CHAPTER 3

Preliminary screening for antifungal activity of six selected plant species

3.1 Introduction

Antimicrobials are compounds that at low concentrations exert an action against microorganisms and exhibit therapeutic toxicity towards them (Goodyear and Threlfall 2004). These can be any substances of natural, synthetic or semi-synthetic origin that may be used to kill microorganisms including bacteria, fungi, protozoa and viruses (Yazaki 2004). The antimicrobial activity of different plant extracts can be detected by observing the growth of various microorganisms that have been placed in contact with extracts of the plants. If the plant extracts inhibit the growth of the test organism, and general toxic effects are not present, then the plant can potentially be used to combat diseases caused by the pathogens. The antimicrobial activities of plant extracts have formed the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies (Lis-Balchin and Deans 1997, Reynolds 1996).

There are several assays that can be used to determine antimicrobial activity in plant extracts, including agar diffusion, bioautography (direct, contact and overlay) and microplate assays (serial dilution assay). The agar diffusion assay is, in general, only suitable for aqueous extracts and can also be used to test up to six extracts per Petri dish against a single microorganism. However, the diffusion method is not suitable for testing non-polar samples or samples that do not easily diffuse into the agar (Cos et al. 2006).

The bioautography assay is used to detect active compounds in a crude plant extract (Cos et al. 2006). An inoculated layer of agar is poured over a developed thin layer chromatography (TLC) plate, and lack of bacterial or fungal growth in certain areas identifies the presence and location of antibacterial compounds on the TLC plate. On the other hand, TLC plates can also be sprayed with a fine suspension of bacteria or fungi and then sprayed with an indicator tetrazolium salt. The inhibition of fungal growth by compounds separated on the TLC plate is visible as white spots against a deep red background (Begue and Kline 1972). In the direct bioautography technique, the microorganism sprayed on the TLC plates will grow directly on

the chromatograms, while in contact bioautography, the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact. In overlay bioautography the agar is applied directly on the TLC plates and this can also be used with microorganisms that grow slowly (Hamburger and Cordell 1987, Rahalison et al. 1991). The advantage of using bioautography is that it can locate separated active compounds easily and also supports a quick search for antimicrobial agents through bioassay-guided isolation. However, there are problems associated with the assay, for example TLC eluent solvents with low volatility such as *n*-butanol (BUOH) and ammonia need to be allowed to evaporate completely so that they cannot inhibit the growth of the microorganism (Cos et al. 2006). The time taken for this may increase the risk of decomposition of active compounds.

In serial dilution assays, plant extract is mixed with water or broth in 96-well microplates and then fungal or bacterial cultures are added to the wells. The minimum inhibitory concentration (MIC) is recorded as the lowest concentration of plant extract resulting in inhibition of fungal growth, shown by a reduction in the red colour of the tetrazolium salt added as an indicator. Dilution techniques require a homogenous dispersion of the sample in water. They are used to determine, principally, the MIC values of an extract or pure compound. In the liquid dilution method, turbidity is often taken as an indication of growth, so where the sample is inactive against the microorganism tested, the liquid will appear turbid (Rios et al. 1998). The assay is quick, and works well with different microorganisms and non-aqueous extracts from different plant species. Moreover, it gives precise, reproducible results and requires just a small volume of extract to determine the minimal inhibitory concentration (MIC) for each bacterial test species against each plant extract or isolated compound. It suffers from one major drawback in that some compounds present in plant extracts may precipitate in the presence of the bacterial growth medium, making it difficult or impossible to use turbidity as a measure of microbial growth. This problem was resolved by adding *p*-iodonitrotetrazolium to the extract and microbial suspension. In the presence of microbial growth this compound is changed to a violet-coloured formazan (Eloff 1998b).

The above-mentioned assays differ in principle, and antimicrobial assay results are in general influenced by the type of assays used (Cos et al. 2006). It is necessary for bioassays to be as simple as possible; in this way sufficiently large numbers of different tests may be performed

so that many biological properties can be screened (Hostettman 1999). In this chapter, serial dilution and bioautography assays will be used to determine antimicrobial activity of the six plant species under investigation.

3.2 Materials and methods

3.2.1 Fungal strains

The seven test fungal species, *Aspergillus niger*, *A. parasiticus, Colletotrichum gloeosporioides*, *Trichoderma harzianum*, *Penicillium expansum*, *P. janthinellum* and *Fusarium oxysporum*, were obtained from the Department of Microbiology and Plant Pathology at the University of Pretoria. These fungi are among the most important pathogenic fungi of economic significance to plants. Fungal strains were maintained on Potato Dextrose (PD) agar. Fungal cultures were subcultured (1% inoculum) in PD broth at 35°C for at least two to four days before being used in the screening assays.

3.2.1.1 Quantification of fungal inoculum

For quantification of fungi, the haemocytometer cell-counting method described by Aberkane et al. (2002) with some modifications was used for counting the number of cells for each fungal culture. The inoculum of each isolate was prepared by first growing the fungus on PD agar slants for 7 days at 35ºC. The slant was rubbed carefully with a sterile cotton swab and transferred to a sterile tube with fresh PD broth (50 ml). The sterile tubes were then shaken for five minutes and appropriate dilutions were made in order to determine the number of cells by microscopic enumeration using a haemocytometer (Neubauer chamber; Merck S.A.). The final inoculum size was adjusted to approximately 1.0×10^6 cells/ml. To confirm the inoculum adjustment, 100 µl of serial dilutions of the conidial suspensions was spread onto PD agar plates. The plates were incubated at 35ºC and observation of the presence of fungal growth was done daily. The colonies were counted after the observation of visible growth and used to calculate the corresponding cells/ml.

3.2.2 Bioassays for antifungal activity

3.2.2.1 Dilution method

The serial microplate dilution method of Eloff (1998b), modified for antifungal activity testing by Masoko et al. (2005), was used to determine the MIC values for plant extracts of *B. buceras*, *B. salicina*, *H. caffrum*, *O. ventosa* and *V. infausta*. The plant extracts were tested in triplicate in each assay, and the assays were repeated once in their entirety to confirm results. Residues of different extracts were dissolved in acetone to a concentration of 10 mg/ml. The plant extracts (100 µl) were serially diluted 50% with water in 96 well microtitre plates (Eloff 1998c), and 100 µl of fungal culture was added to each well. Amphotericin B was used as the reference antibiotic and 100% acetone as the negative control. As an indicator of growth, 40 µl of 0.2 mg/ml *p*-iodonitrotetrazolium violet (INT) dissolved in water was added to the microplate wells. The covered microplates were incubated for three to five days at 35ºC at 100% relative humidity after sealing in a plastic bag to minimize fungal contamination in the laboratory. The MIC was recorded as the lowest concentration of the extract that inhibited antifungal growth. The colourless tetrazolium salt acts as an electron acceptor and was reduced to a red-coloured formazan product by biologically active organisms (Eloff 1998b). Where fungal growth is inhibited, the solution in the well remains clear or shows a marked reduction in intensity of colour after incubation with INT.

In order to determine which plants can be used for further testing, not only the MIC value is important, but also the total activity. Since the MIC value is inversely related to the quantity of antifungal compounds present, the quantity of antifungal compounds present was calculated by dividing the quantity extracted in milligrams from 1g leaves by the MIC value in mg/ml. The total activity is used to determine to what volume an extract from 1 g of plant material can be diluted and still inhibit the growth of the test organism (Eloff 1999). It can also be used to evaluate losses during isolation of active compounds and the presence of synergism (Eloff 2004).

The total activity can be calculated as:

$$\text{Total activity} = \frac{\text{Quantity of material in mg extracted from 1 g of plant material}}{\text{Minimum inhibitory concentration (mg/ml)}}.$$

In the case of bioassay guided fractionation, the total activity in the crude extract and fractions can be calculated by dividing the mass in mg in the fraction with the MIC in mg/ml. Total activity in this case [x ml/fraction] provides an indication of the volume to which the crude extract or fraction can be diluted and still kill the microorganism.

3.2.2.2 Bioautography

TLC plates (10 × 10 cm) were loaded with 100 µg of each of the extracts with a micropipette. The prepared plates were each run using different mobile systems: CEF, BEA and EMW. The chromatograms were dried at room temperature under a stream of air overnight or up to five days until the remaining solvent were removed. Fungal cultures were grown on Potato Dextrose agar for 3 to 5 days. Cultures were transferred into PD broth from agar with sterile swabs. The developed TLC plates were sprayed with concentrated suspension containing c. 1.0×10^6 cells/ml of actively growing fungi. The plates were sprayed until they were wet, incubated overnight and then sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet and further incubated overnight or longer at 35°C in a clean chamber at 100% relative humidity in the dark. White areas indicated where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of the tested fungi. The plates were sealed in plastic to prevent the spreading of the fungi in the laboratory and to retain the humidity and then scanned to produce a record of the results.

3.3 Results and discussion

3.3.1 Quantification of fungal inoculum

The number of fungal cells in the two diagonally opposite corner grids of the haemocytometer were counted and averaged. If the cell number was more than 100, a calculated volume of fresh broth was added to obtain an approximate average of 100 cells. Hence, the cell

concentration for use in the bioassay was maintained at 100×10^4 cells/ml = 1.0×10^6 cells/ml. The same procedure was used for all other tested fungal species under study.

3.3.2 Microplate dilution assay

Plant pathogenic fungi were used as test organisms for testing antifungal activity of extracts of the six selected plant species (*B. buceras*, *B. salicina*, *H. caffrum*, *O. ventosa*, *V. infausta* and *X. kraussiana*). Extracts using solvents of different polarities (acetone, hexane, dichloromethane and methanol) were prepared from the six selected plants. Hexane, DCM and methanol extracts were re-dissolved in acetone since acetone was reported not to be toxic to microorganisms at the concentrations used in the assay (Masoko et al. 2007). The extracts were tested for antifungal activity against seven fungal species: *Aspergillus niger*, *Aspergillus parasiticus*, *Colletotrichum gloeosporioides*, *Penicillium janthinellum*, *Penicillium expansum, Trichoderma harzianum* and *Fusarium oxysporum*. The minimum inhibitory concentration (MIC) results presented in Table 3-1 indicate that plant pathogens are more susceptible than animal pathogens in this case. The plant extracts were tested in a preliminary screening test against two animal pathogens, *Candida albicans* and *Cryptococcus neoformans*, and the lowest MIC values obtained were 0.03 mg/ml.

Hexane and methanol extracts of *B. salicina* had the best activities against the three most sensitive organisms, *P. janthinellum*, *T. harzianum* and *F. oxysporum*. The acetone and methanol extracts of *H. caffrum* had good antifungal activity against the three most sensitive test organisms (with MIC values ranging between 0.02 and 0.08 mg/ml). The DCM extract of *H. caffrum* had lower activity against *T. harzianum* (MIC = 0.63 mg/ml). Four extracts of *O. ventosa* had the best activity against *T. harzianum* (MIC values of 0.04 and 0.08 mg/ml). The acetone and DCM extracts of *V. infausta* also had activity against *A. parasiticus* and *T. harzianum* (MIC values of 0.16 mg/ml). All extracts of *O. ventosa* had the highest activity compared to the other plant extracts against the tested fungi. On the basis of these results *B. salicina* and *O. ventosa* were selected for further investigation in the next chapter.

Table 3-1 Minimum inhibitory concentration (MIC) of six plant species against plant pathogenic fungi (values duplicate SD = 0)

Micr[1]	MIC (mg/ml)																										
	Bucida buceras					*Breonadia salicina*				*Harpephyllum caffrum*				*Olinia ventosa*				*Vangueria infausta*				*Xylotheca kraussiana*					Amp
	Time[2] (h)	Extractant[3] A	H	D	M	A	H	D	M	A	H	D	M	A	H	D	M	A	H	D	M	A	H	D	M	Ave	
A. p	24	0.63	1.25	1.25	1.25	0.63	2.50	0.63	1.25	1.25	2.50	1.25	0.63	1.25	1.25	0.63	1.25	1.25	2.50	0.63	0.63	0.16	0.63	0.32	0.32	1.06	0.02
A. n	48	0.63	2.50	2.50	1.25	2.50	1.25	1.25	2.50	1.25	2.50	1.25	0.63	1.25	2.50	1.25	2.50	1.25	2.50	2.50	2.50	0.63	2.50	1.25	1.25	1.63	0.02
C. g	48	0.63	2.50	2.50	0.63	1.25	2.50	1.25	1.25	1.25	0.63	1.25	2.50	1.25	2.50	1.25	2.50	1.25	2.50	2.50	2.50	1.25	2.50	0.63	0.63	1.54	<0.02
P. e	24	0.08	0.63	0.32	0.32	1.25	2.50	2.50	2.50	2.50	1.25	2.50	0.63	0.32	1.25	1.25	0.63	2.50	1.25	1.25	1.25	0.63	2.50	1.25	1.25	1.27	<0.02
P. j	48	0.02	0.08	0.32	0.02	0.08	0.08	0.08	0.08	0.04	0.32	0.16	0.08	0.08	0.32	0.16	0.32	0.63	0.63	0.63	0.32	0.32	0.63	0.63	0.63	0.28	<0.02
T. h	48	0.02	0.08	0.63	0.02	0.63	0.32	0.63	0.63	0.08	0.63	0.63	0.08	0.04	0.04	0.04	0.08	0.32	0.32	0.32	0.32	0.16	0.32	0.16	0.32	0.28	<0.02
F. o	24	0.02	0.63	0.32	0.04	0.32	0.08	0.16	0.16	0.02	0.32	0.16	0.04	0.08	0.63	0.32	0.16	0.32	0.63	0.32	0.32	0.63	0.32	0.32	0.32	0.28	<0.02
Ave		0.29	1.02	1.05	0.5	0.92	1.25	0.89	1.12	0.88	1.09	0.99	0.62	0.61	1.14	0.70	0.99	1.04	1.37	1.09	1.05	0.54	1.23	0.65	0.67	0.91	

[1]Microorganisms: *A. p.* = *Aspergillus parasiticus*, *A. n.* = *Aspergillus niger*, *C. g.* = *Colletotrichum gloeosporioides*, *P. e.* = *Penicillium expansum*, *P. j.* = *Penicillium janthinellum*, *T. h.* = *Trichoderma harzianum* and *F. s.* = *Fusarium oxysporum*

[2]Time: MIC values after 24 h were sometimes not distinct, so the plates were left to incubate for a further 24 h before MIC was read

[3]Extractant: A = acetone, H = hexane, D = dichloromethane, M = methanol

The total activity values of the six plant species extracted with acetone, hexane, dichloromethane and methanol are given in Table 3-2. The highest total activity was found in the acetone extract of *H. caffrum* (22 000 ml/g) against *F. oxysporum*. The lowest total activity was observed in the methanol extract of *O. ventosa* (133 ml/g) against both *A. niger* and *C. gloeosporioides*. These observations are consistent with the variation of the MIC values in Table 3-2, that is, where the MIC value is low, the total activity is high.

Table 3-2 Total activity in ml/g of six plant species extracted with acetone, hexane, dichloromethane and methanol tested against seven fungi

Micr	Total activity (ml/g)																									
	Bucida buceras					*Breonadia salicina*				*Harpephyllum caffrum*				*Olinia ventosa*				*Vangueria infausta*				*Xylotheca kraussiana*				
	Time (h)	Extractant[2] A	H	D	M	A	H	D	M	A	H	D	M	A	H	D	M	A	H	D	M	A	H	D	M	Ave
A. p	24	746	140	762	360	540	272	248	208	352	151	312	730	328	264	619	240	376	218	651	762	2938	556	1063	1438	595
A. n.	48	209	280	384	200	540	151	138	208	352	151	312	730	328	147	312	133	376	218	182	213	746	156	272	368	296
C g.	48	376	155	384	360	540	151	138	413	352	540	312	204	328	147	312	133	376	218	182	213	376	156	540	730	318
P. e.	24	376	156	213	200	4250	540	969	813	196	272	173	730	1281	264	312	476	209	392	328	384	746	156	272	368	587
P. .j	48	5875	4375	6000	5625	17000	4250	969	13000	11000	1063	2438	5750	5125	1031	2438	938	746	777	651	1500	1469	556	540	730	3910
T. .h	48	746	1094	762	714	17000	4250	492	13000	5500	540	619	5750	10250	8250	9750	3750	1469	1531	1281	1500	2938	1094	2125	1438	3993
F. o	24	1467	4375	3000	2813	17000	540	969	6500	22000	10625	2438	11500	5125	524	1219	1875	1469	778	1281	1500	746	1094	1063	1438	4222
Ave		1400	1510	1644	1467	8124	1451	560	4877	5679	1906	943	3628	3252	1518	2137	1078	717	590	651	868	1423	538	839	930	1989

[1]Microorganisms: *A. p.* = *Aspergillus parasiticus*, *A. n.* = *Aspergillus niger*, *C. g.* = *Colletotrichum gloeosporioides*, *P. e.* = *Penicillium expansum*, *P. j.* = *Penicillium janthinellum*, *T. h.* = *Trichoderma harzianum* and *F. s.* = *Fusarium oxysporum.*

[2]Extractant: A = acetone, H = hexane, D = dichloromethane, M = methanol

Acetone extracts in general had the lowest average MIC value of 0.71 mg/ml against the tested microorganisms, followed by DCM extracts (0.83 mg/ml), methanol extracts (0.89 mg/ml) and finally hexane extracts (1.19 mg/ml) as shown in Table 3-3. Plant extracts with low MIC values could be a good source of bioactive components with antimicrobial potency. In particular, acetone extracts have shown potentially interesting activity compared to extracts prepared using other solvents. Based on the MIC results, acetone was the best extractant, and additional positive features include its volatility, miscibility with polar and non-polar solvents and its relative low toxicity to test organisms (Eloff 1999).

In the current study, negative controls showed that acetone alone was not harmful to the plant pathogens at the highest percentage tested, confirming previous results (Eloff et al. 2007). However, plant extracts are traditionally prepared with water as infusions, decoctions and macerations. Therefore, it would be difficult for the traditional healer to be able to extract those compounds which are responsible for activity in the acetone and methanol extracts (Aliero and Afolayan 2005). Many traditional healers use water to extract plant material, since water is not toxic, not expensive and is the only extractant available. In some cases animal fat is mixed with plant material and under these conditions the non-polar compounds could become available.

Table 3-3 Average MIC values (mg/ml) of extracts prepared with different extractants on all test organisms

Extractants	MIC
Acetone	0.71
Hexane	1.19
Dichloromethane	0.89
Methanol	0.83

Out of the seven plant pathogens used, only three fungi (*P. janthinellum*, *T. harzianum* and *F. oxysporum*) had significant sensitivity to the plant extracts (average MIC values of 0.28 mg/ml, Table 3-4). *Fusarium oxysporum* was reasonably sensitive. This fungus has been reported to cause vascular wilt and damping off in plants which could result in substantial stand reduction and yield (Kishi 1974). The results showed that the other four fungi

(*Aspergillus niger*, *Aspergillus parasiticus*, *Colletotrichum gloeosporioides* and *Penicillium expansum*) were more resistant to all of the extracts (Table 3.4). *Aspergillus niger* was also reported to be resistant to DCM, aqueous and methanolic extracts of 14 plants used traditionally in Paraguay (Portillo et al. 2001).

Table 3-4 Average MIC values of acetone, hexane, dichloromethane and methanol extracts against seven plant pathogens

Microorganism	Average
Aspergillus parasiticus	1.06
Aspergillus niger	1.63
Colletotrichum gloeosporioides	1.54
Penicillium expansum	1.27
Penicillium janthinellum	0.28
Trichoderma harzianum	0.28
Fusarium oxysporum	0.28
Average	0.91

The average MIC values of each plant species using different extractants are shown in Table 3-5. Based on the results obtained, *B. buceras* was the most active plant extract (average MIC value 0.72 mg/ml), followed by *X. kraussiana* (0.78 mg/ml), *O. ventosa* (0.86 mg/ml), *H. caffrum* (0.89 mg/ml) and *V. infausta* (1.14 mg/ml).

In isolating antimicrobial compounds from plant extracts, bioautography plays an enormously important role to facilitate the isolation of antimicrobial compounds. Without good bioautography data it is very difficult to ensure success in isolating antimicrobial activity especially if more than one compound is required to express activity. The bioautography results will be discussed in the next section.

Table 3-5 The average MIC values (mg/ml) of plant extracts prepared using different extractants against seven plant pathogens

Extractants	Plant species					
	Bucida buceras	*Breonadia salicina*	*Harpephyllum caffrum*	*Olinia ventosa*	*Vangueria infausta*	*Xylotheca kraussiana*
A	0.29	0.92	0.88	0.61	1.04	0.54
H	1.02	1.25	1.09	1.14	1.37	1.23
D	1.05	0.89	0.99	0.7	1.09	0.65
M	0.5	1.12	0.62	0.99	1.05	0.67
Average	0.72	1.05	0.89	0.86	1.14	0.78

3.3.3 Bioautography assay

Bioautography was used to determine the number of active compounds in different plant extracts, and representative bioautograms are shown in Figures 3-1 and 3-2. Three solvent systems were used as eluents in the TLC separation, but only the results of CEF and EMW bioautography are given since the compounds in the extracts did not separate using BEA as expected from earlier results. However, the antifungal compounds separated well in both CEF and EMW solvent systems. Their R_f values were also calculated by dividing the distance moved by the compound of interest with the distance moved by the solvent front. The TLC chromatograms developed in CEF (Figure 3-1) of acetone, DCM and methanol extracts of *O. ventosa* showed the same number of active compounds (R_f value of 0.95). The antifungal compounds were clearly visible and more active compounds were observed against *A. parasiticus* than *T. harzianum* and *P. janthinellum*. Furthermore, three active compounds separated with CEF had the same R_f values (0.70, 0.85 and 0.95) in the acetone, hexane, DCM and methanol extracts of *B. salicina*. Three other active compounds with common R_f values of 0.54, 0.72 and 0.95 were visible in the acetone, DCM and methanol extracts of *O. ventosa* against *A. parasiticus* and *P. janthinellum* (Table 3-6).

Data for *B. salicina.*

In EMW (separates more polar compounds) bioautograms, the compound present in the acetone, hexane, DCM and methanol extracts (R_f 0.17) inhibited the growth of three fungi, i.e. *P. janthinellum, A. parasiticus* and *A. niger*. Acetone and hexane extracts had similar active compounds against *P. janthinellum* and *A. niger* with R_f value 0.17. Furthermore, active compounds with the same R_f value of 0.13 were observed in the acetone and hexane extracts against *C. gloeosporioides* and *A. parasiticus*. Bioautograms produced using *P. expansum* and *T. harzianum* showed active compounds in the acetone and hexane extracts, while in the case of DCM and methanol extracts, no active compounds were clearly visible.

Data for *O ventosa.*
In CEF bioautograms, acetone extracts inhibited the growth of fungi i.e *P. janthinellum* with R_f value of 0.17 while no compound were observed in hexane, DCM and methanol extracts. Surprisingly, the acetone extract of *B. salicina* and *O. ventosa* showed similar antifungal compound (R_f 0.13 and 0.17) which were active against *A. parasiticus* and *P. janthinellum*. All extracts of *O. ventosa* did not inhibit the growth of the other three fungi, *A. niger*, *C. gloeosporioides* and *P. expansum*.

Breonadia salicina and *O. ventosa* had the most promising number of antifungal compounds in all four extracts (acetone, hexane, DCM and methanol) because they showed compounds that inhibit the fungi. In summary, the results obtained showed that there were more active compounds separated by EMW (total of 35) than by CEF (total of 17).

Data for other plant extracts.
The remaining four plant species (*B. buceras*, *H. caffrum*, *V. infausta* and *X. kraussiana*) showed no activity in the bioautography screening against the seven test organisms, thus the results are considered not significant. However, these plant extracts had good activity in the microplate assay. Possible reasons may be that some of the active compounds were volatile and evaporated during the drying period of the TLC chromatograms prior to bioautography. Biological activity synergism between different compounds in the extracts is also a possible reason.

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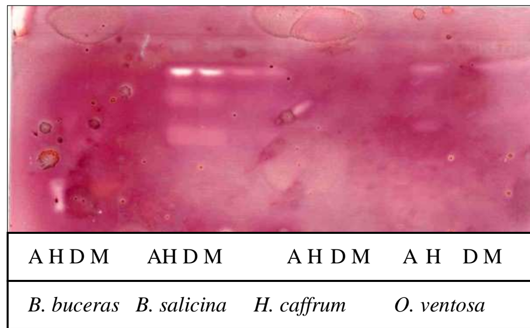
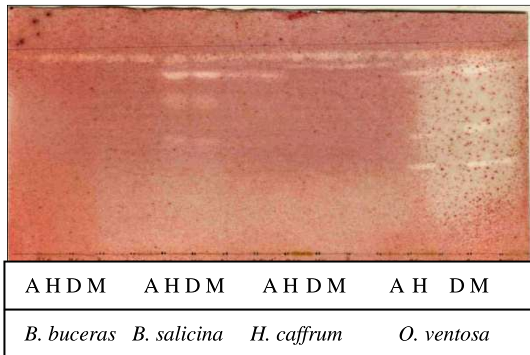




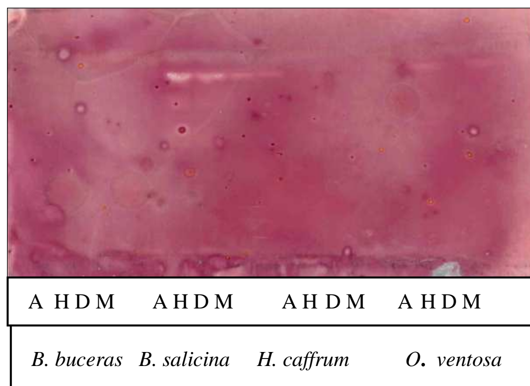
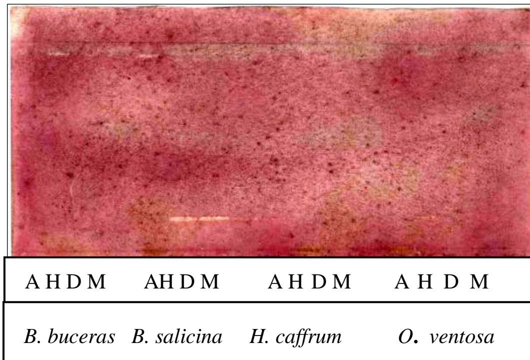
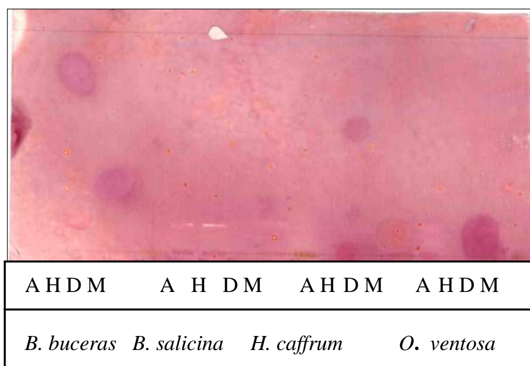


Figure 3-1 Bioautograms of extracts of *Bucida buceras*, *Breonadia salicina*, *Harpephyllum caffrum* and *Olinia ventosa*. Chromatograms were developed in CEF and sprayed with *Penicillium janthinellum* (top), centre (*Aspergillus parasiticus*), bottom (*Trichoderma harzianum*). White areas indicate inhibition of fungal growth. **Lanes from left to right:** acetone (A), hexane (H), DCM (D) and methanol (M)





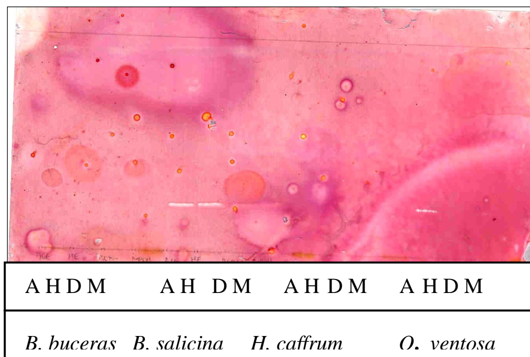
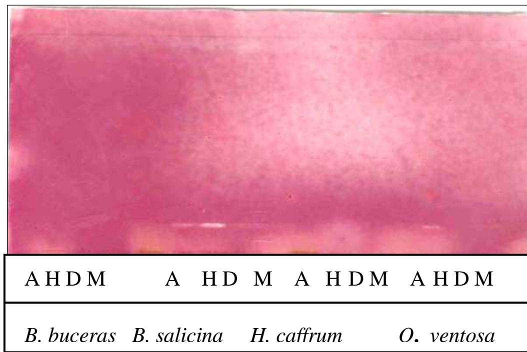
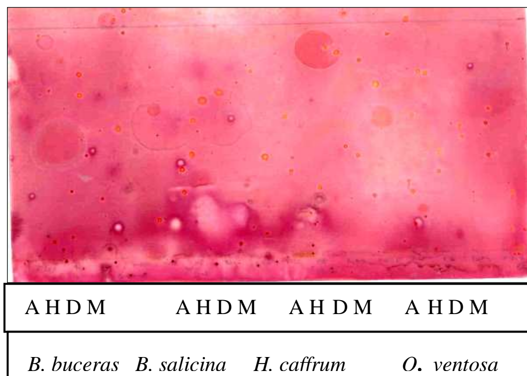


Figure 3-2 Bioautograms of extracts of *Bucida buceras*, *Breonadia salicina*, *Harpephyllum caffrum* and *Olinia ventosa*. Chromatograms were developed in EMW and sprayed with (*Aspergillus niger*) top, centre (*Colleotothrichum gloeosporioides*) and bottom (*Penicillium janthinellum*). White areas indicate inhibition of fungal growth. **Lanes from left to right:** acetone (A), hexane (H), DCM (D) and methanol (M)





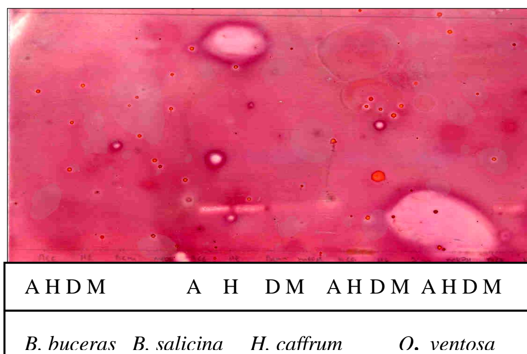


Figure 3-2 (continuation) Bioautograms of extracts of *Bucida buceras*, *Breonadia salicina*, *Harpephyllum caffrum* and *Olinia ventosa*. Chromatograms were developed in EMW and sprayed with (*Aspergillus parasiticus*) top, centre (*Penicillium expansum*) and bottom (*Trichoderma harzianum*). White areas indicate inhibition of fungal growth. **Lanes from left to right:** acetone (A), hexane (H), DCM (D) and methanol (M).

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Table 3-6 The inhibition of fungal growth by bioautography of different plant extracts separated by TLC (CEF solvent system). R_f values of active compounds are shown.

R_f values	Extractants	Microorgansims							TOTAL
		A. p	*A. n*	*C. g*	*P. e*	*P. j*	*T. h*	*F. o*	
Breonadia salicina									
0.70	A	1				1			2
0.85		1				1			2
0.95		1				1	1		3
0.70	H	1				1			2
0.85		1				1			2
0.95		1				1	1		3
0.95	D	1				1	1		3
0.95	M	1				1	1		3
Olinia ventosa									
0.54	A	1							1
0.72		1							1
0.95		1				1	1		3
	H								
0.54	D	1							1
0.72		1							1
0.95		1				1	1		3
0.54	M	1							1
0.72		1							1
0.95		1				1	1		3
Total									35

Microorganisms: *A. p.* = *Aspergillus parasiticus*, *A. n.* = *Aspergillus niger*, *C. g.* = *Colletotrichum gloeosporioides*, *P. e.* = *Penicillium expansum*, *P. j.* = *Penicillium janthinellum*, *T. h.* = *Trichoderma harzianum* and *F. s.* = *Fusarium oxysporum*.

Table 3-7 The inhibition of fungal growth in the bioautography assay of different plant extracts separated by TLC (EMW solvent system). R_f values of active compounds are shown.

R_f values	Extractants	Microorgansims							**TOTAL**
		A. p	*A. n*	*C. g*	*P. e*	*P. j*	*T. h*	*F. o*	
Breonadia salicina									
0.13	A	1	1	1					3
0.17						1			1
0.22							1		1
0.36					1				1
0.13	H	1	1	1	1				4
0.17						1			1
0.22							1		1
0.36					1				1
	D		1						1
	M								
Olinia ventosa									
0.13	A	1							1
0.17						1			1
0.22							1		1
	H								
	D								
	M								
Total									17

Microorganisms: *A. p.* = *Aspergillus parasiticus*, *A. n.* = *Aspergillus niger*, *C. g.* = *Colletotrichum gloeosporioides*, *P. e.* = *Penicillium expansum*, *P. j.* = *Penicillium janthinellum*, *T. h.* = *Trichoderma harzianum* and *F. s.* = *Fusarium oxysporum.*

3.4 Conclusion

The serial microdilution and bioautography assays were used to determine antifungal activity and number of active compounds present in the plant extracts respectively. The results obtained in this preliminary work showed that extracts of *O. ventosa* and *B. salicina* possess the best antifungal activity with MIC values of 0.04 and 0.08 mg/ml compared to the other four plant species tested.

Based on MIC results, acetone was the best extractant. It is also low in toxicity to the test organisms and further studies will be carried out using acetone as extracting solvent. In bioautography, several active compounds were visible in acetone, hexane, dichloromethane and methanol extracts of *B. salicina* while only one antifungal compound was observed in the acetone and hexane extracts of *O. ventosa*. The other four plant species (*B. buceras*, *H. caffrum*, *V. infausta* and *X. kraussiana*) showed no activity in the bioautography screening against the test organisms *Aspergillus niger*, *A. parasiticus*, *Colletotrichum gloeosporioides*, *Penicillium janthinellum*, *P. expansum*, *Trichoderma harzianum* and *Fusarium oxysporum*. In TLC chromatograms developed in CEF (non-polar) more active compounds were observed (total of 35) than in the chromatograms developed using EMW (total of 17). No active compounds were visible in the TLC chromatograms developed in BEA for all four extracts of *V. infausta* and *X. kraussiana*. In summary, *B. salicina* was selected as the best plant species for comprehensive investigation and only two solvent systems, CEF and EMW were recommended for further studies. In the next chapter I will investigate the qualitative antioxidant activity from leaf extracts of six selected plant species.

CHAPTER 4

Antioxidant activity

4.1 Introduction

Plants contain a wide variety of free radical scavenging molecules such as flavonoids, anthocyanins, carotenoids, vitamins and endogenous metabolites. More importantly, these natural products are rich in antioxidant activities (Hertzog et at. 1992). Free radicals can be defined as species with unpaired electrons (Wettasinghe and Shahidi 2000). Antioxidants are known as free radical scavengers and they tend to retard or prevent the oxidation of other molecules by capturing free radicals (Breton 2008). They have various biological activities such as anti-inflammatory, anticarcinogenic and anti-atherosclerotic, antibacterial, antiviral, antimutagenic, antiallergic and antiulcer activity activities (Ikken et al. 1999, Noguchi et al. 1999). These activities may be due to their antioxidant activity (Chung et al. 1998).

Antioxidant compounds also help delay and inhibit lipid oxidation. They play an important role in the maintenance of health and prevention of several diseases. The best way to help prevent these diseases is consumption of an optimal diet containing natural antioxidants. When these constituents are added to foods they tend to minimize rancidity, retard the formation of toxic oxidation products, help maintain the nutritional quality and increase their shelf life (Fukumoto and Mazza 2000). The consumption of food such as fruit, vegetables, red wines and juices helps protect the body from being afflicted with diseases such as cancer and coronary heart disease. This protection is due to the capacity of antioxidants in the plant foods to scavenge free radicals, which are responsible for the oxidative damage of lipids, proteins, and nucleic acids.

Many medicinal plants contain large amounts of antioxidants such as polyphenols, which plays an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Djeridane et al. 2006). A polyphenol antioxidant is a type of antioxidant containing a polyphenolic substructure. Polyphenol antioxidants are found in a wide array of phytonutrient-bearing foods. For example, most legumes, fruits (such as apples, grapes, pears, plums, raspberries and strawberries), vegetables (such as broccoli,

cabbage and onion) are rich in polyphenol antioxidants (Breton 2008). Previously, it has been reported that polyphenolic compounds have antioxidant activity, free-radical scavenging capacity, coronary heart disease prevention, and anticarcinogenic properties (Satora et al. 2008).

Two free radicals, 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) are commonly used to determine antioxidant activity in plant extracts and isolated compounds. The ABTS assay measures the relative ability of antioxidant to scavenge the ABTS generated in aqueous phase, as compared with a Trolox (water soluble vitamin E analogue) standard (Miller and Rice Evans 1997). The method is rapid and can be used over a wide range of pH values (Arnao 1999, Lemanska et al. 2001), in both aqueous and organic solvent systems. On the other hand, it also preferred since it has good repeatability and is easy to perform. In this chapter, a qualitative DPPH method will be used to determine the number of antiradical/antioxidant compounds in plant extracts. The advantage of this qualitative method is that it has good repeatability and is used frequently.

Phenolic compounds are commonly found in both edible and non-edible plants and they have been reported to have multiple biological effects, including antioxidant activity (Kähkönen et al. 1995) and they are considered to provide a major contribution to the total antioxidant activity. This activity of phenolics is mainly due to their redox properties, which can function as hydrogen donors, and singlet or triplet oxygen quenchers. In addition, they have a metal chelation potential (Rice-Evans et al. 1995).

Flavonoids are one of the well known groups of polyphenols. They are found in edible plant products, especially fruits and vegetables (Bravo 1998). Previously, it has been reported that no less than 1-2 g of polyphenols (including flavonoids) should be consumed daily. Food products that contain flavonoid compounds are green leafed, yellow and red vegetables (e.g. onion, cabbage, tomatoes and peppers), fruit (e.g. grapefruits, oranges, dark grapes and apples), red wine and also green tea (Oleszek et al. 1988, Pelegrinin et al. 2000).

4.2 Materials and methods

4.2.1 Extraction

Six plant species were extracted with the following solvents of varying polarities: acetone, hexane, DCM and methanol. The extraction procedure is explained in detail in **section 2.2.4**

4.2.2 Assay for free radical scavenging (DPPH)

The antioxidant activities of plant extracts were determined using the qualitative method 2, 2-diphenyl-1-picrylhydrazyl (DPPH). This assay is preferred because it is used to provide stable free radicals (Fatimi et al. 1993). The effect of antioxidants on DPPH radical scavenging is due to their hydrogen donating ability.

4.2.2.1 TLC fingerprint and antioxidant activity

The TLC chromatograms were developed in three eluent systems BEA, CEF and EMW as described in **section 2.2.4**. The prepared TLC chromatograms were visualized under UV light 254 and 364 and the compounds were identified and highlighted by light pencil circles. A solution of 0.2% DPPH in methanol was prepared and then sprayed on the plates (until it became wet) and allowed to dry in a fume cupboard. The presence of antioxidant compounds was indicated by yellow bands which showed radical scavenger capacity against a purple background. The intensity of the yellow band depends on the quantity and nature of the radical scavenger present in the plant extracts.

4.3 Results and discussion

Figure 4-1 shows the TLC chromatograms of plant extracts sprayed with DPPH. The chromatograms developed in BEA had no zones with antioxidant activity in most of the extracts, probably because antioxidant compounds are usually too polar to be separated well with the BEA solvent system (Figure 4-1). Only the methanol extract of *X. kraussiana* showed a yellow band with an R_f value of 0.14. However, the activity was not strong since the yellow band is not very clear.

In general, the methanol extract had a higher antioxidant activity than the acetone, hexane and DCM extracts. This is again due to the polar nature of most antioxidant compounds, as methanol extracts largely polar compounds. In comparison to members of the Combretaceae (Masoko and Eloff 2007) the extracts of these species contain very few antioxidant compounds and inhibition of microbial infections by stimulating the immune system of the host does not appear to be a realistic mechanism for its activity and traditional use.

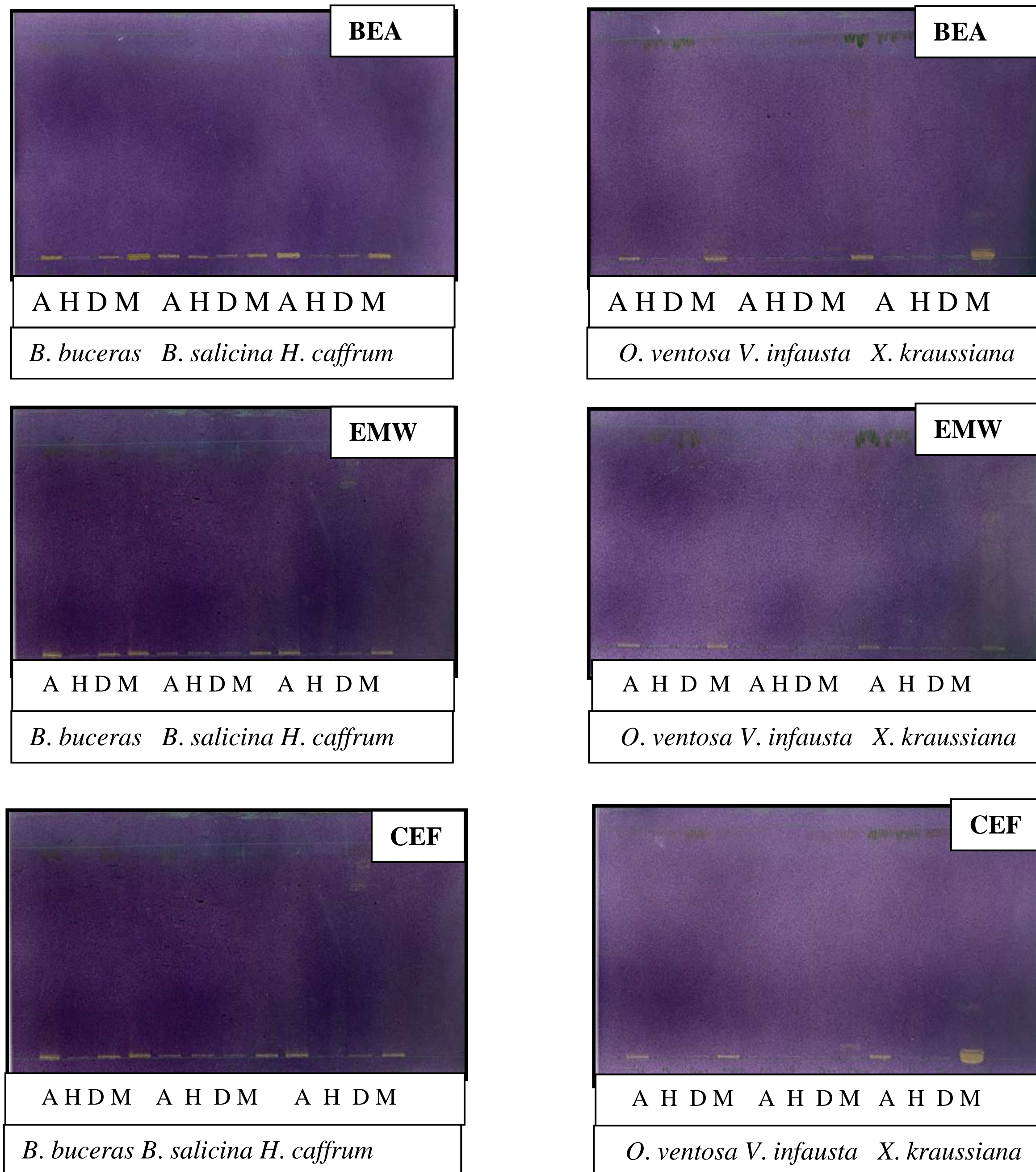

Figure 4-1 TLC chromatograms of six plant species (left to right: *Bucida buceras*, *Breonadia salicina*, *Harpephyllum caffrum*, *Olinia ventosa*, *Vangueria infausta* and *Xylotheca kraussiana*) extracted with acetone, hexane, DCM and MeOH (left to right), developed in BEA, CEF and EMW, and sprayed with DPPH solution.

4.4 Conclusion

The plant extracts of five of the six medicinal plants did not possess strong antioxidant activity. As expected, the polar methanol extracts of *X. kraussiana* showed the antioxidant activity. Due to the limited activity visualised in the qualitative assays, it was deemed unnecessary to include quantitative antioxidant assays in the screening procedure.

It appears that the compounds with antifungal activity, shown using bioautography techniques did not have any antioxidant activity. If they did have such activity, it would have been easier to isolate the active compounds by using the DPPH assay rather than the more complicated and time consuming antifungal bioautography assay for bioassay fractionation to isolate the antifungal compounds. Although *Olinia ventosa* extracts gave very promising results in the earlier antifungal results, the plant was not readily available for collection of test material. In the next chapter the species selected for further work, *Breonadia salicina*, will be investigated in more depth.