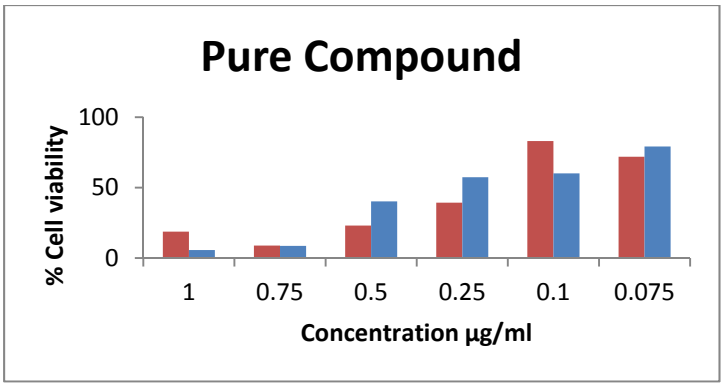


Fig. 7.8: Cytotoxicity of the isolated compound

8.3.5 Identification of the purified active compound

The NMR spectra were run at the CSIR by Ms Teresa Faleschini. Dr Ahmed Aroke interpreted the chemical structure.

Twenty mg of a white powder were isolated. To determine the structure, 2-dimensional NMR (HMBC-NMR, HSQC-NMR, ^{1}H-NMR, ^{13}C-NMR and COSY-NMR) spectra were interpreted and compared with available literature data (Mahato and Kundu, 1994). The correlation of the results can be seen in Tables 7.2 and 7.3.

The structure was determined to be a mixture of the two compounds 3β-OH-α-amyrin and a 3β-OH-β -amyrin, two triterpenoids. The structures can be seen in Figure 7.9.

Table 7.2: NMR-Spectra 1 of the isolated bioactive compound

C	H	HSQC	HMBC (C->H)
1	1.64 (CH_2)	38.76	H 28, H 16
2	1.60 (Ch_2)	27.22	H 15
3	3.2 (CH)	29.00	H 28, H 19, H 16
4	- (C)	42.07	H 18, H 16, H 29
5	0.715 (CH)	55.00	H 18, H 16, H 15, H19
6	1.54 (CH_3)	18.00	H 15
7	- (C)	-	-
8	- (C)	40	H 27, H 24
9	1.53 (CH)	48	H 18, H 16, H 15
10	- (C)	36.9	H 16
11	1.07 (CH_2)	25.35	-
12	5.10 (CH)	124	-
13	- (C)	139	H 23.3
14	- (C)	42	H 27, H 24
15	1.25 (CH_2)	29	-
16	0.87 (CH_2)	24	H 33
17	- (C)	33	H 24, H 29
18	1.31 (CH)	59	H 29 (CH_3)
19	- (CH)	39.6	H 16

20	- (CH)	39.6	H 33
21	- (CH_2)		-
22	- (CH_2)	41.57	-
23	0.81 (CH_3)	29	H 16
24	0.748 (CH_2)	15.48	H 55
25	0.96 (CH_3)	15.37	H 18
26	- CH_3	12	-
27	- CH_3	24	-
28	- CH_3	-	-
29	- CH_3	-	-
$_3$0	- CH3	-	-

Table 7.3: NMR-Spectra 2 of the isolated bioactive compound

C	H	HSQC	HMBC (C->H)
1	1.64 (CH_2)	38.76	H 28, H 16
2	1.60 (Ch_2)	27.22	H 15
3	3.2 (CH)	29.00	H 28, H 19, H 16
4	- (C)	42.07	H 18, H 16, H 29
5	0.715 (CH)	55.00	H 18, H 16, H 15, H19
6	1.54 (CH_3)	18.00	H 15
7	- (C)	-	-
8	- (C)	40	H 27, H 24
9	1.53 (CH)	48	H 18, H 16, H 15
10	- (C)	36.9	H 16
11	1.07 (CH_2)	25.35	-
12	5.10 (CH)	122	-
13	- (C)	145	H 23.3
14	- (C)	42	H 27, H 24
15	1.25 (CH_2)	29	-
16	0.87 (CH_2)	24	H 33
17	- (C)	33	H 24, H 29
18	1.31 (CH)	45	H 29 (CH_3)
19	- (CH)	39.6	H 16
20	- (CH)	39.6	H 33
21	- (CH_2)		-
22	- (CH_2)	41.57	-
23	0.81 (CH_3)	29	H 16
24	0.748 (CH_2)	15.48	H 55
25	0.96 (CH_3)	15.37	H 18
26	- CH_3	12	-
27	- CH_3	24	-
28	- CH_3	-	-
29	- CH_3	-	-
$_3$0	- CH3	-	-

The literature survey showed that this compound has not been isolated from the genus *Deinbollia* before.

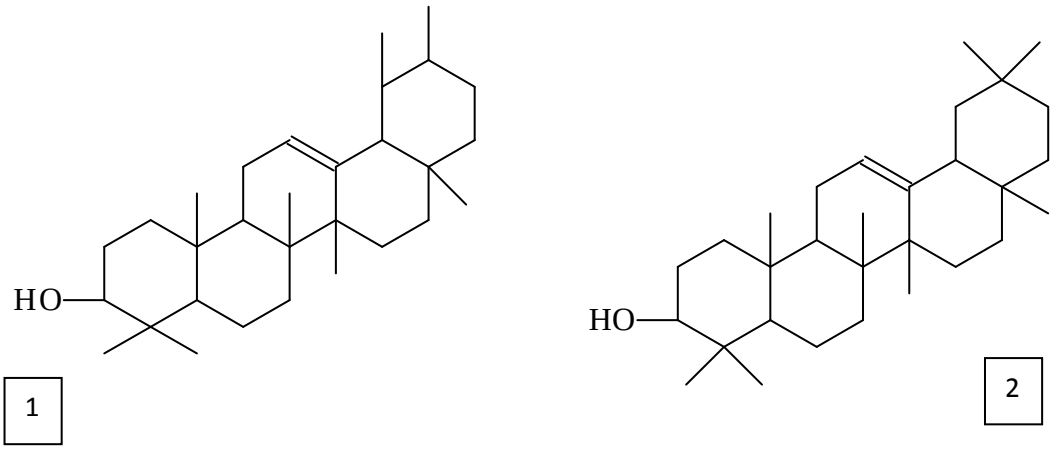


Fig. 7.9: Structures of 3β-OH-α-amyrin (1) and a 3β-OH-β-amyrin (2)

The ^{13}C chemical shift analysis made possible the identification of the structural features, namely oxygen-bearing methane carbons at δc=79.055 and 79.024 (C-3), and twoolefinic carbons atδc=124.411 and 121.713 (C-12) respectively. In the HMBC spectra, the signal at δc=124.411 correlates with 138.02, which is a characteristic feature of ursene derivatives, while δc= 121.713 correlates with 144, acharacteristic feature of oleanene derivatives. The carbon resonating at δc=59.056 (C-18) confirmed the presence of an ursene derivative with no carbonyl functional group at C-17. Also, a carbon resonating at δc=47.6 (C-18) confirmed the presence of an oleanene derivative with no carbonyl functional group at C-17. These observations indicate the presence of two closely related isomers as all signals were duplicated with minor differences. Detailed analysis of the^{13}C NMR spectra, HMBC, HSQC and COSY and comparison of the data reported in the literature (Mahato and Kundu, 1994) confirmed the mixture to be 3β-OH-α-amyrin (1)and 3β-OH-β-amyrin (2).

7.4 Conclusions

One compound that was active against the ATCC strain of *E. coli* and that also showed a distinct band in the bioautographic plates was isolated. Its toxic effect on Vero cells was also investigated and it has a LC_{50} value of 0.042 mg/ml which is a more toxic value than that obtained from the potentized fraction. The compound's activity against *E. coli* was also much lower than the activity of the potentized extract, 0.74 mg/ml opposed to 0.08 mg/ml. This proves that the activity of the plant is more owing to a synergistic effect of a number of compounds than being caused by the presence of a single compound. The structure of the compound was elucidated to be mixture of the two compounds 3β-OH-α-amyrin and a 3β-OH-β-amyrin,two triterpenoids.

Chapter 8

Efficacy of the potentized extract of *Deinbollia oblongifolia* against different *E. coli* strains

8.1 Introduction

All the tests performed to evaluate the antibacterial activities of the plant extracts thus far were done using only the ATCC 25922 strain of *E. coli*. To see whether the extracts are also active when tested against pathological *E. coli* strains, four of these strains were obtained and the extracts were tested against them. Bioautographic assays were also done to see whether the active compounds in the extract and the potentized version changed. The pure compound was also tested against the four pathological strains.

8.2 Materials and Methods

8.2.1 Bacterial cultures

Four *E. coli* strains that were isolated from different organs in different animals by the Department of Veterinary Tropical Diseases (Section Bacteriology, University of Pretoria) were used in the screening process. These included one strain from the sinus of poultry (B 1295), one from the large intestine of a pig (B1297), one isolated from a bovine liver and spleen (B 1314) and the last one was a bursal swab from poultry (B 1279).

The bacteria were cultured on blood agar plates. For the assay, liquid cultures were used. The preparation of the liquid medium and cultures was done as described in section 2.2.4.

8.2.2 Minimum inhibitory concentration determination

The activity of the crude extract, the potentized extract and the isolated compound against the different pathological *E. coli* strains was measured by the use of the microdilution assay described in section 2.2.6.The assay was repeated three times to ensure reproducibility.

8.2.3 Bioautographic assays

The method described in section 2.2.5 was used to show the activity of the crude extract, the potentized extract and the isolated compound against the different pathological *E. coli* strains.

8.3 Results and Discussion

8.3.1 Minimum inhibitory concentration

The minimum inhibitory concentration of the crude extract, the potentized extract and the compound was determined against all four pathogenic bacterial strains. The results show that there were noticeable differences in the minimum inhibitory concentrations of the different extracts against the four different pathogenic bacteria.

The crude extract had the same activity as previously determined with the ATCC 25922 strain against the pathological strains isolated from the poultry sinus B 1295 and the poultry bursal swab B1279 (both 0.16 mg/ml). The activity against the *E. coli* strains isolated from the large intestine from the pig B 1297 and the liver and spleen of a bovine B 1314 was not as good as previously determined (0.31 mg/ml and 0.28 mg/ml respectively).

The chloroform fraction had the same activity against the *E. coli* strain isolated from the poultry sinus (B 1295) as it had against the ATCC 25922 strain (0.08 mg/ml). The activity against the other three pathological strains B 1279, B 1297 and B 1314 was not as good as it was against the ATCC 25922 strain (all 0.16 mg/ml). This difference in activity however only correlated to a difference in one well and was therefore not statistically significant.

The pure compound had a better activity against all four pathogenic strains than against the ATCC 25922 strain (B1279: 0.31 mg/ml; B1295: 0.31 mg/ml; B1297: 0.42 mg/ml and B1314: 0.36 mg/ml compared to ATCC 25922: 0.74 mg/ml value).

The values for all the minimum inhibitory concentrations against all four pathological bacterial strains are given in Table 8.1.

Table 8.1: MIC values in mg/ml (± SD) of the crude extract (C), the chloroform fraction (CF) and the pure compound (P) isolated from *Deinbollia oblongifolia* after 60 or 120 minutes incubation against four strains of pathogenic bacteria

Bacterial strain	MIC 60 C	MIC 120 C	MIC 60 CF	MIC 120 CF	MIC 60 P	MIC 120 P
B 1279	0.16 ± 0.00	0.16 ± 0.00	0.08 ± 0.00	0.16 ±0.00	0.31 ± 0.00	0.31 ± 0.00
B 1295	0.08 ± 0.00	0.16 ± 0.00	0.04 ± 0.00	0.08 ± 0.00	0.29 ± 0.01	0.31 ± 0.00
B1297	0.15 ± 0.03	0.31 ± 0.00	0.16 ± 0.00	0.16 ± 0.00	0.31 ± 0.00	0.42 ± 0.17
B1314	0.28 ± 0.08	0.28 ± 0.08	0.16 ± 0.00	0.16 ± 0.00	0.31 ± 0.00	0.36 ± 0.13

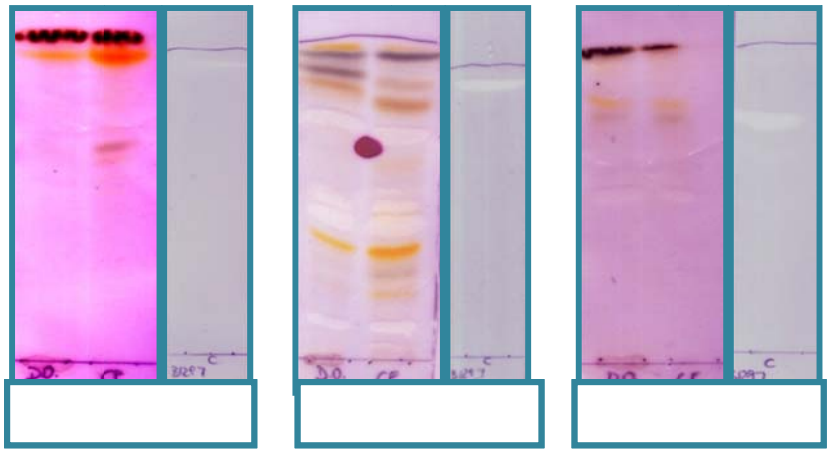
8.3.2 Bioautographic assays

The crude extract (C) was active in all the bioautographic plates sprayed with the four bacterial strains. However some of the assays resulted in more visible inhibition bands than others. Against the bacterial strain isolated from the poultry bursal swab, four different compounds in the chloroform fraction were active in the CEF bioautogram. Against the bacterial strain isolated from the poultry sinus, two compounds in the chloroform fraction (CF) were active when run with the CEF solvent system and 3 compounds of the chloroform fraction were active when run with the BEA solvent system. Against the bacteria isolated from the porcine large intestine and the one isolated from the bovine spleen and liver, two compounds of the fraction were active when run with the EMW solvent system.

The chloroform fraction of the extract was active in all the bioautographic plates sprayed with the four bacterial strains. However some of the assays had more visible inhibition bands than others. Against the bacterial strain isolated from the poultry bursal swab, four different compounds in the chloroform fraction were active when the plate was run with the CEF system. Against the bacterial strain isolated from the poultry sinus, two compounds of the chloroform fraction were active when run with the CEF solvent system and three compounds of the chloroform fraction were active when run with the BEA solvent system. Against the bacteria isolated from the porcine large intestine and the one isolated from the bovine spleen and liver, two compounds of the fraction were active when run with the EMW solvent system.

The pure compound (P) exhibited its activity in all the bioautographic assays for the bacterial strain isolated from the poultry bursal swab and against the strain isolated from the large porcine intestine. The activity was also visible in the plates sprayed with the bacterial strains isolated from the bovine liver and spleen and the poultry sinus that had been developed with CEF and EMW. However, the active band was not visible in the plates eluted with BEA and sprayed with these two bacterial strains.

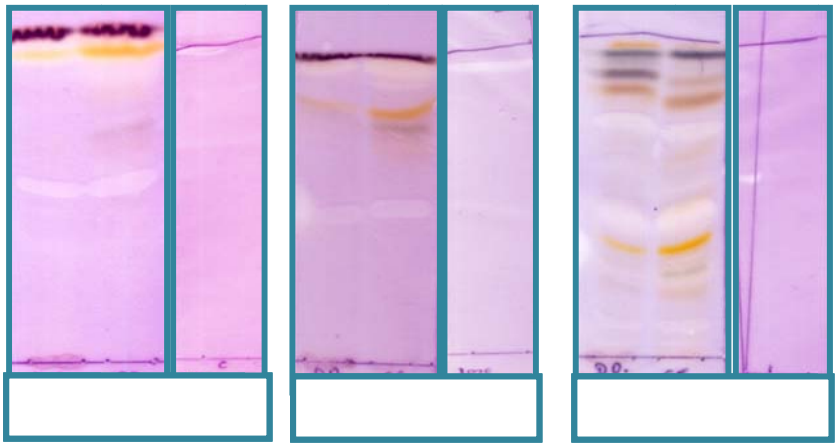
Allthe bioautographic assays are shown in Figures 8.1 to 8.4.



A B C

Fig. 8.1: Bioautography against B 1279

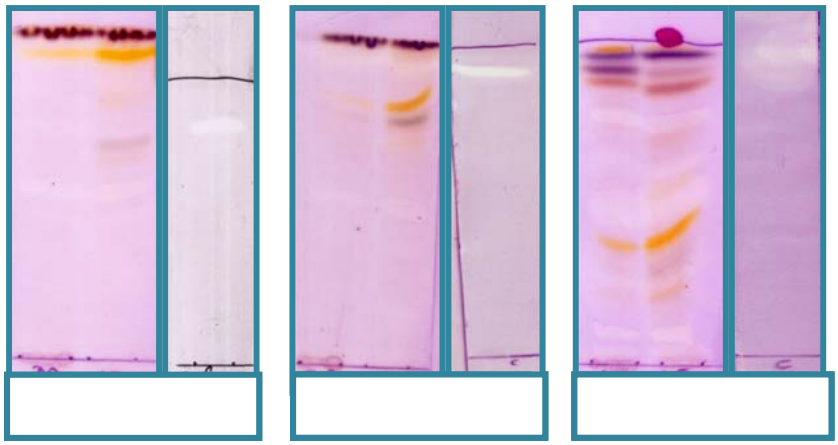
Fig. 8.1 A: Bioautography against B 1279 for C, CF and P EMW; Fig. 8.1 B: Bioautography against B 1279 for C, CF and P CEF; Fig. 8.1 C: Bioautography against B 1279 for C, CF and P BEA



A B C

Fig. 8.2 Bioautography against B 1295

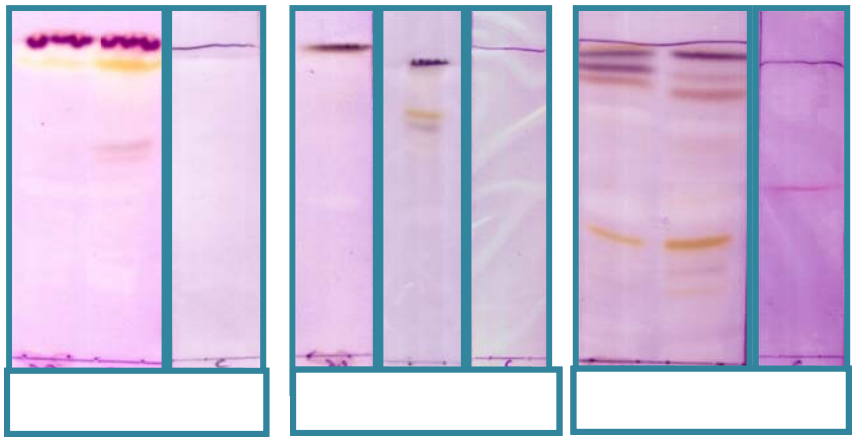
Fig. 8.2 A: Bioautography against B 1295 for C, CF and P EMW; Fig. 8.2 B: Bioautography against B 1295 for C, CF and P CEF; Fig. 8.2 C: Bioautography against B 1295 for C, CF and P BEA



A B C

Fig. 8.3: Bioautography against B 1297

Fig. 8.3 A: Bioautography against B 1297 for C, CF and P EMW; Fig. 8.3 B: Bioautography against B 1297 for C, CF and P CEF; Fig. 8.3 C: Bioautography against B 1297 for C, CF and P BEA



A B C

Fig. 8.4 Bioautography against B 1314

Fig. 8.4 A: Bioautography against B 1314 for C, CF and P EMW; Fig. 8.4 B: Bioautography against B 1314 for C, CF and P CEF; Fig. 8.4 C: Bioautography against B 1314 for C, CF and P BEA

8.4 Conclusion

The crude extract and the chloroform fraction were as active as in the preliminary screening results against at least one of the four pathological *E. coli* strains. The pure compound was even more active against all the four pathological strains than it was against the ATCC strain.

These results prove that the crude extract and the chloroform fraction could be used to combat at least some pathogenic bacteria *in vitro*, and possibly in stock animals. Now the next step was to evaluate the safety of this extract in a living organism.

Chapter 9

Acute, subacute and larval toxicity of the extract of *Deinbollia oblongifolia* and its chloroform fraction

9.1 Introduction

The next step in order to develop a safe product is to evaluate the safety of the extract and its chloroform fraction in a living organism. The OECD method for determining acute and subacute toxicity was used (OECD/OCDE, adopted 17th of December 2001) in trial rats.

It is the principle of the test that, based on a stepwise procedure with the use of a minimum number of animals per step, sufficient information is obtained on the acute and subacute toxicity of the test substance to enable its classification. In the acute and subacute toxicity tests, animals were randomly allocated to 2 groups, one group for the crude extract and one group for the chloroform fraction. For the acute toxicity test, animals were dosed in a step-wise fashion to determine the LD_{50}. For the subacute toxicity test, the two groups of rats were each divided into four groups, which received varying dosages of the crude plant extract or chloroform fraction before being evaluated.

9.2 Materials and Methods

9.2.1 General overview of the study design

9.2.1.1 STUDY DESIGN

The experiment was conducted at the University of Pretoria Biomedical Research Centre (UPBRC), at the Faculty of Veterinary Science, University of Pretoria, in a conventional experimental animal room. Permission to perform the trial was obtained from the Animal Use and Care Committee (AUCC) of the University of Pretoria.

9.2.1.2 TRIAL ANIMALS

Animals were housed in pairs and individually identified by ear notching. The animals were randomly selected and kept in their cages for at least 5 days prior to dosing to allow for acclimatisation to the laboratory conditions. A total of 6 animals per step were used.

Housing and management

Upon arrival at the research site, the rats were examined and only healthy rats were put into the prepared housing. Conventional rat cages (Tekniplast Type III) with 2 rats per cage were used. Enrichment for these cages was provided (tissue, toilet rolls, apples and carrots). The temperature in the experimental room was 22°C (± 3°C) and the relative humidity 50-60%. Artificial lightning was provided with a sequence of 12 hours light, 12 hours dark. A conventional laboratory diet was used for feeding (EPOL rat pellets) as well as an unlimited supply of potable water.

9.2.1.3 STUDY TERMINATION

For the acute toxicity the rats were weighed and humanely sacrificed a minimum of 14 days after each dosage step.

Forthe subacute toxicity the rats were intended to be killed on day 14.

All test animals were subjected to gross necropsy. All gross pathological changes were recorded for each animal and a microscopic examination of the organs showing evidence of gross pathology in animals surviving 24 hours or more were deducted. Terminal cardiac exsanguinations were done for serum chemistry for the subacute test whilst the animals were under isoflurane anaesthesia.

9.2.1.4 COLLECTION AND STORAGE OF SAMPLES

On all animals, gross pathological and histological changes were recorded. Pathology was done by Vetpath, a contracted private company located on the Onderstepoort Campus, that provides veterinary pathological services for, among others, projects done at the Faculty of Veterinary Science, University of Pretoria. Terminal cardiac exsanguinations were done for haematology and serum chemistry in the subacute study (performed by the Department of Companion Animals Clinical Studies, Section of Clinical Pathology), whilst the animals were under isoflurane anaesthesia.

9.2.1.5 MEASUREMENTS AND OBSERVATIONS

1. ***Rats:*** Origin and disease status were obtained from the supplier. Rats were weighed daily on an individual basis. Mass was also determined after euthanasia.

2. ***Mortality:*** Cages were checked twice daily for mortalities. All dead rats were weighed and submitted for *post mortem* examination.

3. ***General observations:*** Records were kept in terms of daily procedures.

9.2.2 Acute toxicity

A total of 24 female rats were used. The animals were randomly allocated to 6 animals per group. Healthy young adult animals of commonly used laboratory strains were employed (nulliparous and non-pregnant). Each animal was approximately 8 weeks old and weighed about 200 g at the start of the study. The dose level used as the starting dose was 50 mg/kg body weight. Depending on the toxic effects on 6 rats, the dose was changed to either 300 mg/kg (for the chloroform fraction) or 5 (for the crude extract) mg/kg body weight. The treatment groups were as follows:

Group 1: The plant material was ground with the Macsalab mill to a fine powder and mixed with acetone in a ratio of 1:10. Then the mixture was shaken on a Labotec Model 20.2 for 3 hours and left to settle overnight. The extract was filtered into preweighed glass vials and dried under constant airflow. The dried extract was then dissolved in acetone to the final concentration that was needed for the particular step of the trial (e.g. 50 mg/ml).

Rats were treated with 50 mg/kg of the plant extract of *Deinbollia oblongifolia* and observed for 3 days. Depending on the toxic effects, the dose was changed to 5 mg/kg.

Group 2: The chloroform fraction was prepared by first making the plant extract of *Deinbollia oblongifolia* (as mentioned in the previous paragraph in **Group 1**). The dried extract was then potentized by solvent-solvent fractionation. Hereby the dried extract was mixed well with equal volumes of chloroform and water and separated in a separatory funnel. The chloroform fraction was then removed from the funnel and concentrated to dryness. Then hexane and 10% water in methanol were added in equal volumes to the dried chloroform component. The 10% water in methanol fraction was diluted to 35% water in methanol and the equal volume of chloroform was added. This resulting chloroform fraction was dried and dissolved in acetone to the final concentration that was needed for the trial (e.g. 50 mg/ml).

Rats were treated with 50 mg/kg of the chloroform fraction of *Deinbollia oblongifolia* and observed for 3 days. Depending on the toxic effects, the dose was changed to 300 mg/kg.

The animals were dosed by oral gavage with the extract and observed individually after dosing (at least once during the first 30 minutes, and periodically during the first 24 hours, with special attention given during the first 4 hours and daily thereafter) for a total of 14 days, except when they needed to be removed from the study and humanely killed for animal welfare reasons or were found dead. If toxic signs were observed, the treatment was repeated with a lower dose under the same conditions.

Depending on the observation of toxic signs, the dosage was adjusted to either 5 mg/kg or 300 mg/kg (limit test). An approximate LD_{50} was assigned according to the OECD method.

The test substances were administered in a constant volume of 1 ml over the range of doses to be tested by varying the concentration of the dosing preparation. The test substance was administered in a single dose by gavage using an intubation canula. The animals were fasted beforehand by withholding food, but not water, overnight for about 8 hours. After fasting, the animals were weighed and the test substance administered. After the administration, food was withheld for a further 3-4 hours. The time interval between treatment groups was determined by the onset, duration, and severity of the toxic signs. The treatment of the animals at the next dose was delayed until the survival of the previously dosed animals was ensured.

At the end of the study (after 14 days) or after the animal died, necropsies and micropathological examinations were performed on all the animals.

Since the acute toxicity trial is designed to assign a LD_{50} to a previously untested chemical substance, no control group is included in this trial.

9.2.3 Subacute toxicity

A total of 21 female rats were used for each group. The animals were purchased following conclusion of the acute toxicity trial, and randomly allocated to 6 animals per dosage group in a total of 3 dosage groups for both the plant extract and the chloroform fraction. There was also one extra group with 6 animals that was treated with just the solvent to serve as a control group. Each animal was between 8 and 12 weeks old and weighed about 200 g. The dose levels used were determined by the acute toxicity study

For the subacute study, there were seven groups. Each group was treated daily with the same dose of the extracts for a time period of 2 days (the initially planned treatment for 2 weeks could not be carried out due to observed toxic signs in the animals).

Group 1: rats were treated with 5 mg/kg of the plant extract of *Deinbollia oblongifolia*

Group 2: rats were treated with 20 mg/kg of the plant extract of *Deinbollia oblongifolia*

Group 3: rats were treated with 50 mg/kg of the plant extract of *Deinbollia oblongifolia*

Group 4: rats were treated with 50 mg/kg of the chloroform fraction of *Deinbollia oblongifolia*

Group 5: rats were treated with 125 mg/kg of the chloroform fraction of *Deinbollia oblongifolia*

Group 6: rats were treated with 300 mg/kg of the chloroform fraction of *Deinbollia oblongifolia*

Group 7: rats were treated with 0 mg/kg of the plant extract of *Deinbollia oblongifolia*

The animals were dosed once daily by oral gavage with the extract (in different concentrations in the different groups) and observed individually after dosing (at least once during the first 30 minutes, and periodically thereafter, with special attention given during the first 4 hours) for a total of 3 days, except when they needed to be removed from the study and humanely killed for animal welfare reasons, or were found dead.

The test substances were administered in a constant volume of 1 ml over the range of doses to be tested by varying the concentration of the dosing preparation. The test substance were administered once daily in a single dose by gavage using an intubation canula. Before dosing, the animal was weighed and the test substance administered. After 14 days the experiment should have been terminated, but it had to be prematurely terminated 3 days into the study.

9.2.4 Larval assay

For the larval assay a modified version of the method developed by Khater and Khater in 2009 was used. The larvae were collected from different vulture restaurants that are run by the Vulture Programme, Rhino and Lion, a Non-profit Organization, South Africa. The emerging adult flies were identified as *Chrysomya marginalis* and *Lucilia cuprina*. Larvae were hatched by placing the flies in an enclosed space and supplying them with matured tenderised steak to lay their eggs in. The eggs were then incubated together with the meat and after hatching early stages of the larvae were harvested for conduction of the assay.

One millilitre of the diluted extracts (in acetone) at a concentration of 50 mg/ml was added to a piece of tenderised steak and left outside until the solvent had evaporated.

Then 15 to 20 of the obtained larvae were counted and transferred onto this piece of meat, which was then placed in a plastic container and covered with tidy roll (paper towel) secured with an elastic band. In the first eight hours checks were done every half an hour on the larvae. Another check was carried out after a total of 12 hours and after 24 hours a final check was performed and the larvae were placed in glass Petri dishes and filmed using a video camera to compare the movement of the larvae of the different dosage groups. A piece of steak where the pure solvent had been added served as negative control and a piece of steak where 1 ml of Virbamec (Virbac, 1% m/v ivermectin) had been added served as positive control. The assay was done in triplicate to increase reproducibility.

9.3 Results and Discussion

9.3.1 Acute toxicity study

Crude extract

The first treatment started with 50 mg/kg dissolved in 1 ml of acetone. Six rats were dosed with this treatment. The taste was apparently not very pleasant since all the rats fought against the gavaging. All the rats showed acute toxic signs immediately after dosing, including dizziness, ataxia and troubled breathing. All the rats seemed very subdued after the dosing. The first animal was euthanized after 30 minutes. After an hour a further toxic sign was observed. The animals started to scratch themselves excessively. After forty minutes the breathing of two of the rats became laboured and they made vocal sounds while trying to breath. One hour and a half after dosing, mucosal discharge out of the nose and eyes was observed as well as warfarin stains (reddish coloured stains on the fur resulting from stress). The second animal was put down two hours after dosing.

Four hours after dosing, food was supplied, but not one of the animals seemed to show any appetite. The animals started eating after a total of eight hours. Ten hours after dosing the fur of the animals was piloerect. Twelve hours after dosing the animals seemed to improve, but after leaving the animals to rest overnight, the symptoms became more severe and all the animals had to be euthanized. They had all lost weight during the trial period, ranging from 5.7%-18.1% reduction of the body mass they had at the starting dose.

In the pathological report all the rats showed congestion of the liver and gas accumulation in the stomach. Two of the rats had a splenomegaly and four had renal congestion. Three rats showed signs of congested ovaries and one had pathological lung symptoms (atelectasis and emphysema). The pathological report stated that these six rats revealed signs of acute shock without aspirated material present in the trachea or aspiration pneumonia. These pathological findings and the clinical signs of disorientation, laboured breathing and a mild degree of nervous signs suggested a possible toxic effect of the dosed extract. A summary of the clinical signs, pathological findings and the percentage of body weight gained or lost is shown in Table 9.1.

After 50 mg/ml showed severe toxic effects, the next group of three animals was treated with a dosage of 5 mg/kg. Since the carrier solvent seemed to not settle well with the animals (they were fighting the gavaging and seemed to aspirate rather than swallow the administered extracts) the carrier solvent was changed to a 5% acetone in water mixture. This was the lowest concentration of acetone the extract was soluble in. Again there were difficulties with getting the animal to swallow the substance and it was

assumed that the extract might cause pharyngeal spasms. Therefore the application needle was changed to a longer needle to try and place the solvent a bit further down into the oesophagus of the animal, so that the pharynx did not get into contact with the extract. The animals were again scratching after dosing, but no other signs were observed and the animals appeared alert and curious and the scratching had subsided by the next morning. The animals were further observed for 14 days and then euthanized and sent for pathological examination.

A second group of three animals was treated with the same dosage (5 mg/kg). Again, scratching during the first day was observed, but otherwise the animals were alert and active and eventually euthanized after 14 days and sent for pathological examination. Upon weighing on the day of euthanasia it was seen that none of the rats had experienced a weight loss, indicating that this dosage had at least a less toxic effect than the previously used dose.

The pathological report of the six animals dosed with 5 mg/kg crude extract stated that all the animals were in a good body condition and that the only consistent morphological finding was the liver congestion and the accentuated lobulation of the liver. This was stated to not be a specific finding and that the correlation to administration of the crude extract was uncertain.

A summary of the clinical signs, pathological findings and the percentage of body weight gained or lost is shown in Table 9.1.

Table 9.1: Dosing regimen, clinical signs and macroscopic lesions and percentage of body weight gained or lost during the trial in animals dosed with *Deinbollia oblongifolia*

Rat	Dosing quantity	Clinical signs	Macroscopic lesions	% (+, -)
1	50 mg/kg	Depression, piloerection, unsteady gait, pulmonary distress; euthanized after 28 hours	Moderate gas accumulation in stomach; moderate splenomegaly, moderate liver and renal congestion; mild ovarian congestion; blood around mouth nose and on front feet; dry content in large intestine	-13.0%
2	50 mg/kg	Depression, piloerection, unsteady gait, pulmonary distress; euthanized after 2 hours	Mild bilateral emphysema; moderate gas accumulation in stomach; mild splenomegaly; severe liver congestion; bilateral renal congestion	-5.7%
3	50 mg/kg	Depression, piloerection, unsteady gait, pulmonary distress; euthanized after 28 hours	Prominent gas accumulation in stomach; moderate ovarian congestion; mild hepatomegaly and liver congestion; bilateral renal congestion; blood on skin and around nose, mouth and one front foot;	-14.2%
4	50 mg/kg	Depression, piloerection, unsteady gait, pulmonary distress; euthanized after 28 hours	Gas accumulation in stomach with petechial haemorrhages in mucosa; mild gas accumulation in the anterior intestine; mild swollen and congested liver; congestion of ovaries; blood around mouth and nose	-18.1%
5	50 mg/kg	Depression, piloerection, unsteady gait, pulmonary distress; euthanized after 1 hour	Moderate gas accumulation in stomach; Severe liver congestion and mild hepatomegaly; bilateral renal congestion; moderate bilateral congestion of ovaries; focal atelectasis and multifocal areas of emphysema in both lungs	-10.2%
6	50 mg/kg	Depression, piloerection, unsteady gait, pulmonary distress; euthanized after 27 hours	Gas accumulation in stomach; moderate liver congestion; small amount of gas in anterior intestine; congested ovaries bilateral; blood on skin, around mouth, nose and front feet; dry contents in the large intestine	-9.8%
8	5 mg/kg	None except for warfarin spot on the day of euthanasing; searched for food after dosing; euthanized on day 14	Liver congestion with paler peripheral lobulation and congested centrilobular areas;	+13.7%
10	5 mg/kg	While dosing spasms; else no clinical signs; euthanized on day 14	Moderate liver congestion with accentuated lobulation, which appears as a pale reticular pattern surrounding sunken dark red central areas of less than1 mm disseminating throughout the parenchyma; bilateral moderate renal congestion; mild splenic white pulp prominence	+15.6%

Rat	Dosing quantity	Clinical signs	Macroscopic lesions	% (+, -)
11	5 mg/kg	None except for some scratching and a light 1 hour head tilt 2 hours after dosing; euthanized day 14	Moderate liver congestion with accentuated lobulation, which appears as a pale reticular pattern surrounding sunken dark red central areas of less than 1 mm disseminated throughout the parenchyma; bilateral moderate renal congestion; mild splenic white pulp prominence	+13.6%
12	5 mg/kg	None; euthanized day 14	Mild liver congestion and mild accentuated lobulation in liver	+0.6%
13	5 mg/kg	Scratching; euthanized day 14	Moderate liver congestion and accentuated lobulation, which appears as a pale reticular pattern surrounding sunken dark red central areas of less than 1 mm disseminate throughout the parenchyma; mild bilateral kidney congestion; spleen slightly enlarged with white pulp prominence; mild bilateral lung congestion; mild extensive haemoragae subcutaneous in the neck area (from handling?)	+15%
14	5 mg/kg	None; euthanized day 14	Moderate liver congestion with severe accentuated lobulation in the liver parenchyma	+11.2%

Chloroform fraction of *Deinbollia oblongifolia*

The first treatment dose was 50 mg/kg in 10% acetone in water (the amount of acetone was determined by the solubility of the extracts). The first group of three rats was dosed. The only obvious sign of possible toxicity was again the occurrence of scratching on the first day in all the dosed animals. The animals were observed for 14 days and then sacrificed and sent for pathological evaluation. According to OECD protocol a second group of 3 animals was dosed with the same treatment and observed for 14 days. They again scratched more than the undosed animals during the first 24 hours. After 14 days the animals were sacrificed and sent for pathological evaluation. Both groups of rats seemed more subdued during the first day after dosing compared to the untreated animals.

The next step was to dose the animals with 300 mg/kg of the chloroform fraction. The extract was dissolved in a mixture of 50% acetone and 50% water (the lowest concentration of acetone that the extracts were soluble in). Right after dosing the animals started to excessively shred the paper in the cage and scratch. The animals also showed intermittently symptoms of laboured breathing, piloerect hair, warfarin stains and a lack of coordination during the first 24 hours. The animals returned back to their normal behaviour after 24 hours and were further observed for 14 days and then sacrificed and sent for pathological evaluation. Upon euthanasia the animals were weighed and it was discovered that there was no weight loss in any of the trial animals. The pathological results showed that all the animals

were in a good body condition and that the only consistent macroscopical finding was the hepatic congestion and the accentuated lobulation of the liver, which was observed in many of the rats. The pathological report stated that this was not a specific finding and that the correlation to the dosed extract was uncertain. Table 9.2 shows a summary of all the clinical signs, pathological findings and the percentage of weight gained or lost during the trial.

A second group of three rats was dosed with 300 mg/kg in a 50% acetone and 50% water mixture. The rats showed again toxic signs during the first 24 hours (shredding of paper, piloerect hair, warfarin stains, laboured breathing and lack of coordination) which again subsided after the first day. The rats were sacrificed after 14 days and sent for pathological evaluation. Upon euthanasia the animals were weighed and it was discovered that there was no weight loss in any of the trial animals. The pathological results showed that all the animals were in a good body condition and that the only consistent macroscopical finding was the hepatic congestion and the accentuated lobulation of the liver, which was observed in many of the rats. The pathological report stated that this was not a specific finding and that the correlation to the dosed extract was uncertain. Table 9.2 records a summary of all the clinical signs, pathological findings and the percentage of weight gained or lost during the trial.

Table 9.2: Dosing regimen, clinical signs and macroscopic lesions and percentage weight gain or loss in animals dosed with the chloroform fraction of *Deinbollia oblongifolia*

Rat	Dosing quantity	Clinical signs	Macroscopic lesions	% (+,-)
7	50 mg/kg	Scratching; warfarin spot on day 14; euthanized day 14	Hepatic liver congestion with prominent accentuated lobulation and congestion	+14.3%
9	50 mg/kg	None; euthanized day 14	Moderate liver congestion and moderate accentuated lobulation in the liver; moderate oedema of the uterus; mild cranial ventral bilateral pulmonary atelectasis	+8.4%
16	50 mg/kg	Scratching; euthanized day 14	Mild accentuated lobulation and moderate congestion of the liver; mild oedema of the uterus and congestion of the ovaries; moderate lung congestion	+13.9%
17	50 mg/kg	Scratches; euthanized day 14	Mild liver congestion with accentuated lobulation of moderate degree	+16.1%
18	50 mg/kg	Scratches; euthanized day 14	Mild hepatocellular congestion with accentuated lobulation on the periphery of the lobules; mild uterine oedema and congestion of the ovaries	+13.4%

Rat	Dosing quantity	Clinical signs	Macroscopic lesions	% (+,-)
19	300 mg/kg	Extensive scratching; frantically trying to eat paper; slight pulmonary distress and disorientation right after dosing for three hours; piloerection; euthanized day 14	Mild liver congestion with moderate accentuated lobulation of the liver parenchyma	+15.0%
21	300 mg/kg	Extensive scratching; frantically trying to eat paper; slight pulmonary distress and disorientation right after dosing for four hours; piloerection; euthanized day 14	Hepatic congestion with moderate accentuated lobular pattern; mild cranial ventral congestion of the lung	+12.1%
25	300 mg/kg	Extensive scratching; frantically trying to eat paper; slight pulmonary distress and disorientation right after dosing for four hours; piloerection; euthanized day 14	Liver congestion with mild accentuated lobulation; mild oedema of the uterus; mild pulmonary congestion	+14.3%
26	50 mg/kg	Scratching; euthanized day 14	Hepatic congestion centrilobular with accentuated pale lobulation of the liver parenchyma; mild bilateral renal congestion	+13.2%
31	300 mg/kg	Extensive scratching; frantically trying to eat paper; slight pulmonary distress and disorientation right after dosing for three hours; piloerection; euthanized day 14	Mild liver congestion with moderate accentuated lobulation of the liver parenchyma	+14.0%
33	300 mg/kg	Extensive scratching; frantically trying to eat paper; slight pulmonary distress and disorientation right after dosing for four hours; piloerection; euthanized day 14	Moderate liver congestion with a pale accentuated lobular pattern of the liver parenchyma; mild lung congestion	15.0%
34	300 mg/kg	Extensive scratching; frantically trying to eat paper; slight pulmonary distress and disorientation right after dosing for four hours; piloerection; euthanized day 14	Moderate liver congestion with moderate accentuated lobulation	15.1%

From these results the dosage groups for the subacute toxicity study were calculated to be the following for the crude extract: 5 mg/kg; 20 mg/kg and 50 mg/kg. The chloroform fraction was less toxic with

dosages for the chloroform fraction calculated as 50 mg/kg, 125 mg/kg and 300 mg/kg for the subacute toxicity study.

9.3.2 Subacute toxicity study

From the results of the acute toxicity study, the dosage groups for the subacute toxicity study were determined to be 5 mg/kg, 20 mg/kg and 50 mg/kg for the crude extract and 0, 50 mg/kg, 125 mg/kg and 300 mg/kg for the chloroform fraction. One group was just dosed with the solvent (50% acetone in water) to serve as negative control. Six rats were allocated to each group. All the animals were dosed once in the mornings of every day.

*Group 1 (5 mg/kg crude extract):*The rats showed clinical signs of pulmonary distress (except for one of them). Some of them also showed signs of neurological irritation (scratching, piloerection, pica), which can be associated with a toxic effect. The pica in rats has been shown to indicate a toxic effect (Mitchell et al, 1976). All the rats lost weight during the trial period (only rats terminated on day 3 were weighed).The pathological findings ranged from gas accumulation in the stomach, adrenal congestion, liver congestion, lung congestion and atelectasis and splenomegaly, but none of these findings was found in all of the rats of the group and so these findings could not be directly associated with a toxic shock. However the animal that was euthanized the day following the first dosing did show consistent signs of toxic shock (gas accumulation in stomach, signs of pulmonary oedema and hepatic blood pooling due to heart failure) that can be associated either to the plant material or the dosing solvent. See Appendix Table B 1 for a summary of all the clinical signs, pathological findings and the percentage of weight gain/loss the animals experienced during the trial. The blood chemistry results indicated that the liver in the rats was affected (especially the ALT, AST, GGT, ALP and ALB values) suggesting a toxic effect (see Appendix Table B 2 for blood chemistry results).

Group 2 (20 mg/kg crude extract): The rats showed clinical signs of pulmonary distress. Some of them also showed signs of neurological impairment (unsteady gait, depression, scratching, piloerection, and pica). All these symptoms can again be linked to a toxic effect. In two of the rats it was noted that the abdomen was swollen, which was most likely due to the splenomegaly found in the pathological examination. The rats did not lose weight during the trial period (only rats that were terminated on day 3 were weighed). All of the rats showed upon pathological examination a spleen with extramedullary haemopoiesis in the red pulp. Other pathological findings ranged from gas accumulation in the stomach, adrenal congestion, liver congestion, lung congestion and atelectasis and splenomegaly, but none of these findings was found in all of the rats of the group and so these findings could not be directly associated with a toxic shock that was directly linked to the dosing of the animals with the plant extract.

However the animals that were euthanized the day following the first dosing did show consistent signs of toxic shock (gas accumulation in stomach, signs of pulmonary oedema and hepatic blood pooling due to heart failure) that can be associated either to the plant material or the dosing solvent. See Appendix Table B 1 for a summary of all the clinical signs, pathological findings and the percentage of weight gain/loss the animals experienced during the trial. The blood chemistry results indicated that the liver in the rats was affected again (especially the ALT, AST, GGT, ALP and ALB values) suggesting a toxic effect. The urea and creatinine values were also elevated suggesting an involvement of the kidneys into the picture of a general toxic shock (see Appendix Table B 3 for blood chemistry).

Group 3 (50 mg/kg crude extract): The rats showed clinical signs of pulmonary distress. Some of them also were neurologically impaired judging from their unsteady gait, their obvious depression, the pathological scratching and the piloerection of the hair as well as the exhibition of pica. This suggests again a toxic effect. Two of the rats again exhibited a swollen abdomen. The rats did not lose weight during the trial period. Five of the rats showed upon pathological examination a spleen with extramedullary haemopoiesis in the red pulp. Other pathological findings ranged from gas accumulation in the stomach, adrenal congestion, liver congestion, lung congestion and atelectasis and splenomegaly, but none of these findings was found in all of the rats of the group and so these findings could not be directly associated with a general toxic shock due to the dosing of the rats with the crude extract. See Appendix Table B 1 for a summary of all the clinical signs, pathological findings and the percentage of weight gain/loss the animals experienced during the trial.
The blood chemistry results again indicated that the liver in the rats was affected (especially the ALT, AST, GGT, ALP and ALB values) suggesting a toxic effect (see Appendix Table B4 for blood chemistry).

Group 4 (50 mg/kg chloroform fraction): The rats showed signs of pulmonary distress and neurological impairment (scratching, depression, unsteady gait, piloerection, warfarin spots and pica). One of the rats had an apparent swollen abdomen. Two of the rats were euthanized the same day after they were first dosed due to the signs of acute shock they showed. Upon euthanasia three of the animals had gained weight during the trial whereas one animal lost weight (only animals terminated on day 3 were weighed). Upon pathological examination it was concluded that the animals that were euthanized the day following the first dosing showed prominent gas accumulation in the stomach as well as signs of a pulmonary oedema and hepatic blood pooling due to heart failure which suggests a shock and is most likely associated with the dosing of the plant material or the dosing solvent. Pathological findings in the other rats included adrenal congestion, kidney congestion, liver congestion, lung congestion and atelectasis as well as extramedullary haemopoiesis in the spleen. See Appendix Table B 5 for a full summary of clinical signs, pathological findings and the percentage of the weight gain or loss the animals

experienced during the trial. The blood chemistry results indicated that the liver in the rats was affected (especially the ALT, AST, GGT, ALP and ALB values) suggesting a toxic effect (see Appendix Table B 6 for blood chemistry).

Group 5 (125 mg/kg chloroform fraction): All the rats showed signs of pulmonary distress and neurological impairment (scratching, depression, unsteady gait, piloerection, warfarin spots and pica). Upon euthanasia it was discovered that 4 of the animals had gained weight during the trial whereas two animals lost weight. Pathological findings in the rats included adrenal congestion, kidney congestion, lung congestion and atelectasis as well as extramedullary haemopoiesis in the spleen, but none of the findings was seen consistently in all the rats and could therefore not be directly associated with the dosing See Appendix Table B 5 for a full summary of clinical signs, pathological findings and the percentage of the weight gain or loss the animals experienced during the trial. The blood chemistry results indicated that the liver in the rats was affected (especially the ALT, AST, GGT, ALP and ALB values) suggesting a toxic effect (see Appendix Table B 7 for blood chemistry).

Group 6 (300 mg/kg chloroform fraction): The clinical signs included pulmonary distress and neurological impairment (scratching, depression, unsteady gait, piloerection, warfarin spots and pica). One of the rats had an apparent swollen abdomen. Upon euthanasia it was discovered that all of the animals except for one had lost weight during the trial. Pathological findings in the rats included mild gas accumulation in the stomach, adrenal congestion, kidney congestion, lung congestion and atelectasis as well as extramedullary haemopoiesis in the spleen, but none of the findings was seen consistently in all the rats and could therefore not be directly associated with the dosing. See Appendix Table B 5 for a full summary of clinical signs, pathological findings and the percentage of the weight gain or loss the animals experienced during the trial. The blood chemistry results indicated that the liver in the rats was affected (especially the ALT, AST, GGT, ALP and ALB values) suggesting a toxic effect (see Appendix Table B 8 for blood chemistry).

Group 7 (Solvent acetone): Three of the animals of the control group showed severe clinical signs of toxic shock after the first day of dosing (pulmonary distress, piloerection, depression, one even died during the night) and had to be euthanized the same or the next day. It was concluded that aspiration of the solvent could be responsible for these severe effects and therefore the rats were dosed intraperitoneally on the second day. All of them showed severe depression after dosing, but got gradually better during the day, however they still showed signs of toxic shock (piloerection, depression, scratching, pica, unsteady gait) and so it was decided to euthanize the rest of the control group along with the other groups and terminate the trial. Two of the animals that were terminated on the third day showed weight loss whereas one gained weight slightly. Upon pathological examination it was

discovered that the animals that were euthanized on the second day showed signs of shock (gas accumulation in stomach, signs of pulmonary oedema and hepatic blood pooling due to heart failure). The other animals showed several symptoms (pulmonary congestion, inflammation in the peritoneum, extramedullary haemopoiesis, and adrenal congestion). See Table B 9 for a full summary of the clinical signs, pathological findings and the percentage of weight gain or loss the rats experienced during the trial. The blood serum chemistry indicated that the extract had had a toxic effect on the liver (especially the ALT, AST, GGT, ALB and ALP values). See Appendix Table B 10 for the full serum chemistry.

It was also discovered that most of the animals had a haemothorax, but this was interpreted as being directly associated with the blood collection upon euthanasia by cardiac puncture.

The original outline of the study also required haematology to be done (full blood count, haematocrit, etc.), but since the rats were euthanized on the weekend and the laboratory was not available, the blood had to be frozen and the haematology work was therefore not possible.

These results showed that an unambiguous conclusion about the toxicity of the extracts cannot be drawn since the solvent also seemed to have had an effect on the rats. Acetone was chosen as solvent based on the solubility of the extract, the LD_{50} value of acetone in rats based on literature reports and because acetone is generally considered relatively safe in rats due to its low level of toxicity. This has been established in a 13-week drinking water study (NTP, 1991; Dietz *et al.*, 1991) where rats were exposed to different levels of acetone in the drinking water where no mortalities or overt clinical signs were observed within the experimental period. It could however be possible that acetone could cause toxic effects since experimental animal data characterizing the effect of long-term oral exposure to acetone are not available, due probably to its low toxicity (EHC 207, 1998). In a study on the mammalian acetone metabolism it was found that acetone is toxic at high doses and that its primary target sites are liver, kidney and bone marrow (Kalapos, 2003).

One of the PhD students Ms Lilian Mukandiwa in the Phytomedicine Programme had developed an assay to test the efficacy of different plant extracts on larval motility and development. Therefore it was decided to perform a larval toxicity assay to investigate the toxic effect of the extracts and acetone on another life form.

9.3.3 Larval assay

The crude extract of *Deinbollia oblongifolia* and the potentized extract of this plant were tested for their larvicidal activity in this assay. The extracts were added in a concentration of 50 mg/ml to pieces of tenderised steaks. Acetone served as a negative control and ivermectin as positive control. Checks

were done every half an hour during the first 8 hours and then once after a total of 12 hours and after 24 hours.

The larvae that were dosed with the crude extract showed the first visible toxic signs (paralysis, partial paralysis) after six hours. These effects lasted until the last check after 24 hours. It was also discovered that the process of the further development was noticeably compromised (slower development, some did not develop further at all and just died).

The larvae that were dosed with the potentized extract showed toxic signs even earlier. The first paralytic effects in the larvae were observed 1.5 hours after dosing. The effects lasted until the final observation 24 hours after dosing. The further development was also seriously impaired. Mortalities and slowed developmental processes occurred in the larvae.

The positive control group that was treated with ivermectin showed toxic effects straight away and all the larvae died.

The negative control group that was treated with acetone did not show any toxic effects, neither right after dosing nor in the further development.

9.4 Conclusion

The results of the acute toxicity study and the larval assay proved that the extracts were of a toxic nature. The results of the subacute toxicity trial did indicate that the solvent control also seemed to have a toxic effect on the rats. In older literature it has been postulated that acetone enhances the hepatotoxic effects of a number of compounds (Plaa and Traiger, 1972; Moldeus and Gergely, 1980; Liu *et al.*, 1991; EHC 207, 1998). It could therefore be a possibility that toxic nature of the extracts was further intensified by the use of acetone as a solvent.

The results of this experiment unfortunately showed that the extract cannot be used safely in a living organism in this form as an antidiarrhoeal agent. The idea of the further study of the efficacy of the extracts in a living organism challenged with *E. coli* therefore had to be abandoned.

Chapter 10

General Conclusions

10.1 Introduction

Since prehistoric times, people have used natural resources for medicinal purposes (Anesini and Perez, 1993) including combating infections. Therefore, it is only logical that in times of growing resistance of pathogenic microorganisms against presently used drugs, research should again turn to these resources.

The existence of diarrhoea in humans and livestock has always been a source of tremendous problems. In livestock, diarrhoea causes huge economic losses for farmers due to the higher mortality rate especially during the weaning of the young animals. Weaning in the young is related to a higher susceptibility to diarrhoea due to their age-related increased susceptibility to bacterial infections. In many cases, this diarrhoea is caused by bacterial agents such as *E. coli*. To deal with this problem, farmers have been using antibiotic feed additives for years. But, as recent research and medical cases show, this usage has led to the creation of drug-resistant bacterial strains, e.g. multiresistant *Enterococcus faecalis*. Because of this, the European Union banned antibiotic feed additives and now the farming community, as well as the veterinary community, are forced to find alternatives to prevent economic losses.

Since plants have always been used, especially in rural areas, for medical purposes and many plant species have proven antibacterial activity – plant extracts provide a potential solution to the problem especially since natural products are the source of many useful pharmaceuticals. It is important to develop a useful system to recommend which plant species would be the most likely to yield a commercial product. To verify the efficacy of selected plant extracts used traditionally different aspects were investigated: good activity against *E. coli* was seen as a positive parameter since this pathogen plays an important role in the occurrence of diarrhoea, while high tannin content was viewed negatively. Species with low tannin content are recommended because even though tannins may have antibacterial activity and have been used traditionally for the treatment of diarrhoea, they do have reported unwanted interactions for prophylactic use. For example, the presence of tannins in a herbal drug may hinder the absorption of proteins and lead to lower productivity. Tannins may affect the induction of enzymes such as cytochrome p450, which may accelerate drug metabolism to result in blood levels of actives too low for a therapeutic effect (Williamson, 2001). Furthermore, tannins produce a bitter taste in the feed. The presence of tannins in a prophylactic drug would therefore be impractical.

Therefore the aim of this project was to develop a formula based on *in vitro* assays to predict the potential value of traditionally used plants. In the introduction a number of objectives were identified to attain this aim.

10.2 Evaluate the magnitude of seasonal variation in the antibacterial activity

Since some scientists are sceptical about the use of plant extracts due to the possible variation of activity during the season. The magnitude of seasonal variation in the antibacterial activity of 5 selected plant species (collected monthly) against *E. coli* and *S. aureus* and the tannin content was determined. A monthly screening of acetone leaf extracts of five chosen plant species (*Acacia karroo*, *Acacia sieberiana* var. *woodii*, *Peltophorum africanum*, *Trichilia emetica*, and *Ziziphus mucronata*) was done over a period of one year. All the samples for the monthly evaluation were collected in the Gauteng area due to the easier accessibility and the quicker transport to the drying room to ensure optimum quality of the dried product. Leaves had the highest activity between the months of January to April (summer to autumn) after they were fully developed and before senescence started. There was little to no correlation between the activity against the bacteria and the tannin content of the plant even though tannins are commonly associated antibacterial activity. Therefore it can be assumed that tannin did not influence the antibacterial activity in the plant, but that other compounds were responsible.

10.3 Evaluate the genetic and geographic variation in antibacterial activity

The next objective was to determine the magnitude of interspecies variations. This is an important step in the rational use of plant material and predicting activity for a species. Leaves from 42 different trees of the same species (*Combretum molle*) were collected in a range of areas (Waterberg, Onderstepoort, SANBI National Botanical Garden Pretoria, Magaliesberg and Lowveld Botanical Garden Nelspruit) and screened for their antibacterial properties and tannin content. There was limited variation in antibacterial activity between different plants of the same species tested in this study. The average MIC against *E. coli* was 0.227 mg/ml. The low standard deviation of 0.07 indicated that there was very little variation in activity. The average value against *S. aureus* was 0.399 mg/ml with a slightly higher standard deviation of 0.16. Although, only plants from Gauteng and Mpumalanga provinces were investigated the climatic and geological conditions were sufficiently different to conclude that at least for *Combretum molle* the possible variation in activity between different plants of the same species is not a major factor to take into consideration. Differences in the tannin content were however detected. The number of antibacterial compounds based on bioautography varied without influencing the overall antibacterial activity. Further investigation in this matter by isolating the different active compounds and comparing their structures might be warranted. Again there was no correlation between the activity of the plant and its tannin

content. Some authors have decided that because many members of the Combretaceae contain large quantities of tannins in roots and bark, it is not a viable option to investigate members of the family for antimicrobial compounds. These results show that at least *Combretum molle* leaves have a low tannin content and that tannin content is not associated with antibacterial activity.

10.4 Verify the efficacy of selected plant extracts used traditionally to treat diarrhoea and develop a ranking system

After it was determined that the season had an effect on antibacterial activity of the plant extracts and different plants of the same species growing in different areas have a similar antibacterial activity, the next objective was to evaluate the efficacy and to develop a ranking system for plants that have been used to treat diarrhoea in ethnomedicine, in order to recommend the most promising species for further investigation. To do that, 53 tree species with reported medicinal use in Zulu medicine as antidiarrhoeal agents, or to treat dysentery that were available were chosen. The antibacterial activity of acetone leaf extracts of these species against the nosocomial agents *S. aureus* and *E. coli* was evaluated. *E. coli* was chosen due to its role in the occurrence of diarrhoea and *S. aureus* was included in the assays to detect possible selective antibacterial activity of the extracts. The tannin content was again also determined. All the investigated species were active to differing degrees against the bacterial pathogens. A formula was designed that would incorporate high activity against *E.coli,* low activity against *S. aureus*, low tannin content and high extractability with acetone to determine the species with the highest potential value: (0.60/MIC *E. coli*) + (0.25/gallic acid equivalent) + (0.10*MIC *S. aureus*) + (0.05*extract yield) = Rank.

10.5 Select species to be used for further analysis

Five plants were chosen for further in depth studies based on the ranking system that was obtained from application of the formula. The most important aspect for choosing these species for further research was that they had a high activity against *E. coli*, but other aspects were taken into account as well, i.e. low tannin content, a lower activity against *S. aureus* and a high extract yield. The five selected species based on these criteria were *Acacia sieberiana* var. *woodii*, *Albizia adianthifolia, Deinbollia oblongifolia*, *Spirostachys africana* and *Tetradenia riparia*.

10.6 Determine the *in vitro* cytotoxicity

Even though it is a very attractive idea to use plant medicines to treat diseases in animals, it is dangerous to assume that because they are natural they should be safe. Many natural products are extremely toxic. Also, because nearly all herbal remedies contain multiple biologically active constituents, interaction with conventional drugs is a matter of concern (Wynn et al, 2007). Therefore it is important to investigate the toxicity of herbal drugs and of their constituent compounds. The determination of the LC_{50} value was also necessary in order to determine the selectivity index (SI) for the plants (SI = LC_{50} / MIC): *Acacia sieberiana* var. *woodii* SI = 0.20; *Albizia adianthifolia* SI = 0.49; *Deinbollia oblongifolia* SI = 0.46; *Spirostachys africana* SI = 0.19; *Tetradenia riparia* SI = 0.31.

10.7 Potentize (enhance the activity of) two extracts

The next objective was to potentize two extracts and to determine their antibacterial activity against *E. coli* as well as their cytotoxicity to recommend which one should be investigated in further detail. The crude extract of *Deinbollia oblongifolia* had the lowest toxicity on the cells and was therefore selected for further study. *Spirostachys africana* was also chosen for further study due to its good activity and total activity against *E. coli*. It may be possible to increase the activity and/or decrease the toxicity of an extract by simple techniques. The two extracts were subjected to solvent-solvent fractionation and in both cases, the chloroform fraction was the most active against *E. coli*. Cytotoxicity of the two fractions was determined and the chloroform fraction of *Deinbollia oblongifolia* was the least toxic. The LC_{50} of 0.188 mg/ml may appear to be quite toxic, but it has to be kept in mind that the kidney cells were directly exposed to the extract in the cytotoxicity test, whereas in a biological system the extract may undergo metabolic changes in the gut or while travelling through the body. Also, relative to other plant extracts tested in the same assay system, this LC_{50} value is among the highest of a large number of extracts tested. The Selectivity Index (2.35 for the potentized extract of *Deinbollia oblongifolia* compared to a value of 0.45 for the crude extract and 0.78 for the potentized extract of *Spirostachys africana* compared to the lower value of 0.19 for the crude extract) showed that a potentization of both extracts was indeed achieved.

10.8 Isolate and characterise the active compound

Another objective of the current research was to isolate and characterise the active compound(s) of the chosen potentized plant extract, so the chloroform fraction of *Deinbollia oblongifolia* was fractionated by chromatographic isolation procedures and an antibacterial compound was successfully isolated. It was determined to be a mixture of the two compounds, 3β-OH-α-amyrin and 3β-OH-β-amyrin, two

triterpenoids. The efficacy of the crude extract, the chloroform fraction and the pure compound was tested against four pathological strains of *E. coli* isolated from different organs of different animals. The activity varied compared to the original activity that was determined against the ATCC 25922 strain. The extracts proved to be less effective against three of these bacterial strains, but showed the same efficacy against one of the strains. The isolated compound was even more active against all the pathological strains than it was against the ATCC strain.

10.9 Determine the acute and subacute toxicity in *in vivo* tests in rats

To determine whether either the crude or the potentized extract could be used safely in a biological system, acute and subacute toxicity studies in rats were done using the protocol recommended by the OECD. Unfortunately it became clear that the crude extract and the chloroform fraction were toxic to the rats. There might also have been an interaction between the carrier solvent and the drug that caused the toxicity, even though the acetone-water carrier mixture has been reported to be relatively safe in rats. Even though the extracts were toxic, they could still be used for medicinal purposes. For example some chemotherapeutic drugs were developed from toxic plant products (e.g. taxol), since many oncology drugs induce cellular toxicity and death through free radical generation (Wynn et al, 2007). In terms of antidiarrhoeal drugs, as with others such as anthelmintics, the balance needs to be achieved between toxicity to the pathogen and toxicity to the host.

It would be very interesting to determine genotoxicity and mutagenicity in these extracts to determine whether they could be used for developing such a chemotherapeutic agent. This however will have to be done in the scope of a different project since it surpasses the scope for this dissertation.

10.10 Test the efficacy of the potentized extract in *in vivo* feeding experiments

Due to the toxicity of the extracts in rats this aspect could not be investigated any further.

10.11 Recommend further steps in the development of a model

The results obtained in this project highlight the general difficulties in the development of a new drug or preparation against diarrhoea and other bacterial-related diseases because of toxicity concerns. Many promising plant species with excellent antibacterial activity have to be rejected owing to unacceptable toxicity, so toxicity is an aspect that needs to be tested early. Another point is that it is absolutely imperative to test the toxicity of plant extracts *in vivo* as well as *in vitro* since the effects can vary following metabolic activity, and an extract that was deemed relatively safe in an *in vitro* cytotoxicity screening can prove to be highly toxic in an animal. Many traditionally used medicines have never been

evaluated for their toxic potential and are being used based on their history of ethnomedicinal use, and are generally regarded as safe. Toxicity issues are probably the major stumbling block to the development of new therapeutic preparations. One may ask why if *Deinbollia* has been traditionally used apparently without ill effects, such toxicity is now found. The answer is probably in the extractant used to extract compounds from the plant material. Rural people would have mainly water as an extractant and water extracts a totally different group of compounds from plants (Kotze and Eloff, 2002).

This work was the first step in the development of a ranking system for the selection of medicinal plants to be used to treat diarrhoea on a rational basis. It resulted in a large quantity of new data that is now available for other scientists to build upon. To improve the ranking system, more weight should possibly be placed on the low activity against*S. aureus* to prevent the selection of general metabolic toxins. Determining the activity of the extracts on other microorganisms such as beneficial microorganisms such as *Lactobacillus spp*. would be very useful. It would be very interesting to perform *in vitro* and *in vivo* toxicity studies on all the plants and include these results in the ranking system. This should definitely increase the potential of developing a useful therapeutic product to combat diarrhoea in stock production.

Much research still has to be done to perfect the system, but this work represents the first step and may help scientists in the future to select the most promising plant species for in depth screening as opposed to the current random selection, or selection based on only one or two appropriate parameters.