Chapter 7

Evaluation of different extracts of selected south african plant species for antifungal activity

7.1. Introduction

The remarkable rise in the incidence of life threatening systemic fungal infections has been a cause for concern in the last two decades. Although most of these pathogens have co-existed with man from time immemorial, the increase in opportunistic infections caused by these pathogens has become more alarming with the advent of HIV-AIDS (Groll *et al.,* 1996; Stevens, 1990; Denning, 1998). For example, oropharyngeal candidosis (OPC) was found to occur in more than 80% of all patients during the course of HIV infection (Torssander *et.al*., 1987; Feigal *et. al.,* 1991) while *Candida* infections following bone marrow transplants are said to occur in up to 11% of patients (Sable and Donowitz, 1994). On the other hand, invasive aspergillosis is said to affect between 10 and 20% of patients with leukaemia and between 5 and 25% of patients that have undergone heart or lung transplantation (Denning, 1994). A similar scenario has also been reported for *Cryptococcus neoformans* infection with an overall death rate in transplant recipients of 20 to 100% (Carlson *et al*., 1987; Jabbour, 1996; Singh *et al.,* 1997).

The common clinically used antifungals are associated with numerous drawbacks such as toxicity, efficacy and cost, and their frequent usage has resulted in the development and emergence of resistant strains of these pathogens. The challenge the world is faced with is the development of effective strategies for the treatment of infections caused by these pathogens. Furthermore, concerns in recent years have been raised about the environmental impact of the use of synthetic fungicides in agriculture and the potential health risks posed by their continuous use (Abad *et al*., 2007).

The existence of such problems generates the need to discover new classes of antifungal compounds to treat fungal infections. In recent years, investigation of active constituents present in plants and natural product-derived compounds has accelerated due to their importance in drug discovery. Currently, three out of the seven classes of clinically available antifungal agents are from natural sources (Arif *et al*., 2011). This places plants as a rich source of bioactive secondary metabolites of a wide variety, which can be exploited for their antifungal properties. The

molecules isolated can be used directly, or as a model for developing better molecules. In this study, different extracts of selected species of plants were evaluated for their antifungal activity against three fungal pathogens of clinical importance in an attempt to identify and isolate active compounds against these pathogens, especially when the prevalence of these pathogens in immunocompromised patients is high.

7.2. Materials and Methods

7.2.1. Thin layer chromatography (TLC) analysis of crude extracts

The plants were collected and extracted using hexane, acetone, dichloromethane and methanol as described in section 3.1 and 3.2. Extracted plant materials were dissolved in acetone and spotted onto TLC plates and eluted in different solvent systems as described in section 3.4 for separation of constituents in the different extracts.

7.2.2. Bioautography on TLC plates

Thin layer chromatography plates (10 x 10 cm) were spotted with 10 µℓ (10 mg/mℓ) of the different extracts of each plant species and eluted in the three different mobile eluting solvent systems of varying polarity: CEF, BEA and EMW (section 3.4) without spraying with vanillin. The chromatograms were dried for 5 days at room temperature under a stream of air to remove the eluent solvent system. Chromatograms were sprayed with fungal pathogens as described (section 3.8.1).The fungal pathogens used in this study were *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. Plates sprayed with pathogens were incubated in a humidified atmosphere at 30°C and thereafter sprayed with INT as described (section 3.8.1).

7.2.3. Microdilution assay for MIC determination

In the antifungal bioassay, the method described by Eloff (1998b) and modified by Masoko *et al*. (2005) using Sabouraud Dextrose (SD) broth as nutrient medium was used to test the activity of extracts on selected fungal pathogens. Two-fold serial dilutions of test substances (initial concentration of 10 mg/mℓ) dissolved in acetone were prepared in 96-well microtitre plates as described (section 3.8.2). Actively growing organisms (section 3.8.1) were transferred from SD agar plates using a sterile cotton swab into fresh SD broth and the assay was conducted as described (section 3.8.2). Amphotericin B (starting concentration of 0.08 mg/mℓ), a standard antifungal agent, was

included as a positive control. The selectivity index (SI) was calculated by dividing the cytotoxic concentration of each extract of the different plant species by the MIC. i.e (CC_{50}) / (MIC).

7.2.4. Determination of total activity

The total activity of plant extracts was determined using the method of Eloff (2004). Total activities of an extract or fraction gives an indication of the efficacy at which active constituents present in one gram can be diluted and still inhibit the growth of test organism. This value is calculated in relation to the MIC value of the extract, expressed mathematically as follows: Total activity = amount extracted from 1g (mg) of plant material divided by the MIC (mg/mℓ)

7.2.5. Determination of cytotoxic effect of the extracts on different cell types

The cytotoxic effect of the different extracts of plants selected for the study was evaluated on Vero cells using the method of Mosmann (1983) as described in section 3.9.

7.3. Results and Discussion

7.3.1. Inhibition of bacterial growth using bioautography

Three solvent systems were used to separate the active components of the different extracts of each plant species. A lot of difficulties were experienced in the growth of pathogens on TLC plates. In some cases growth was poor depending on the solvent system used for elution, which was more evident in plates eluted in the non-polar and basic eluent system, BEA, as well as the polar and neutral eluent system, EMW. As such, zones of inhibition were not evident in a majority of extracts of the different plant species (Table 7.1). The non-activity recorded for most extracts eluted in BEA may be associated with the presence of more active polar constituents in the extracts that could not separate well in BEA. It is also possible that the poor growth of fungal pathogens observed in this study may be associated with residual traces of eluent solvent on chromatograms that inhibit fungal growth (Masoko and Eloff, 2005). In other cases, although growth was evident, the lack of zones of inhibitions on chromatograms may be associated with non-activity of the compounds, a possible evaporation or break-down of active compounds during the removal of TLC eluents or disruptions of synergism between active constituents caused by TLC (Masoko and Eloff,

2005). CEF and EMW were the solvent systems that best separated the active constituents and also supported growth of organisms, with the intermediately polar system CEF recording the highest number of active constituents (Table 7.1).

In general, where visible zones of inhibition were recorded, DCM extracts showed the highest number of active constituents against tested pathogens (although variation was observed between different species of plants) followed by the acetone, methanol and hexane extracts with the least number of active compounds. Between species of plants, some compounds were detected with similar R_f values in extracts of different plant species as could be seen with the acetone, methanol and DCM extracts of *A. schimperi* and *C. edulis* against *C. albicans* as well as *S. alata* against *A. fumigatus* (R_f 0.87) and *A. schimperi* and *A. senegalensis* against *C. neoformans* (R_f 0.91) on chromatograms eluted in CEF (Table 7.1). A similar phenomenon was also observed with extracts of some species of plants eluted in EMW.

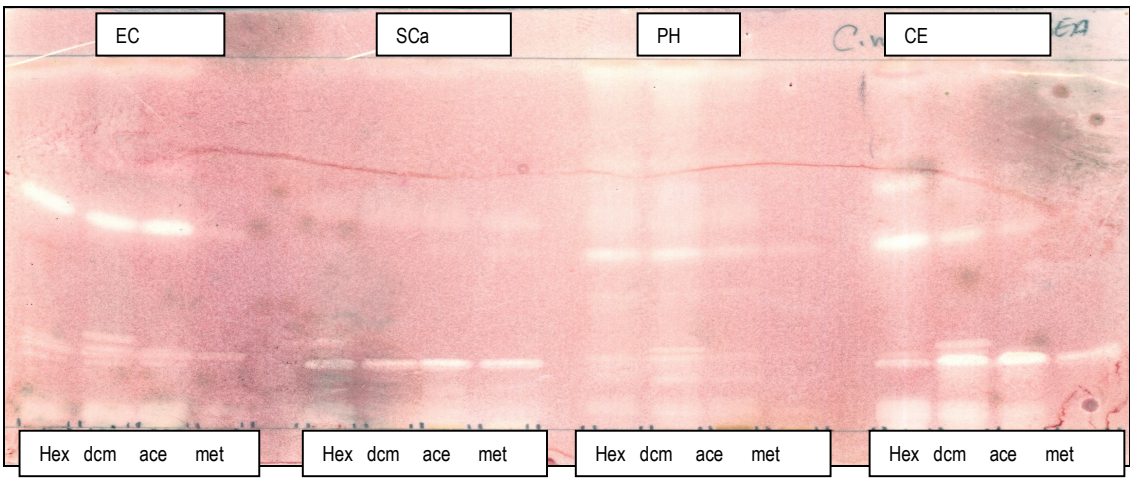

Figure 7.1. A representative bioautograph eluted in BEA indicating inhibition of growth of *C.neoformans.*

White zones on a purple background represent inhibition of growth of the pathogen.

PH = Podocarpus henkelii, EC = Ekebergia capensis, Schrebera alata, Carissa edulis

Hex = Hexane, DCM =Dichloromethane, Ace = Acetone, Met = Methanol

Table 7.1. R_f values of antifungal compounds detected[illegible] ts in bioautography

		BEA				CEF				EMW			
Organism	Plants	Acetone	Methanol	Hexane	DCM	Acetone	Methanol	Hexane	DCM	Acetone	Methanol	Hexane	DCM
	Acocanthera schimperi					0.87	0.87		0.87	0.93	0.93	-	0.93
	Carissa edulis			-					0.49				
						0.87	0.87		0.87	0.93	0.93	-	0.93
	Annona senegalensis			-		0.94			0.94				
	Podocarpus henkelii												
	Schrebera alata	0.33	0.33		0.33	0.51			0.51				
C. albicans						0.86	0.86		0.86				
	Ekebergia capensis									-	0.37	-	-
	Plumbago zeylanica			-				-					
	Acokanthera shimperi					0.85	0.85	0.85	0.85	0.93	0.93	-	0.93
						0.91	0.91	0.91	0.91				
	Carissa edulis	0.2	0.2	0.2	0.2	0.43	0.43	N/A	0.43				
		0.43		0.43	0.43		N/A		0.46				
				0.68								-	
						0.84	0.84	0.84	0.84				
				-		0.9	0.9	0.9	0.9				
	Annona senegalensis					0.5	0.5	N/A	0.5				
						0.91	0.91	0.91	0.91				
C.neoformans								-					
								-					
	Schrebera alata	0.2	0.2	0.2	0.2								
	Ekebergia capensis	0.2	0.2	0.2	0.2								
		0.54		0.24	0.24								
				0.54	0.54								
	Podocarpus henkelii	0.43		0.2	0.2								
				0.43	0.43								
					0.23								
	Plumbago zeylanica												
	Acokanthera schimperi					0.89	0.89	N/A	0.89	0.96	0.96	0.96	0.96
						0.93	0.93	N/A	0.93		N/A		
	Carissa edulis						N/A		0.52	0.96	0.96		0.96
						0.81	0.81	N/A	0.81				
						0.93	0.93	0.93	0.93				
	Annona senegalensis					0.26	0.26	N/A	0.26	0.97	0.97	0.97	0.97
				-		0.41	0.41	N/A	0.41				
A. fumigatus						0.5	0.5	0.5	0.5				
						0.93	0.93	N/A	0.93				
	Podocarpus henkelii					0.95	-	0.95	0.95				
	Schrebera alata					0.46				0.95	-	0.95	
						0.87	0.87	-	0.87				
	Ekebergia capensis								0.5	0.95			0.95
						0.96	0.96	-	0.96				
	Plumbago zeylanica												

Table 7.2. Antifungal activity of selected plant species against animal fungal pathogens (MIC mg/mℓ)

		Hexane			Dichloromethane			Acetone			Methanol		
Plant	Part	C.n	C.a	A.f	C.n	C.a	A.f	C.n	C.a	A.f	C.n	C.a	A.f
Acokanthera schimperi	Leaves	0.16	2.5	0.32	0.08	0.32	0.64	0.08	0.32	1.25	0.08	0.43	0.64
Carissa edulis	Leaves	1.25	2.5	0.64	0.32	2.5	0.64	0.16	2.5	0.64	0.16	2.5	1.25
Annona senegalensis	Leaves	0.04	2.5	0.64	0.16	0.32	0.08	0.04	0.64	0.16	0.08	0.32	0.32
Podocarpus henkelii	Leaves	0.64	2.5	0.64	0.64	0.32	0.64	0.32	0.08	0.64	0.16	0.32	0.32
Schrebera alata	Leaves	0.64	2.5	1.25	0.32	0.32	0.64	0.64	0.16	0.64	1.25	0.16	1.25
Ekebergia capensis	Leaves	1.25	2.5	1.25	0.32	0.32	1.25	0.64	0.32	0.64	1.25	0.32	0.64
Plumbago zeylanica	Leaves	1.25	1.25	2.5	0.16	0.32	0.08	0.16	0.32	0.16	0.32	0.32	0.64

C.n = Cryptococcus neoformans, *C.a = Candida albicans*, *A.f = Aspergillus fumigatus*

(Table 7.1). Although these compounds were active against the same pathogens, the presence of compounds with similar R_f values in the different species of plants may not necessarily indicate the presence of the same active compounds in the different plants.

7.3.2. Antifungal activity of extracts in terms of MIC values

Various authors have used different assays to evaluate the antifungal activity of crude plant extracts. Tadeg *et al*. (2005) for instance used the agar well diffusion method while Abdillahi *et al.* (2008) used the M27-P broth dilution described by Espinel-Ingroff and Pfaller (1995) in evaluating the antifungal activity of plant extracts. While the agar well diffusion method is marked with limitations, especially when it comes to diffusion of extracts into the agar, MIC values obtained using the M27-P broth dilution method were found to be less senstive when compared with the serial microdilution method described by Eloff (1998b) in that study. On the other hand, Motsei *et al.* (2003) also used a serial microplate dilution assay in determining the antifungal activity of some South African medicinal plants against selected *C. albicans* isolates. In that study, an ELISA reader was used to measure the turbidity of organism exposed to plant extracts. Earlier reports by our group indicated several complications associated with measurement of growth using turbidity (Eloff, 1998b). In this study, we therefore used the serial microdilution method (Eloff, 1998b) as modified by Masoko *et al.* (2005) to evaluate the different extracts for antifungal activity since this method has been shown to be more reliable (Masoko *et al*., 2008).

In this study, MIC values were recorded following 24 and 48 hours of incubation. However, no differences in MIC values were observed with time of incubation and as such, only results for 24 hours are presented (Table 7.2). Since no validated criteria exist for MIC end points for *in vitro* testing of plant extracts, the proposed classification of Aligiannis *et al*., (2001) in this study was considered to be too high and as such, plant extracts with MIC values of < 0.1 mg/mℓ are considered as potential candidates for consideration. Minimum inhibitory concentration < 0.1 mg/mℓ in this study are considered to be of moderate antifungal activity, and < 0.08 mg/mℓ and below as excellent activity.

At 24 and 48 hours of incubation, MIC values of the different plant species ranged between 0.16 and 0.04 mg/mℓ. The acetone extract of the different plant species had the highest antifungal activity followed by the methanol and DCM, and hexane the lowest. Some extracts exhibited antifungal activity against one or more pathogens, which was

not observed in the other extracts. This could be seen with the acetone and methanol extracts of *S. alata* and *C. edulis* that was not the case with the DCM and hexane extracts (Table 7.2), indicating the presence of active constituents in some extracts depending on the type of solvent used for extraction.

The lowest MIC value indicating high activity for acetone extracts was obtained for *A. senegalensis* against *C. neoformans* at a concentration of 0.04 mg/mℓ (Table 7.2). Excellent activity with MIC = 0.08 mg/mℓ was also obtained with these extracts for *A. schimperi* and *P. henkelii* against *C. neoformans* and *C. albicans* respectively (Table 7.2). Although different assay methods were used, the non-activity observed with *A. schimperi* against *C. albicans* in this study, is consistent with previous reports (Tadeg *et al*., 2005) where the hydroalcoholic extract of this plant was inactive against the said pathogen. The finding further confirms the presence of consituents in *P.henkelii* active against *C. albicans* as reported by Abdillahi *et al*. (2008). Moderate antifungal activity (MIC = 0.16 mg/mℓ) was also observed with the acetone extracts of *C.edulis* and *P. zeylanica* against *C. neoformans, A. senegalensis* and *P. zeylanica* against *A. fumigatus* and *S. alata* against *C. albicans*.

Antifungal activities exhibited by the methanol extracts of the different plant species were most notable against *C. neoformans* in those plants that showed activity. *A. schimperi* and *A. senegalensis* exhibited excellent antifungal activity against *C. neoformans* with MIC = 0.08 mg/mℓ while moderate activity (MIC = 0.16 mg/mℓ) was obtained for *C. edulis* and *P. henkelii* against the same pathogen (Table 7.2). Of all the methanol extracts of the different plant species, only that of *S.alata* exhibited moderate activity against *C. albicans* while none showed activity against *A. fumigatus* (Table 7.2).

With DCM extracts, moderate to excellent activity was obtained for *A. senegalensis* and *P. zeylanica* against *C. neoformans* and *A. fumigatus* with MIC of 0.16 mg/mℓ and 0.08 mg/mℓ respectively, while *A. schimperi* exhibited excellent activity against *C. neoformans.* As observed with the methanol extracts of the different plant species against *A. fumigatus*, none of the DCM extracts of these plants exhibited activity against *C. albicans* (Table 7.2). Extracts prepared using hexane of the different plant species were less active against the different pathogens with moderate to excellent activity of MIC = 0.16 mg/mℓ and 0.04 mg/mℓ against *C.neoformans* obtained for *A. schimperi* and *A. senegalensis* respectively.

The total activity value, which was calculated by dividing extract quantity in mg from 1g crude material of the different extracts by the MIC are presented in Table 7.3. This value represents the volume at which active constituents present in 1g of crude plant extract can be diluted and still be potent enough to kill the pathogen. Despite the poor MIC values obtained with some extracts of the different plants, good total activity values were observed in some extracts as could be seen with the methanol extracts against *C. albicans* (Table 7.3). The highest total activity value was obtained for the methanol extract of *C. edulis*, acetone extract of *A. schimperi*, methanol extract of *P. henkelii* and DCM extract of *A. schimperi* against *C. neoformans* in that order. The total activity, on the other hand, of extracts against *C. albicans* were highest for the acetone extracts of *P. henkelii*, methanol extract of *S. alata* and the methanol extract of *P. henkelii*, while for *A. fumigatus* the DCM extract of *A. senegalensis* and the methanol extract of *A. schimperi* were most active in that sequence.

Table 7.3. Total activity values of different extracts of plants on pathogenic fungi after 24 hours of incubation

	Hexane			Dichloromethane			Acetone			Methanol		
Plant	C.n	C.a	A.f	C.n	C.a	A.f	C.n	C.a	A.f	C.n	C.a	A.f
Acokanthera schimperi	187.5	12	93.75	656.25	164.06	82.03	1312.5	328.13	84	3125	581.4	390.63
Carissa edulis	180	90	351.6	132.8	17	66.41	281.3	20	78.13	1640.6	105	210
Annona senegalensis	312.5	5	19.53	203.13	101.56	406.3	500	31.25	125	937.5	234.38	234.38
Podocarpus henkelii	54.69	14	54.69	46.88	93.75	46.88	203.13	812.5	101.6	1093.8	546.87	273.4
Schrebera alata	11.72	3	6	78.13	78.13	39.6	50.78	203.13	50.78	94	734.38	94
Ekebergia capensis	8	4	8	70.31	70.31	18	39.06	78.13	39.06	60	234.38	117.19
Plumbago zeylanica	6	6	3	78.13	39.06	156.3	46.88	23.44	66.88	351.56	351.56	351.56
Average	108.6	19.1429	76.65286	180.8	80.55286	116.503	347.66	213.797	77.9214	1043.21	398.281	238.7371

C.n = *Cryptococcus neoformans,* C.a = *Candida albicans*, A.f = *Aspergillus fumigatus*

Table 7.4. Selectivity index values using Vero cell toxicity of extracts

	Solvents															
	Hexane				Dichloromethane				Acetone				Methanol			
Plants	CC_{50}	C.n	C.a	A.f	CC_{50}	C.n	C.a	A.f	CC_{50}	C.n	C.a	A.f	CC_{50}	C.n	C.a	A.f
Acocanthera schimperi	30	0.02	0.02	0.01	0.4	0.00	0.00	0.01	<1	0.00	0.00	0.00	<1	0.00	0.00	0.00
Carissa edulis	75	0.12	0.03	0.06	0.4	0.00	0.00	0.00	>1000	24.23	96.9	24.2	10	0.01	0.06	0.01
Annona senegalensis	15	0.01	0.01	0.01	27	0.08	0.08	0.02	8	0.01	0.03	0.01	<1	0.00	0.00	0.00
Podocarpus henkelii	56	0.09	0.02	0.09	42	0.07	0.13	0.07	45	0.14	0.56	0.07	42	0.26	0.13	0.13
Schrebera alata	22	0.14	0.01	0.07	32	0.40	0.10	0.05	30	0.38	0.09	0.02	25	0.31	0.06	0.04
Ekebergia capensis	42	0.03	0.02	0.07	27	0.08	0.01	0.04	30	0.19	0.01	0.05	670	4.19	0.27	0.54
Plumbago zeylanica	36	0.90	0.01	0.06	42	0.26	0.13	0.53	32	0.80	0.05	0.20	>1000	17.88	4.47	4.47

C.n = *Cryptococcus neoformans*, C.a = *Candida albicans*, A.f = *Aspergillus fumigatus*

Despite less potent activity observed when evaluating MIC values, the SI value against test pathogens was highest for the acetone extract of *C. edulis* against *C. albicans* with SI value of 96.9, followed by those obtained for *C. neoformans* and *A. fumigatus* respectively (Table 7.4). With *P. zeylanica,* the methanol extract against *C.neoformans* had SI = 17.88 and 4.47 against *C. albicans* and *A. fumigatus.* On the other hand, only the methanol extract of *E. capensis* against *C. neoformans* showed a good SI value of 4.19. The high SI values obtained for some extracts of these plants indicate that these extracts are relatively less toxic with potent inhibitory activity on these pathogens.

Overall, going by MIC values, acetone extracts of these plant species comparatively had the best activity with more than one plant species having activity against all the pathogens used in the study followed by the methanol. With DCM extracts, activity was only observed against *C.albicans* while with the hexane extracts of the different plants, no activity was observed against any of the pathogens. The activity of the acetone extract is consistent with previous report (Eloff, 1999) were acetone is considered as the best extract for antimicrobial activity. Similarly, the acetone extracts had the best selectivity index value, followed by methanol. The susceptibility of pathogens was generally highest with methanol and acetone extracts of plants against *C.neoformans* respectively. *A.fumigatus* was the most non-susceptible pathogen against hexane, DCM and methonal extracts of the different plant species.

7.4. Conclusion

Plants are a relatively cheap source of biological material, consisting of a vast mixture of metabolites, primary or secondary, available for selecting molecules of desired biological activity. Hence in this study, solvents of varying polarity were used to extract selected plant species and each extract of the same plant was tested against three fungal pathogens. The antifungal activity of extracts of the different plant species varied with the type of organism. The acetone extracts of the different plant species exhibited the highest antifungal activity followed by the methanol and DCM extracts, and the hexane extracts were the least active. Except for *Ekebergia capensis*, extracts exhibited antifungal activity against one or more pathogens. *C. neoformans* was the most susceptible pathogen, followed by *A. fumigatus.* Despite the poor MIC values obtained with some extracts of the different plants, good total activity values were observed in some extracts suggesting the relevance of concentration of toxic principles present in an extract. It is also very important to determine selectivity index values so as to be able to differentiate between selective activity and activity due to toxic effects of the extracts. Thus, in the selection of plants for isolation of active compounds, the selectivity index value should also be considered.