African Journal of Biotechnology Vol. 4 (12), pp. 1425-1431, December 2005
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2005 Academic Journals

Full Length Research Paper

The diversity of antifungal compounds of six South African *Terminalia* species (Combretaceae) determined by bioautography

P. Masoko, J.N. Eloff*

Phytomedicine Programme, Department of Paraclinical Sciences, University of Pretoria, Private Bag X04, Onderstepoort, 0110, South Africa.

Accepted 31 October, 2005

A bioautography method was developed to determine the number of antifungal compounds in *Terminalia* species extracts. Acetone, hexane, dichloromethane and methanol leaf extracts of six *Terminalia* species (*T. prunioides*, *T. brachystemma*, *T. sericea*, *T. gazensis*, *T. mollis* and *T. sambesiaca*) were tested against five fungal animal pathogens (*Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Microsporum canis* and *Sporothrix schenkii*). The R_f values and relative activities of separated compounds were determined. Hexane and dichloromethane extracts had at least three times more antifungal compounds than the other extracts indicating the nonpolar character of the antifungal compounds. From the R_f values, the non-polar character of the antifungal compounds was confirmed indicating that the antifungal activity is not due to tannins. *M. canis* had the highest number, up to ten, of antifungal compounds. All *Terminalia* species contained a compound (R_f = 0.46 in benzene/ethanol/ammonium hydroxide (90/10/1) active against all tested pathogens. *T. sericea* and *T. brachystemma* were the most promising candidates for isolating antifungal compounds. The results demonstrate the value of bioautography in examining plant extracts with antifungal activity, selecting species for further study and dereplicating the isolation of compounds.

Key words: Combretaceae, *Terminalia* species, antifungal activity, bioautography, R_f value, dereplication.

INTRODUCTION

Opportunistic fungal infections represent a significant cause of morbidity and mortality in immunocompromised patients, including those with AIDS, cancer and organ transplants (Fisher-Hoch and Hutwagner, 1995). Despite the increase in fungal infections, therapeutic options are very limited and are often unsatisfactory because of elevated toxicity and an inability to eradicate infections (Sheehan et al., 1999). In the last few years the incidence of fungal infections in the immunocompromised host has increased greatly. The emergence of these pathogens has been followed by both primary drug resistance and the secondary development of azole-resistant isolates of *Candida albicans* and *Cryptococcus neoformans* (Poeta et al., 1998).

Amphotericin B (AMB) is considered the “gold standard” for treatment of these infections. AMB is however associated with a number of severe and sometimes life threatening side effects including fever, chills and nephrotoxicity. Other treatment regimens include azole antifungal drugs, of which fluconazole and itraconazole are the most widely used (Graybill, 1989). The emergence of resistance to FLC and lack of efficacy of fluconazole and the limited efficacy of traconazole against pulmonary aspergillosis have highlighted the need for a broad-spectrum antifungal agent (Salama et

*Corresponding author. E-mail: kobus.eloff@up.ac.za, Tel.: +27 12 529 8244. Fax: +27 12 529 8252.

Abbreviations: AMB, Amphotericin B; DCM, dichloromethane; EMW, Ethyl acetate/methanol/water (40/5.4/4); CEF, chloroform/ethyl acetate/formic acid (5/4/1); BEA, benzene/ethanol/ammonium hydroxide (90/10/1); INT, *p*-iodonitrotetrazolium violet; C.a, *Candida albicans;* C.n, *Cryptococcus neoformans*; M.c, *Microsporum canis*; S.s, *Sporothrix schenckii;* A.f, *Aspergillus fumigatus*.

al., 2001). It is clear that new antifungal agents with potent and broad spectrum fungicidal activities are needed for the effective management of these infections.

The search for new antifungal agents led us to the screening of antifungal compounds in *Terminalia* L. species (Combretaceae). The selection of this genus is based on uses in traditional medicine, since *Terminalia* species are widely used medicinal plants both in Africa and in Asia. *Terminalia sericea* has recently been selected as one of the 50 most important medicinal plants in Africa by the Association for African Medicinal Plant Standards [www.aamps.net]. The use of decoctions of several *Terminalia* species is wide-spread in Africa, and many species are reputed to contain antimicrobial constituents (Silva et al., 1996; Baba-Moussa et al., 1999). Based on preliminary phytochemical screening, Baba-Moussa et al. (1999) concluded that tannins were probably responsible for antifungal effect. Because tannins have low bioavailability, the potential value of tannins as a systemic antifungal compound is low. Some scientists have concluded that there is therefore not much scope for investigating the Combretaceae for antimicrobial compounds.

We could show that *Terminalia* species occurring in southern Africa have substantial antifungal activity. Most of the crude extracts had minimum inhibitory concentration (MIC) values of c. 0.08 mg/ml, and some had MIC's as low as 0.02 mg/ml (Masoko et al., 2005). *Microsporum canis* was the most susceptible microorganism and *T. sericea* extracts were the most active against nearly fungi tested.

In this contribution we improved a technique for bio-autography of fungal extracts. Hostettmann et al. (2000) stated that direct bioautography with *Candida albicans* is not possible and they developed an agar overlay technique. In our hands many difficulties were obtained with agar overlay techniques. We succeeded in developing a direct bioautography technique with several yeasts and molds. With this direct bioautography technique we could determine how many antifungal compounds are present in different extracts, to what degree the same compounds occur in different extracts and to what degree the same compounds inhibit different fungi. This information helps in deciding which species should be selected for further study. The results also made it possible to determine if tannins are responsible for antifungal activity as concluded by Baba-Moussa et al. (1999).

MATERIALS AND METHODS

Plant collection

Leaves were collected in summer of 2003, from trees in the Lowveld National Botanical Garden in Nelspruit, South Africa. Voucher specimens in the garden herbarium verified the identity of the plants. Leaves collected were from the following five species with the section of the genus (Carr, 1988) in brackets: *T. prunioides* M.A.Lawson (Abbreviatae), *T. brachystemma* Welw. ex Hiern (Psidioides), *T. sericea* Burch ex DC (Psidioides), *T. gazensis* Bak.f. (Platycarpae), *T. mollis* Laws. (Platycarpae) and *T. sambesiaca* Engl.& Diels.(Platycarpae).

Plant storage

Leaves were separated from stems, and dried at room temperature. Most scientists have tended to use dried material because there are fewer problems associated with large-scale extraction of dried plants rather than fresh plant material (Eloff, 1998a). The dried plants were milled to a fine powder in a Macsalab mill (Model 200 LAB), Eriez®, Bramley, and stored at room temperature in closed containers in the dark until used.

Extraction procedure

Dried plant leaves from each species were individually extracted by weighing samples of 1 g of finely ground plant material and extracting with 10 ml of acetone, hexane, dichloromethane (DCM) or methanol (technical grade-Merck) in polyester centrifuge tubes. Tubes were vigorously shaken for 3 to 5 min in a Labotec model 20.2 shaking machine at high speed. After centrifuging at 3500 rpm for 10 min the supernatant was decanted into pre-weighed labelled containers. The process was repeated 3 times to exhaustively extract the plant material and the extracts were combined. The solvent was removed under a stream of air in a fume cupboard at room temperature, to quantify the extraction efficiency.

Phytochemical analysis

Chemical constituents of the extracts were analyzed by thin layer chromatography (TLC) using aluminium-backed TLC plates (Merck, silica gel 60 F_{254}). The TLC plates were developed under saturated conditions with one of the three eluent systems developed in our laboratory that separate components of Combretaceae extracts well i.e.: ethyl acetate/methanol/water (40:5.4:5): [EMW] (polar/neutral); chloroform/ethyl acetate/for-mic acid (5:4:1): [CEF] (intermediate polarity/acidic); benzene/ethanol/ammonium hydroxide (90:10:1): [BEA] (non-polar/basic) (Kotze and Eloff, 2002).

Fungal test organisms

Five species of fungi were obtained from the Central Microbiology Laboratory, Faculty of Veterinary Science and used as test organisms. These fungi represent the different morphological forms of fungi, namely yeasts (*Candida albicans* and *Cryptococcus neoformans*), thermally dimorphic fungi (*Sporothrix schenckii*) and moulds (*Aspergillus fumigatus* and *Microsporum canis*) and are the most common and important disease-causing fungi of animals. *Candida albicans* was isolated from a Goldian finch, *C. neoformans* from a cheetah, and *Aspergillus fumigatus* from a chicken, all of which suffered from a systemic mycosis. *Microsporum canis* was isolated from a cat with dermatophytosis and *S. schenckii* from a horse with cutaneous lymphangitis. Not one of the animals had been treated prior to sampling. All fungal strains were maintained on Sabouraud dextrose agar (Oxoid, Basingstoke, UK).

Bioautographic methods

TLC plates (10 x 10 cm) were loaded with 100 µg (5 µl of 20 mg/ml) of each of the extracts. The prepared plates were developed in the three different mobile systems used: CEF, BEA and EMW. The

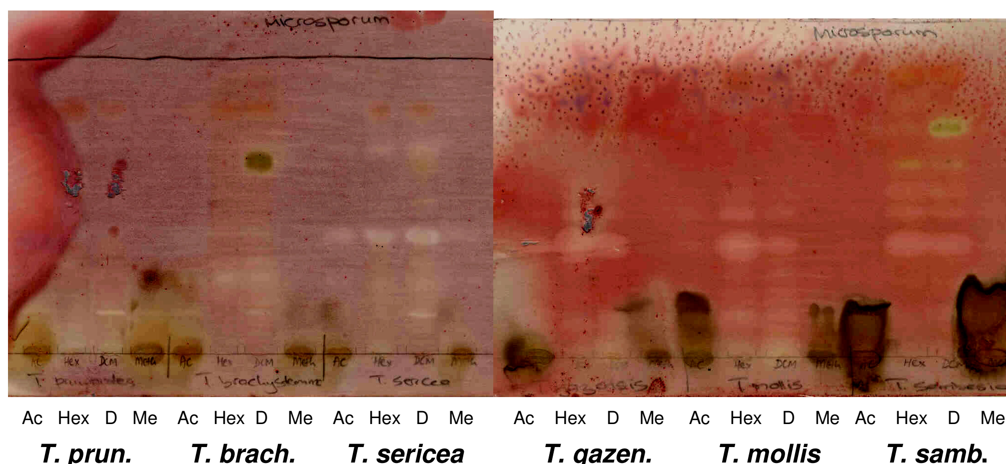

Figure 1. Bioautography of *Terminalia* species extracted with acetone (Ac), hexane (Hex) and dichloromethane (D) in lanes from left to right for each group, separated by BEA and sprayed with *M. canis*. White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of *Microsporum canis*. Methanol (Me) extracts yielded no inhibition zones.

chromatograms were dried for up to a week at room temperature under a stream of air to remove the remaining solvent.

Cultures were grown on Sabouraud agar for 3 to 5 days. Sabouraud broth was prepared in 250 ml bottles. Cultures were transferred into broth from agar with sterile swab. The TLC plates developed were inoculated with a fine spray of the concentrated suspension containing approximately 10^9 organisms per ml of actively growing fungi e.g. conidia for filamentous fungi and yeast cells for the other fungi in a Biosafety Class II cabinet (Labotec, SA) cupboard. The plates were sprayed until they were just wet, incubated overnight and then sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet (Sigma®) (INT) and further incubated overnight or longer in the case of *S. schenckii* and *M. canis* at 35°C in a clean chamber at 100% relative humidity in the dark. White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of tested fungi. We tried to use digital photography to record results and it did not work well, therefore we decided to use a scanner. To minimize fungal spreading and infections in our laboratory, the bioautograms were sealed in the clear plastic envelopes before scanning in for a permanent record.

RESULTS AND DISCUSSION

Method development

During this study we experienced a number of difficulties. Firstly we found that preparing cultures some days before spraying them, makes it difficult to get good results, possibly due to quick mycelial overgrowth and blockage of the spray gun with mycelia. We then prepared cultures on agar and after strong growth mainly conidia were collected by light swabbing and transferred to sterile fresh broth. After broth incubation for an hour spraying with this culture led to good results. This procedure led to lower overgrowth of the mycelia.

In the bioautography process with bacteria, chromatograms covered with bacteria are incubated and then sprayed with INT. Because the fungi grow slower and at different rates there is a danger of overgrowth of mycelia and disappearance of zones of inhibition. We sprayed the bioautograms with INT three to four hours after spraying with tested microorganisms. This makes it easier to see positive results immediately they appear. *A. fumigatus* was inhibited by some extracts, but if we did not check results early during incubation, we found that there was growth of mycelia on top of active band, which made it difficult to see inhibition.

There are number of bioautography techniques described for fungi. Homma et al. (1989) poured molten agar seeded with fungal mycelium or conidia onto the surfaces of TLC plates, Motsei et al. (2003) dipped developed TLC plates in a tank containing a fungal culture and placed it on damp paper towels in a metal tray. The trays were covered with cling wrap, and incubated in an oven at 33°C overnight or until growth was observed. Both these methods are prone to difficulties with moulds. In our procedure, a fungal inoculum was applied by spraying a suspension of mainly conidia in Sabouraud dextrose broth, and growth was indicated by spraying with INT. This procedure worked well with both yeast and moulds.

Bioautography results

Bioautography was used to screen for antifungal compounds to obtain more information on the diversity of antifungal compounds present in different extracts. Inhibition zones of antifungals are observed as white spots on a purple-red background (Figure 1). White areas indicate where reduction of INT to the coloured

Table 1. The inhibition of fungal growth by bioautography of extracts of different *Terminalia* species separated by TLC with BEA as eluent. R_f values of active components and relative degree of inhibition shown.

	BEA/Acetone					**BEA/Hexane**					**BEA/Dichloromethane**				
R_f values	**C.a**	**C.n**	**M.c**	**S.s**	**A.f**	**C.a**	**C.n**	**M.c**	**S.s**	**A.f**	**C.a**	**C.n**	**M.c**	**S.s**	**A.f**
T. prunioides															
0.46						XX					XX				
0.26						XX					XX	X			
0.16				XX			XX						XXX	XXX	
T. brachystemma															
0.79									XXX					XXX	
0.73							XX	XX				XX	XXX		XXX
0.46						XXX	XXX	XXX	XXX		XX	XX	XXX	XXX	
0.28									XXX					XXX	
0.26						XX	XX	XX			XX	XX	XX		
0.12			XX										XXX		
T. sericea															
0.79									XXX					XXX	
0.73		XX				XX	X	XX		XX	X	X	XXX		X
0.53								XXX					XXX		
0.46						XXX	XX		XXX	XX	XXX	XX		XXX	XX
0.28								XX	XXX				XX	XXX	
0.26						XX	X			XX	XX	X			
0.12			XX					XX	XXX				XX	XXX	
T. gazensis															
0.36			XXXX				XX	XXXX				X	XXX		
0.03			XX				XX	XXXX					XXX		
T. mollis															
0.44			XXX					XXXX		XX			XX		XX
0.36			XXX				X	XXXX		XX		X	XXXX		XX
0.03								XX					XX		
T. sambesiaca															
0.44			XXX					XXXX		X			XX		X
0.36			XXX					XXXX		X			XXX		X
0.03								XX					XX		

C.a = *C. albicans*, C.n = *C. neoformans*, M.c = *M. canis*, S.s = *S. schenckii*, A.f = *A. fumigatus*.
Relative degree of inhibition: X = slight inhibition, XXXX = very high inhibition.
BEA = benzene:ethyl acetate:formic acid.

formazan did not take place due to the presence of compounds that inhibited the growth of tested fungi. A representative bioautograms also indicates the scoring used to determine relative activity of fungal compounds (Figure 1).

In some cases organisms did not grow too well and it was difficult to detect inhibition zones. These cases were not included in this report because bioautograms with no fungal growth possibly do not present a true picture. The most likely explanation is that there were still traces of formic acid left on the chromatogram that inhibited the fungal growth.

In other cases there were growth, but no inhibition was observed even though MIC values indicated antifungal activity (Masoko at al., 2005). The non-activity of these extracts in bioautography could be explained by evaporation of active compounds during removal of the TLC eluents or by the disruption of synergism between active constituents caused by TLC.

Three separation systems were used, but only results of BEA and CEF were given, because antifungal compounds were relatively non-polar and therefore did not separate well in the polar eluent EMW. In all cases R_f values of active compound were 0.94 – 0.96 (EMW). With the exception of two methanol extracts from *T. prunioides* and two from *T. brachystemma*, no antifungal compounds could be observed in the EMW system, confirming the relative non-polarity of the antifungal compounds. These results confirm the low activity found when MIC values were determined for *Terminalia* methanol extracts (Masoko et al., 2005).

Acetone extracts of six *Terminalia* species separated with BEA (Table 1) possessed few compounds with high activity against tested pathogens. *T. gazensis, T. mollis*

Table 2. The inhibition of fungal growth by bioautography of extracts of different *Terminalia* species separated by TLC with CEF as eluent. R_f values of active components and relative degree of inhibition shown.

R_f values	CEF/Acetone					CEF/Hexane					CEF/Dichloromethane				
	C.a	C.n	M.c	S.s	A.f	C.a	C.n	M.c	S.s	A.f	C.a	C.n	M.c	S.s	A.f
T. prunioides															
0.97	XXXX									X					X
0.57						XX		XX		X	XX		XX		XX
0.55				XX			XX	XX	XX		XX	XX	X	XX	
0.50													XX		
0.24												XX			
T. brachystemma															
0.95						XXXX			XXX	X	XXX			XXX	X
0.88						XXXX			XXXX	XX	XXXX			XXXX	
0.86	X					X					X				
0.81								XXX	XXX				XX	XXX	
0.78	X					X					X				
0.55									XX	X				XX	XX
T. sericea															
0.95						XXX		XXX	XXX	X			XXX	XX	X
0.88	X	X				X	X	XXX	XX	XX	X	XX	XXX	XX	XX
0.78	X					X		XXX			X		XXX		
0.57								XX		X			XX		X
0.55							X		X			XX		XX	
T. gazensis															
0.95			XXXX							XX					XX
0.9			XXX					XXXX	XX				XXXX	XX	
0.86			XX				XXX	XXX				XX	XXX		
0.75			XX					XXX		XX			XXX		X
0.53							XX	XXX				XX	XXX		
T. mollis															
0.95			XX					XXXX	XX	X			XXXX	XX	X
0.9								XXXX	XX				XXXX	XX	
0.86			XX				XXX	XXXX				XX	XXXX		
0.75			XXX					XXX		XX			XXXX		X
T. sambesiaca															
0.97						XX					X		XXXX		
0.95			XXX					XXXX	XXX	XX			XXXX	X	X
0.9			XXX					XXXX					XXXX		
0.86			XXX				XXX	XXXX				XXX	XXXX		
0.75			XXX					XXXX					XXXX		

C.a = *C. albicans*, C.n = *C. neoformans*, M.c = *M. canis*, S.s = *S. schenckii*, A.f = *A. fumigatus*.
Relative degree of inhibition: X = slight inhibition, XXXX = very high inhibition.
CEF = chloroform:ethyl acetate:formic acid.

and *T. sambesiaca* extracts had two very active compounds against *M. canis*. In all cases the three extracts had active compounds with R_f values of 0.36 and 0.44. Hexane and dichloromethane (DCM) extracts of *T. brachystemma* and *T. sericea* had a number of antifungal compounds that were very active against all tested pathogens. *T. brachystemma* hexane extract, however did not show activity against *A. fumigatus*. *T. prunioides* hexane extract displayed activity against *C. albicans* and *C. neoformans*. DCM extract did not show activity against *A. fumigatus*. Hexane and DCM extracts of *T. gazensis, T. mollis* and *T. sambesiaca* had high activity against *M. canis* and fewer active compounds against *A. fumigatus.* In the case of *T. gazensis* extracts there were no active compounds against *A. fumigatus*.

Acetone extracts of *Terminalia* species were also separated with CEF (Table 2) and they shared few active compounds. However *T. gazensis, T. mollis* and *T. sambesiaca* were very active against *M. canis* at R_f values of 0.75, 0.86, 0.90 and 0.95. Hexane and DCM extracts displayed number of compound, which were very active against *M. canis*. Hexane and DCM extracts sepa-

Table 3. Number of antifungal bands at different R_f values in all six *Terminalia* species separated by BEA.

Rf values	BEA/Acetone					BEA/Hexane					BEA/Dichloromethane					Total
	C.a	**C.n**	**M.c**	**S.s**	**A.f**	**C.a**	**C.n**	**M.c**	**S.s**	**A.f**	**C.a**	**C.n**	**M.c**	**S.s**	**A.f**	
0.79									2					2		**4**
0.73		1				1	2	2		1	1	2	2		2	**14**
0.53								1					1			**2**
0.46						3	2	1	2	1	3	2	1	2	1	**18**
0.44			2					2		2			2		2	**10**
0.36			3				2	3		2		2	3		2	**17**
0.28								1	2				1	2		**6**
0.26						3	2	1		1	3	3	1		1	**15**
0.16				1			1						1	1		**4**
0.12			2					1	1				2	1		**7**
0.03			1				1	3					3			**8**
Total		**1**	**8**	**1**	**0**	**7**	**10**	**15**	**7**	**7**	**7**	**9**	**17**	**8**	**8**	
Grand total			**10**					**46**					**49**			

Table 4. Number of antifungal bands at different R_f values in all six *Terminalia* species separated by CEF.

Rf values	CEF/Acetone					CEF/Hexane					CEF/Dichloromethane					Total
	C.a	**C.n**	**M.c**	**S.s**	**A.f**	**C.a**	**C.n**	**M.c**	**S.s**	**A.f**	**C.a**	**C.n**	**M.c**	**S.s**	**A.f**	
0.97	1					2				2	1		1		2	**9**
0.95			3					3	4	4	1		3	3	4	**25**
0.9			2					3	2				3	2		**12**
0.88	1	1				2	1	1	2	2	2	1	1	2	1	**17**
0.86	1		3			1	3	3			1	3	3			**18**
0.81									1	1			1	1		**4**
0.78	2					2		1			2		1			**8**
0.75			3					3		2			3		2	**13**
0.57						1		2		2	1		2		3	**11**
0.55				1			1	1	3	1	1	1	1	2		**12**
0.53							2	1				2	1			**6**
0.50													1			**1**
0.24												1				**1**
Total	**5**	**1**	**11**	**1**	**0**	**8**	**7**	**18**	**12**	**14**	**9**	**8**	**21**	**10**	**12**	
Grand total			**18**					**59**					**50**			

rated with CEF showed similar compounds in all *Terminalia* species tested. BEA separation (Table 3) shows that hexane extracts have 46 active compounds and DCM have 49, a difference of 3, and in CEF (Table 4) hexane extracts have 59 and DCM 60, a difference of 1. From this it appears that either hexane or DCM can be used for extraction of antifungal compounds in *Terminalia* species.

A compound with an R_f value of 0.46 from different *Terminalia* extracts separated with BEA (Table 3) inhibit fungal growth of all tested pathogens, and compounds with R_f values of 0.88 and 0.55 separated with CEF (Table 4) inhibit fungal growth of all pathogens. Antifungal compounds with R_f values of 0.86, 0.88 and 0.95 in CEF separation were present in 18, 17 and 25 cases, respectively. In BEA, compounds with R_f values of 0.46 and 0.36 were present 8 and 17 cases, respectively.

T. sericea holds promise for isolating antifungal compounds, because hexane and DCM extracts of *T. sericea* have compounds inhibiting growth of all

pathogens, especially the compound at R_f value of 0.46. It is followed by *T. brachystemma*, which also had compounds (R_f = 0.46), which inhibited growth of all tested pathogens.

This study showed that there are a number of active compounds against fungi present in *Terminalia* species. Activity may not only be attributable to tannins found in *Terminalia,* as was previously postulated (Baba-Moussa et al., 1999). The results obtained here are in line with the low MIC values obtained in different extracts in previous work (Masoko et al., 2005).

It appears that bioautography is an important detection method for a new or unidentified antifungal compounds, because it is based on the biological effects of the substances under study. Bioautography in different solvent systems makes it possible to dereplicate the isolation of compounds previously isolated from related taxa.

CONCLUSION

The isolation of antifungal compounds from plant extracts appears to be an important research activity (Hostettman et al., 2000). The low number of papers that have appeared on screening and isolation of antifungal compounds compared to work on antibacterial compounds from plants may be due to difficulties in antifungal assay techniques. These results may contribute to a resolution of these difficulties.

ACKNOWLEDGEMENT

The National Research Foundation (NRF) and the Research Committee, Faculty of Veterinary Science, University of Pretoria provided financial assistance.

REFERENCES

Baba-Moussa F, Akpagana K, Bouchet P (1999). Antifungal activities of seven West African Combretaceae used in traditional medicine. J. Ethnopharmacol. 66: 335-338.

Carr JD (1988). Combretaceae in southern Africa. Tree Society of southern Africa, Johannesburg.

Eloff JN (1998a). Conservation of Medicinal Plants: Selecting Medicinal Plants for research and gene banking. Monographs in systematic Botany from the Missouri Garden. 71: 209-222 In : Conservation of plants Genes III: Conservation and utilisation of African plants. Robert P. Adams and Janice E. Adams, eds., Missouri Botanical Garden Press, St.Louis, USA.

Fisher-Hoch SP, Hutwagner L (1995). Opportunistic candidiasis: an epidemic of the 1980's. Clin. Infect. Dis. 21: 897-904.

Graybill JR (1989). New antifungal agents. Eur. J. Clin. Microbiol. Infect. Dis. 5: 402-412.

Homma Y, Sato Z., Hirayama F, Konno K, Shirahama H, Suzui T (1989). Production of antibiotics by *Pseudomonas cepacia* as an agent for biological control of soilborne plant pathogens. Soil Biol. Biochem. 21: 723–728.

Hostettmann K, Marston A, Ndjoko K, Wolfender JL. (2000). The potential of African plants as a source of drugs. Curr. Org. Chem. 4: 973-1010.

Kotze M, Eloff JN (2002). Extraction of antibacterial compounds from *Combretum microphyllum* (Combretaceae). S. Afr. J. Bot. 68: 62-67.

Masoko P, Picard J, Eloff JN (2005). Antifungal activities of six South African *Terminalia* species (Combretaceae). J. Ethnopharmacol. 99: 301-308.

Motsei ML, Lindsey KL, van Staden J, Jäger AK (2003). Screening of traditionally used South African plants for antifungal activity against Candida albicans. J.Ethnopharmacol. 86: 235-241.

Poeta MD, Schell WA, Dykstra CC, Jones S, Tidwell RR, Czarny A., Bajic M, Perfect JR (1998). Structure *in vitro* activity relationships of pentamidine analogues and dication-subtituted Bis-benzimidazoles as new antifungal agents. Antimicrobial agents and chemotherapy 42, 2495-2502.

Salama SM, Atwal H, Gandhi A, Simon J, Poglod M, Montaseri H, Khan JK, Furukawa T, Saito H , Micetich RG (2001). *In vitro* and *In vivo* activities of Syn2836, Syn2869, Syn2903, and Syn2921: New series of triazole antifungal agents, Antimicrobial Agents and Chemotherapy 45: 2420-2426.

Sheehan DJ,Hoelscher AC,Sibley MC (1999). Current and emerging azole antifungal agents. Clin. Microbiol. Rev. 12: 40-79.

Silva O, Duarte A, Cabrita J, Pimentel M, Diniz A, Gomes E (1996).Antimicrobial activity of Guinea-Bissau traditional remedies. J. Ethnopharmacol. 50: 55-59.

Silva O, Duarte A, Cabrita J, Pimentel M, Diniz A, Gomes E (1996). Antimicrobial activity of Guinea-Bissau traditional remedies. J. Ethnopharmacol. 50: 55-59.