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The antifungal activity of twenty-four southern African *Combretum* species (Combretaceae)

P. Masoko [a], J. Picard [b], J.N. Eloff [a,*]

[a] *Phytomedicine Programme, Department of Paraclinical Sciences, South Africa*

[b] *Department of Veterinary Tropical Diseases, Faculty of Veterinary Sciences, University of Pretoria, Private Bag X04, Onderstepoort 0110, South Africa*

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Abstract

The antifungal activities of acetone, hexane, dichloromethane and methanol leaf extracts of 24 South African *Combretum* species were determined against five fungal animal pathogens (*Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Microsporum canis* and *Sporothrix schenckii*) representing yeasts, moulds and dimorphic fungi. MIC's determined after 48 h were usually two times higher than values determined after 24 h. Most of the antifungal extracts had MIC values of c. 0.08 mg/ml, some with MIC values as low as 0.02 mg/ml. These are substantially better values that reported in the literature to date. *M. canis* was the most susceptible microorganism followed by *S. schenckii*. *A. fumigatus* was the most resistant of the pathogens tested. Methanol extracted the highest quantity from leaves, but the acetone extracts had the highest antifungal activity in practically all cases. The methanolic extracts of *C. moggii* and *C. petrophilum* were however most active against all the pathogens. All extracts of *C. nelsonii* were also very effective against all the pathogens. Based on these results and work done earlier, *C. nelsonii* was selected for fractionation and bioassay-guided isolation of the antifungal compounds followed by *C. albopuntactum*, and *C. imberbe*.

Keywords: Combretaceae; *Combretum* species; Antifungal activity; MIC

1. Introduction

During the past decade there has been an increase in the number of patients with weakened immune status associated with human immunodeficiency virus (HIV). This has been associated with an increase in the incidence of human systemic mycoses. Even in a rich country such as the USA the number of deaths due to mycoses increased from 1557 in 1980 to 6534 in 1997 (McNeil et al., 2001; White et al., 1998). In spite of their expense, Amphotericin B and the azole group of antifungal agents are extensively used in the treatment of fungal infections. Unfortunately, the widespread and incorrect use of these antifungals has led to the emergence of drug resistance in several common pathogenic fungi (Graybill, 1996). Due to this emergence of antibiotic resistant human pathogenic fungi, it is important to develop new antifungal agents. The field of ethnobotanical research has expanded greatly in recent years. Plants may yield valuable antimicrobials.

In South Africa plants are widely used by all sections of the population either directly as folk remedies or in different indigenous systems of medicine or indirectly in the pharmaceutical preparations of modern medicines. Plants used in traditional medicine may constitute an important source of new biologically active compounds.

Traditional healers throughout Africa use species of the Combretaceae for many medicinal purposes. This includes treating fever, headaches, abdominal disorders, abdominal pains, gallstones, diarrhoea, dysentery, gastric ulcers, bilharziasis, hookworm, nosebleeds, sore throats, colds, chest coughs, pneumonia, conjunctivitis, dysmenorrhoea, infertility in women, venereal diseases including syphilis, earache, fattening babies, leprosy, scorpion and snake bites, swelling caused by

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.

* Corresponding author.

E-mail address: kobus.eloff@up.ac.za (J.N. Eloff).

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mumps, toothache, heart diseases, cleanse the urinary system, backache, jaundice, stomach and gastric problems, blennorrhagia, constipation and general weakness (Hutchings et al., 1996; Neuwinger, 1996; Iwu, 1993; Bever, 1986). Some of these uses may be attributed to antifungal activity of extracts.

The Combretaceae consists of 18 genera, the largest of which are *Combretum*, with about 370 species, and *Terminalia*, with about 200 species (Lawrence, 1951). Species from the genus *Combretum* and to a lesser extent *Terminalia* are most widely used for medicinal purposes. As they are common and widely distributed throughout western and southern Africa, (Rogers and Verotta, 1996), they are readily available for use. The leaves and bark of the *Combretum* species are predominantly used.

In Alexandra et al., 1992 found several antimicrobial compounds in 12 different *Combretum* species. Martini and Eloff (1998) found evidence for at least 14 unidentified bacterial inhibitors from the leaves of *Combretum erythrophyllum*. Eloff (1999) quantified the antibacterial activities of the leaf extracts of 27 members of Combretaceae, and in 2002 Fyhrquist et al. found activity in extracts of the roots and stembark of *Combretum* and *Terminalia* species used in Tanzania. The antibacterial properties of *Combretum* species (Silva et al., 1996; Baba-Moussa et al., 1999) have been well investigated, this is not the case regarding their antifungal properties (Bhatt and Saxena, 1979; Baba-Moussa et al., 1999). Our aim in this work is to address this gap by investigating the antifungal activities of 24 *Combretum* species occurring in southern Africa. We have shown that extracts of South African *Terminalia* species (another member of the Combretaceae) have substantial antifungal activities, with MIC's as low as 20 μg/ml (Masoko et al., 2005).

Resistance to azole compounds, especially among *Candida* species, has been well investigated over the past few years. As a consequence of the AIDS epidemic, during the past decade there was a striking increase in mucosal infections caused by *Candida* species that was associated with a worrying emergence of resistance to azoles. Primary resistance to amphotericin B has emerged in parallel with the increase in the number of invasive infections due to the so-called emerging fungi. Usually included in these emerging fungi are yeasts such as *Trichosporon beigelii*, *C. lusitaniae* or *C. guillermondii*. Many of these fungi show primary or intrinsic resistance to amphotericin B, and may cause invasive infections, usually associated with a high mortality (Tritz and Woods, 1993). Although *C. glabrata* and *C. krussei* are usually considered to be susceptible to amphotericin B, they tend to have higher MIC's to polyenes than *C. albicans,* and a growing body of data suggests that a significant proportion of isolates of both species can be resistant to amphotericin B (Rex et al., 2000).

There is still a high mortality associated with some invasive fungal infections, especially those produced by filamentous fungi. Most antifungal agents are expensive and have serious side effects. Other sources of antifungal agents should also be investigated. Hostettmann et al. (2000) stressed the importance of investigating plants for new antifungal agents. The aim of this report is to investigate the antifungal activity of different leaf extracts of 24 *Combretum* species in order to determine which species have good potential as antifungal agents.

2. Materials and methods

2.1. Plant collection

Leaves were collected, in Summer, from plants in the Lowveld National Botanical Garden in Nelspruit in 2003. Voucher specimens and origins of the trees are kept in garden herbarium. Plants used are listed in Table 1 below. More information on the origin and references of these plants are presented elsewhere (Eloff, 1999).

2.2. Plant drying and 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.

2.3. Extraction procedure

Plant samples from each species were individually extracted by weighing four aliquots of 1 g of finely ground plant material and extracting with 10 ml of acetone, hexane, dichloromethane

Table 1
Combretum species used for antifungal screening

Combretum L	
Section	Species
Hypocrateropsis Engl. & Diels	*C. celastroides* Welw. Ex Laws
	(i) *C. celastroides* ssp. *celastroides*
	(ii) *C. celastroides* ssp. *orientale*
	C. imberbe Wawra
	C. padoides Eng. & Diels
Angustimarginata Engl. & Diels	*C. caffrum* (Eckl. & Zeyh) Kuntze
	C. erythrophyllum (Burch.) Sond.
	C. kraussii Hochst
	C. woodii Duemmer
	C. nelsonii Duemmer
Metallicum Excell & Stace	*C. collinum* Fresen
	(i) *C. collinum* ssp. *suluense*
	(ii) *C. collinum* ssp. *taborense*
Spathulipetala Engl. & Diels	*C. zeyheri* Sond.
Ciliatipetala Engl. & Diels	*C. albopunctactum* Suesseng.
	C. apiculatum Sond.
	(i) *C. apiculatum* ssp. *apiculatum*
	C. edwardsii Exell
	C. moggii Excell
	C. molle R. Br.
	C. petrophilum Retief
Breviramea Engl. & Diels	*C. hereroense* Schinz
Conniventia Engl. & Diels	*C. microphyllum* Klotzsch
	C. paniculatum Vent.
Poivrea (Comm. Ex DC)	*C. bracteosum* (Hochst)
	C. mossambicense (Klotzsch)
	C. acutifolium

Infra-generic classification from Carr (1988).

(DCM) or methanol (technical grade-Merck) in centrifuge tubes. These tubes were vigorously shaken for 3–5 min in a Labotec model 20.2 shaking machine at high speed. After centrifugation at 3500 rpm for 10 min the supernatant was decanted into labelled containers. This 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 before dissolving extracts in acetone to a concentration of 10 mg/ml, to quantify the assay. Preliminary experiments have shown that acetone diluted according the MIC bioassay procedure does not inhibit the growth of any of the fungi tested (Manuscript by Eloff and Masoko in preparation).

2.4. 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 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/formic acid (5:4:1): [CEF] (intermediate polarity/acidic); Benzene/ethanol/ammonium hydroxide (90:10:1): [BEA] (non-polar/basic) (Kotze and Eloff, 2002). Development of the chromatograms was in a closed tank in which the atmosphere had been saturated with the eluent vapor by lining the tank with filter paper wetted with the eluent.

To detect the chemical components of each extract, vanillin-sulphuric acid (0.1 g vanillin (Sigma): 28 ml methanol: 1 ml sulphuric acid) was sprayed on the chromatograms and heated at 110 °C to optimal colour development.

2.5. Fungal test organisms

Five fungi were obtained from the bacteriology laboratory, Department of Veterinary Tropical Diseases, 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. *C. albicans* was isolated from a Goldian finch, *C. neoformans* from a cheetah, and *A. fumigatus* from a chicken, all of which suffered from a systemic mycosis. *M. 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).

2.6. Antifungal assays

2.6.1. Microdilution assay

A serial microdilution assay (Eloff, 1998c) was used to determine the minimum inhibitory concentration (MIC) values for plant extracts using tetrazolium violet reduction as an indicator of growth. This method had previously been used only for antibacterial activities (Eloff, 1998c; McGaw et al., 2001). To apply it to measuring antifungal activities, a slight modification was made to suit fungal growth conditions. Residues of the 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

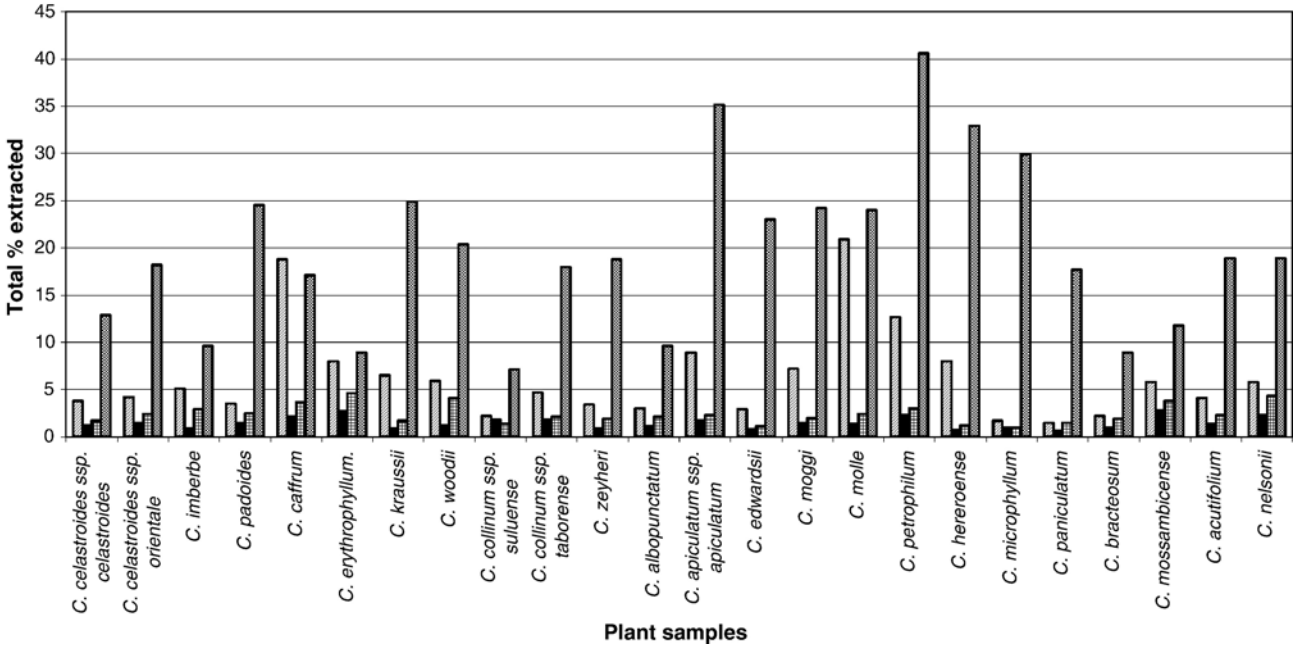

Fig. 1. Total percentage of *Combretum* species extracted by acetone ▨, hexane ■, dichloromethane ▤, and methanol ▩. Order from left to right represents infra-generic classification (Carr, 1988).

Table 2
MIC values in mg/ml of *Combretum* species after 24 and 48 h incubation

Organisms	Time (h)	MIC values (mg/ml)																					
		C. cela. ssp. *celastroides*[a]				*C. cela.* ssp. *orientale*[b]				*C. imberbe*				*C. padoides*				*C. caffrum*				Average	Amphotericin B
		Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol		
C. albicans	24	0.16	0.64	0.32	0.64	0.16	0.32	0.16	0.32	>2.5	0.16	0.16	>2.5	0.32	0.32	0.32	>2.5	>2.5	0.16	0.64	>2.5	0.4	
	48	0.16	0.64	0.32	0.64	0.16	0.32	0.16	0.32	>2.5	0.16	0.16	>2.5	0.32	0.32	0.32	>2.5	>2.5	0.16	0.64	>2.5	0.4	0.4
C. neoformans	24	0.16	0.16	0.08	0.32	0.08	0.32	0.08	0.16	0.16	0.16	0.32	0.32	0.32	0.64	0.32	0.32	0.32	0.16	0.16	0.32	0.21	
	48	0.16	0.16	0.08	0.32	0.08	0.32	0.08	0.16	0.16	0.16	0.32	0.32	0.32	0.64	0.32	0.32	0.32	0.32	0.16	0.32	0.27	0.3
A. fumigatus	24	0.32	>2.5	0.64	0.64	0.32	2.5	0.64	0.64	2.5	2.5	0.64	0.64	0.16	0.32	0.32	0.32	>2.5	>2.5	1.25	1.25	1.25	0.2
	48	0.64	>2.5	1.25	0.64	0.32	2.5	2.5	2.5	2.5	2.5	2.5	2.5	0.32	2.5	2.5	0.32	>2.5	>2.5	1.25	1.25	1.25	
S. schenckii	24	0.32	0.32	0.16	0.16	0.08	0.16	0.16	0.16	>2.5	>2.5	0.32	>2.5	0.32	>2.5	>2.5	0.64	0.64	0.32	0.32	0.32	0.32	
	48	0.32	0.32	0.16	0.16	0.08	0.16	0.16	0.16	>2.5	>2.5	0.32	>2.5	0.32	>2.5	>2.5	0.64	0.64	0.64	0.64	0.32	0.53	0.4
M. canis	24	0.02	0.08	0.08	0.02	0.02	0.04	0.08	0.08	0.04	0.04	0.04	0.16	0.02	0.08	0.08	0.02	0.02	0.04	0.02	0.02	0.03	
	48	0.32	0.64	0.64	0.08	0.04	0.32	0.32	0.08	0.32	0.64	0.16	0.32	0.08	0.64	0.16	0.08	0.08	0.32	0.32	0.16	0.27	0.2
Average		0.26	0.37	0.37	0.36	0.13	0.7	0.43	0.46	0.95	0.79	0.49	0.71	0.25	0.68	0.54	0.33	0.34	0.27	0.54	0.5		

Organisms	Time (h)	MIC values (mg/ml)																					
		C. erythrophyllum				*C. kraussii*				*C. woodii*				*C. coll.* ssp. *suluense*[c]				*C. coll.* ssp. *taborense*[d]				Average	Amphotericin B
		Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol		
C. albicans	24	>2.5	0.64	0.64	2.5	2.5	0.08	0.32	1.25	0.16	0.08	0.16	0.32	0.08	2.5	0.08	0.16	0.64	0.64	0.64	0.64	0.74	
	48	>2.5	0.64	0.64	2.5	2.5	0.08	0.32	1.25	0.16	0.08	0.16	0.32	0.08	2.5	0.08	0.16	0.64	0.64	0.64	0.64	0.74	0.4
C. neoformans	24	>2.5	0.64	0.32	0.64	0.64	0.32	0.16	0.32	0.32	0.16	0.16	2.5	0.16	2.5	0.08	0.08	0.08	0.16	0.32	0.32	0.52	
	48	>2.5	0.64	0.32	0.64	0.64	0.32	0.16	0.32	0.32	0.16	0.16	2.5	0.16	2.5	0.08	0.08	0.08	0.16	0.32	0.32	0.52	0.3
A. fumigatus	24	1.25	>2.5	>2.5	1.25	0.32	0.32	0.32	0.16	0.32	1.25	0.64	0.32	0.64	2.5	2.5	0.32	0.64	2.5	1.25	1.25	0.99	0.2
	48	2.5	>2.5	>2.5	2.5	0.64	2.5	2.5	0.16	1.25	2.5	1.25	2.5	2.5	2.5	2.5	2.5	0.64	2.5	2.5	2.5	2.02	
S. schenckii	24	>2.5	0.32	0.32	1.25	0.64	0.16	0.16	0.32	0.08	0.16	0.16	0.32	0.08	0.08	0.08	0.16	0.32	0.16	0.16	0.16	0.27	
	48	>2.5	0.32	0.32	1.25	0.64	0.32	0.32	0.64	0.32	0.32	0.32	1.25	0.16	2.5	0.16	0.32	0.64	0.32	0.32	0.64	0.58	0.4
M. canis	24	0.02	0.16	0.32	0.02	0.02	0.02	0.02	0.02	0.04	0.02	0.02	0.02	0.08	0.04	0.08	0.04	0.02	0.04	0.08	0.02	0.06	
	48	0.02	1.25	0.32	0.16	0.32	0.16	0.64	0.04	0.32	0.32	0.32	0.32	0.64	1.25	0.64	0.32	0.32	1.25	0.64	0.16	0.47	0.2
Average		0.95	0.58	0.4	1.27	0.89	0.43	0.49	0.45	0.33	0.51	0.34	1.04	0.46	1.89	0.63	0.41	0.4	0.84	0.69	0.67		

Organisms	Time (h)	MIC values (mg/ml)																					
		C. zeyheri				*C. albopuctatum*				*C. api.* ssp. *apiculatum*[e]				*C. edwardsii*				*C. moggi*				Average	Amphotericin B
		Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol		
C. albicans	24	0.02	0.64	0.64	0.04	0.64	2.5	0.32	0.32	0.08	0.16	0.04	0.04	0.04	1.25	1.25	0.04	0.04	1.25	1.25	0.02	0.53	
	48	0.16	2.5	1.25	0.16	0.64	2.5	1.25	1.25	0.32	1.25	0.32	0.32	0.32	1.25	1.25	0.64	0.64	1.25	1.25	0.02	0.93	0.4
C. neoformans	24	0.08	0.08	0.16	0.16	0.08	0.08	0.16	0.16	0.08	0.08	0.08	0.04	0.08	0.16	0.32	0.16	0.08	0.32	0.32	0.02	0.14	
	48	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.08	0.08	0.08	0.08	0.04	0.32	0.32	0.16	0.08	0.32	0.32	0.04	0.22	0.3
A. fumigatus	24	0.32	2.5	0.16	0.64	0.08	0.64	0.16	0.32	0.08	2.5	0.16	0.04	0.08	0.64	0.16	0.16	0.32	2.5	0.16	0.16	0.59	0.2
	48	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	

S. schenckii	24	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02		
	48	0.02	0.08	0.04	0.08	0.08	0.16	0.16	0.32	0.02	0.04	0.02	0.02	0.04	0.08	0.08	0.04	0.02	0.16	0.08	0.02	0.08	0.4	
M. canis	24	0.02	0.02	0.02	0.04	0.02	0.02	0.02	0.04	0.02	0.02	0.02	0.02	0.04	0.02	0.04	0.02	0.02	0.02	0.04	0.02	0.03		
	48	0.02	0.02	0.02	0.04	0.02	0.02	0.04	0.04	0.02	0.02	0.02	0.02	0.04	0.02	0.04	0.04	0.04	0.08	0.04	0.02	0.03	0.2	
Average		0.35	0.87	0.51	0.4	0.44	0.88	0.5	0.53	0.32	0.67	0.33	0.31	0.32	0.63	0.6	0.38	0.38	0.84	0.6	0.28			

Organisms	Time (h)	MIC values (mg/ml)																				Average	Amphotericin B
		C. molle				*C. petrophilum*				*C. hereroense*				*C. microphyllum*				*C. paniculatum*					
		Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol		
C. albicans	24	0.04	1.25	0.32	0.04	0.02	0.32	0.32	0.02	0.02	0.32	0.32	0.02	1.25	1.25	1.25	0.16	1.25	1.25	1.25	1.25	0.6	
	48	0.04	1.25	0.32	0.32	0.04	2.5	2.5	0.04	0.32	0.32	2.5	0.04	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	1.51	0.4
C. neoformans	24	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	
	48	0.04	1.25	0.16	0.08	0.02	0.32	2.5	0.02	0.16	0.08	0.32	0.08	0.16	0.64	0.08	0.16	0.32	1.25	0.16	0.16	0.4	0.3
A. fumigatus	24	0.32	2.5	0.64	0.64	0.64	2.5	2.5	0.64	0.64	2.5	0.64	0.64	0.64	2.5	0.64	2.5	0.64	2.5	2.5	1.25	1.4	0.2
	48	1.25	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	1.25	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.38	
S. schenckii	24	0.08	0.08	0.08	0.08	0.04	0.04	0.04	0.02	0.04	0.04	0.08	0.08	0.04	0.04	0.04	0.08	0.02	0.04	0.02	0.04	0.05	
	48	0.08	0.32	0.32	0.08	0.08	0.32	0.32	0.04	0.16	0.16	0.32	0.16	0.64	0.64	0.32	0.32	0.32	0.32	0.04	0.04	0.24	0.4
M. canis	24	0.02	0.02	0.02	0.02	0.02	0.04	0.04	0.02	0.04	0.02	0.02	0.04	0.04	0.02	0.02	0.08	0.02	0.02	0.02	0.04	0.03	
	48	0.02	0.02	0.04	0.02	0.02	0.04	0.04	0.02	0.04	0.02	0.02	0.04	0.04	0.02	0.04	0.16	0.02	0.02	0.02	0.08	0.04	0.2
Average		0.19	0.92	0.44	0.38	0.34	0.83	1.08	0.33	0.39	0.6	0.67	0.24	0.78	1.01	0.74	0.85	0.76	1.04	0.9	0.79		

Organisms	Time (h)	MIC values (mg/ml)																Average	Amphotericin B
		C. bracteosum				*C. mossambicense*				*C. acutifolium*				*C. nelsonii*					
		Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol		
C. albicans	24	0.16	0.16	0.08	0.08	0.08	0.16	0.08	0.04	0.02	0.16	0.16	0.02	0.04	0.08	0.32	0.16	0.11	
	48	1.25	2.5	2.5	1.25	1.25	2.5	2.5	1.25	0.16	2.5	2.5	0.04	0.04	0.16	0.32	0.16	1.31	0.4
C. neoformans	24	0.04	0.08	0.02	0.02	0.04	0.02	0.08	0.08	0.02	0.04	0.04	0.04	0.04	0.02	0.08	0.08	0.05	
	48	0.16	0.16	0.32	0.32	1.25	1.25	0.64	0.64	0.04	0.16	0.16	0.08	0.16	0.32	0.32	0.16	0.38	0.3
A. fumigatus	24	0.16	2.5	0.08	0.16	0.16	2.5	0.16	0.16	0.04	0.16	0.04	0.04	0.16	0.32	0.16	0.16	0.44	0.2
	48	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	0.08	2.5	0.16	0.16	0.64	2.5	0.64	0.64	1.71	
S. schenckii	24	0.04	0.02	0.02	0.02	0.04	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.08	0.02	0.03	
	48	0.16	0.08	0.16	0.16	0.64	0.16	0.16	0.16	0.04	0.32	0.32	0.08	0.08	0.32	0.16	0.16	0.2	0.4
M. canis	24	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.16	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.03	
	48	0.02	0.02	0.02	0.02	0.08	0.04	0.02	0.32	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.04	0.2
Average		0.45	0.8	0.57	0.46	0.61	0.92	0.62	0.53	0.05	0.59	0.34	0.05	0.12	0.38	0.21	0.16		

[a] *C. celastroides* ssp. *celastroides*.
[b] *C. celastroides* ssp. *orientale*.
[c] *C. collinum* ssp. *suluense*.
[d] *C. collinum* ssp. *taborense*.
[e] *C. apiculatum* ssp. *apiculatum*.

Table 3
Total activity in ml/g of *Combretum* species extracted after 24 and 48 h incubation

Organisms	Time (h)	Total activity (ml/g)																			
		C. cela. ssp. *celastroides*[a]				*C. cela.* ssp. *orientale*[b]				*C. imberbe*				*C. padoides*				*C. caffrum*				Average
		Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	
C. albicans	24	238	42	119	127	263	106	294	175	20	150	650	28	109	138	256	52	75	419	58	28	167
	48	238	42	119	127	263	106	294	175	20	150	650	28	109	138	256	52	75	419	58	28	167
C. neoformans	24	238	169	475	253	525	106	588	350	319	150	325	219	109	69	256	403	588	419	231	219	300
	48	238	169	475	253	525	106	588	350	319	150	325	219	109	69	256	403	588	209	231	219	290
A. fumigatus	24	119	11	59	127	131	14	73	88	20	10	163	109	219	138	256	403	75	27	30	56	106
	48	59	11	30	127	131	14	19	22	20	10	42	28	11	18	33	403	75	27	30	56	58
S. schenckii	24	119	84	238	506	525	213	294	350	20	10	325	28	109	18	33	202	294	209	116	219	195
	48	119	84	238	506	525	213	294	350	20	10	325	28	109	18	33	202	294	105	58	219	187
M. canis	24	1900	338	475	4050	2100	850	588	700	1275	600	2600	438	1750	550	1025	6450	9400	1675	1850	3500	2106
	48	119	42	59	1013	1050	106	147	700	159	38	650	219	438	69	513	1613	2350	209	116	438	502
Average		338	99	229	709	604	183	318	326	219	128	605	134	307	122	292	1018	1381	372	278	498	

Organisms	Time (h)	Total activity (ml/g)																			
		C. erythrophyllum				*C. kraussii*				*C. woodii*				*C. coll.*ssp. *suluense*[c]				*C .coll.* ssp. *taborense*[d]				Average
		Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	
C. albicans	24	32	128	58	42	26	325	122	116	369	425	244	238	22	12	413	256	73	22	50	127	155
	48	32	128	58	42	26	325	122	116	369	425	244	238	22	12	413	256	73	22	50	127	155
C. neoformans	24	32	128	116	163	102	81	244	453	184	213	244	30	22	12	413	513	588	88	100	253	199
	48	32	128	116	163	102	81	244	453	184	213	244	30	22	12	413	513	588	88	100	253	199
A. fumigatus	24	64	33	15	83	203	81	122	906	184	27	61	238	22	12	13	128	73	6	26	65	118
	48	32	33	15	42	102	10	16	906	47	14	31	30	22	12	13	16	73	6	13	32	73
S. schenckii	24	32	256	116	83	102	163	244	453	738	213	244	238	688	375	413	256	147	88	200	506	278
	48	32	256	116	83	102	81	122	227	184	106	122	61	22	12	206	128	73	44	100	127	110
M. canis	24	4000	513	116	5200	3250	1300	1950	7250	1475	1700	1950	3800	1375	750	413	1025	2350	350	400	4050	2161
	48	4000	66	116	650	203	163	61	3625	184	106	122	238	44	24	52	128	147	11	50	506	525
Average		829	167	84	655	422	261	325	1451	392	344	350	514	226	123	276	322	419	72	109	605	

Organisms	Time (h)	Total activity (ml/g)																			
		C. zeyheri				*C. albopuctatum*				*C. api.* ssp. *apiculatum*[e]				*C. edwardsii*				*C. moggi*				Average
		Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	
C. albicans	24	1700	38	73	575	47	9	103	172	1113	313	1825	2450	2300	18	23	1725	2500	34	37	6400	1073
	48	213	10	38	153	47	9	26	44	278	40	228	306	288	18	23	108	156	34	37	6400	423
C. neoformans	24	425	300	294	144	375	275	206	344	1113	625	913	2450	1150	144	91	431	1250	134	144	6400	860
	48	106	75	147	72	94	69	103	172	1113	625	913	1225	2300	72	91	431	1250	134	144	3200	617
A. fumigatus	24	106	10	294	36	375	34	206	172	1113	20	456	2450	1150	36	181	431	313	17	288	800	424
	48	14	10	19	9	12	9	13	22	36	20	29	39	37	9	12	28	40	17	18	51	22

S. schenckii	24	1700	1200	2350	1150	1500	1100	1650	2750	4450	2500	3650	4900	4600	1150	1450	3450	5000	2150	2300	6400	2770
	48	1700	300	1175	288	375	138	206	172	4450	1250	3650	4900	2300	288	363	1725	5000	269	575	6400	1776
M. canis	24	1700	1200	2350	575	1500	1100	1650	1375	4450	2500	3650	4900	2300	1150	725	3450	5000	2150	1150	6400	2464
	48	1700	1200	2350	575	1500	1100	825	1375	4450	2500	3650	4900	2300	1150	725	1725	2500	538	1150	6400	2131
Average		936	434	909	358	582	384	499	660	2256	1039	1896	2852	1872	404	368	1350	2301	548	584	4885	

Organisms	Time (h)	Total activity (ml/g)																				
		C. molle				*C. petrophilum*				*C. hereroense*				*C. microphyllum*				*C. paniculatum*				Average
		Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	
C. albicans	24	5225	62	166	3600	6350	181	88	4150	4000	63	106	3350	34	11	16	769	34	10	16	46	1414
	48	5225	62	166	450	3175	23	11	2075	250	63	14	1675	17	6	8	49	17	5	8	23	666
C. neoformans	24	10450	3900	2650	7200	6350	2900	1400	4150	4000	1000	1700	3350	2150	700	1000	6150	2100	650	1000	2850	3283
	48	5225	62	331	1800	6350	181	11	4150	500	250	106	838	269	22	250	769	131	10	125	356	1087
A. fumigatus	24	653	31	83	225	198	23	11	130	125	8	53	105	67	6	31	49	66	5	8	46	96
	48	167	31	21	58	51	23	11	33	32	8	14	54	17	6	8	49	17	5	8	23	32
S. schenckii	24	2613	975	663	1800	3175	1450	700	4150	2000	500	425	838	1075	350	500	1538	2100	325	1000	1425	1380
	48	2613	244	166	1800	1588	1813	88	2075	500	125	106	419	67	22	63	384	131	41	500	1425	708
M. canis	24	10450	3900	2650	7200	6350	1450	700	4150	2000	1000	1700	1675	1075	700	1000	1538	2100	650	1000	1425	2636
	48	10450	3900	1325	7200	6350	1450	700	4150	2000	1000	1700	1675	1075	700	500	769	2100	650	1000	713	2470
Average		5307	1317	822	3133	3994	949	372	2921	1541	402	592	1398	585	252	338	1206	880	235	467	833	

Microorganisms	Time (h)	Total activity (ml/g)																Average
		C. bracteosum				*C. mossambicense*				*C. acutifolium*				*C. nelsonii*				
		Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	Acetone	Hexane	DCM	Methanol	
C. albicans	24	344	113	550	363	725	256	300	1300	2050	244	388	2750	1450	688	125	538	761
	48	44	7	18	23	46	16	10	42	256	16	25	1375	1450	344	125	538	271
C. neoformans	24	1375	225	2200	1450	1450	2050	300	650	2050	975	1550	1375	1450	2750	500	1075	1339
	48	344	113	138	91	46	33	38	81	1025	244	388	688	363	172	125	538	276
A. fumigatus	24	344	7	550	181	363	16	150	325	1025	244	1550	1375	363	172	250	538	466
	48	22	7	18	12	23	16	10	21	513	16	388	344	91	22	63	134	106
S. schenckii	24	1375	900	2200	1450	1450	2050	1200	2600	2050	1950	3100	2750	2900	2750	500	4300	2095
	48	344	225	275	181	91	250	150	325	1025	122	194	688	725	172	250	538	347
M. canis	24	2750	900	2200	1450	2900	2050	1200	325	2050	1950	3100	2750	2900	2750	2000	4300	2223
	48	2750	900	2200	1450	725	1025	1200	163	2050	1950	3100	2750	2900	2750	2000	4300	2013
Average		969	340	1035	665	782	776	456	583	1409	771	1378	1684	1459	1257	594	1680	

[a] *C. celastroides* ssp. *celastroides*.
[b] *C. celastroides* ssp. *orientale*.
[c] *C. collinum* ssp. *suluense*.
[d] *C. collinum* ssp. *taborense*.
[e] *C. apiculatum* ssp. *apiculatum*.

(Eloff, 1998c). Fungal cultures were transferred into fresh Sabouraud dextrose broth, and 100 µl of this was added to each well. Amphotericin B was used as the reference antibiotic and positive control, and appropriate solvent blanks were included as negative control. As an indicator of growth, 40 µl of 0.2 mg/ml of *p*-iodonitrotetrazolium violet (Sigma®) (INT) dissolved in water was added to each of the microplate wells. The covered microplates were incubated for 2 to 3 days at 35 °C and 100% relative humidity. The MIC was recorded as the lowest concentration of the extract that inhibited antifungal growth after 24 and 48 h.

One is tempted to consider the 48 h value as a minimal fungicidal concentration especially since no growth was apparent in the particular well after 120 h. When cells from wells showing no growth after 48 h were incubated in fresh growth medium, however fungal growth resumed. The inhibition therefore appears to be fungistatic rather than fungicidal at the levels tested. Motsei et al. (2003) used a different technique to determine MIC values of medicinal plants traditionally used against *C. albicans* infections and it was not possible to distinguish between fungistatic and fungicidal activities.

3. Results and discussion

Twenty-four *Combretum* species were selected for antifungal activity screening based on their use in traditional medicinal treatments for both domestic animals and humans and availability in southern Africa. Success in isolating compounds from plant material is largely dependent on the type of the solvent used in the extraction procedure (Lin et al., 1999). The total percentages extracted using different solvents (acetone, hexane, DCM and methanol) are shown in Fig. 1. Methanol was the quantitatively the best extractant, extracting a greater quantity of plant material than any of the other solvents. Total percentages extracted with methanol of *C. apiculatum* subspecies *apiculatum*, *C. petrophilum*, *C. hereroense* and *C. microphyllum* were between 25 and 41%. Hexane and dichloromethane are more selective extractants for *Combretum* species, because for all the species, the total percentage extracted was below 5% (Fig. 1). The total percentage extracted with acetone was better in 10 of the *Combretum* species tested, ranging from 5 to 21%.

After evaporation of extracting solvents, the hexane, dichloromethane and methanol extracts were redissolved in acetone because this solvent was found not to be harmful towards bacteria (Eloff, 1998b). We found that acetone was also not harmful towards fungi at the final concentration (25%) the fungi were subjected to (manuscript by Eloff and Masoko in preparation). Of the four solvents used, methanol extracted more chemical compounds from leaves of the *Combretum* species, but the extract probably contained highly polar compounds and tannins that may not be that interesting for clinical application.

The separated compounds on TLC plates were made visible by spraying with vanillin-sulphuric acid. There was some similarity in the chemical composition of the non-polar components of extracts using extractants of varying polarity.

MIC values were determined by checking growth after 24 and 48 h to determine the end point. The MIC values of most of the extracts were in the order of 0.08 mg/ml and some had values as low as 0.02 mg/ml, especially against *C. neoformans*, *S. schenckii* and *M. canis* (Table 2). The methanolic extracts of *C. moggii* and *C. petrophilum* were very active against all the tested pathogens. All extracts of *C. nelsonii* were very effective against all the pathogens. Acetone and methanol extracts of *C. acutifolium* were active against all pathogens after 24 h of incubation, with MIC values ranging from 0.02 and 0.04 mg/ml. Only the MIC against *A. fumigatus* increased to 0.16 mg/ml after 48 h. The acetone extracts of *C. molle* and *C. celastroides* ssp. *orientale* were the most active against all the fungi tested as they had average MIC values, of 0.19 and 0.13 mg/ml respectively.

The hexane extracts of *C. collinum* ssp. *suluense*, *C. microphyllum*, *C. paniculatum*, and the methanolic extracts of *C. erythrophyllum*, *C. woodii*, and the dichloromethane extract of *C. petrophilum* were the least active against all the fungi tested as the MIC values ranged from 1.01 to 1.89 mg/ml.

To determine which plants can be used for further testing and isolation, not only the MIC value is important, but also the total activity (Eloff, 1999). Because the MIC value is inversely related to the quantity of antifungal compounds present, an arbitrary measure of the quantity of antifungal compounds present was calculated by dividing the quantity extracted in mg from 1 g leaves by the MIC value in mg/ml. This total activity (Table 3) value indicates the volume to which the biologically

Table 4
Average MIC values of different *Combretum* species after 24 and 48 h incubation of all extracts against all test pathogens

Combretum species	Average MIC values (mg/ml)				
	24 and 48 h	24 h	24 h[a]	48 h	48 h[a]
C. celastroides ssp. *celestroides*	0.34	0.28	0.23	0.4	0.32
C. celastroides ssp. *orientale*	0.43	0.32	0.15	0.54	0.18
C. imberbe	0.74	0.57	0.17	0.9	0.28
C. padoides	0.54	0.29	0.27	0.62	0.35
C. caffrum	0.39	0.36	0.27	0.43	0.35
C. erythrophyllum	0.8	0.65	0.49	0.95	0.57
C. kraussii	0.56	0.4	0.43	0.72	0.54
C. woodii	0.55	0.36	0.29	0.74	0.46
C. collinum ssp. *suluense*	0.85	0.61	0.39	1.08	0.73
C. collinum ssp. *taborense*	0.65	0.5	0.28	0.79	0.43
C. zeyheri	0.53	0.28	0.13	0.78	0.35
C. albopunctatum	0.59	0.28	0.28	0.89	0.49
C. apiculatum ssp. *apiculatum*	0.41	0.18	0.05	0.64	0.17
C. edwardsii	0.48	0.23	0.22	0.73	0.29
C. moggi	0.53	0.33	0.22	0.72	0.27
C. molle	0.48	0.31	0.13	0.66	0.27
C. petrophilum	0.65	0.37	0.06	0.93	0.53
C. hereroense	0.48	0.28	0.07	0.67	0.3
C. microphyllum	0.85	0.53	0.27	1.16	0.83
C. paniculatum	0.87	0.61	0.33	1.14	0.8
C. bracteosum	0.57	0.19	0.05	0.96	0.57
C. mossambicense	0.67	0.19	0.06	1.14	0.8
C. acutifolium	0.26	0.05	0.04	0.47	0.41
C. nelsonii	0.22	0.09	0.07	0.37	0.15
Average	0.56	0.34	0.21	0.77	0.44

[a] Without *A. fumigatus*.

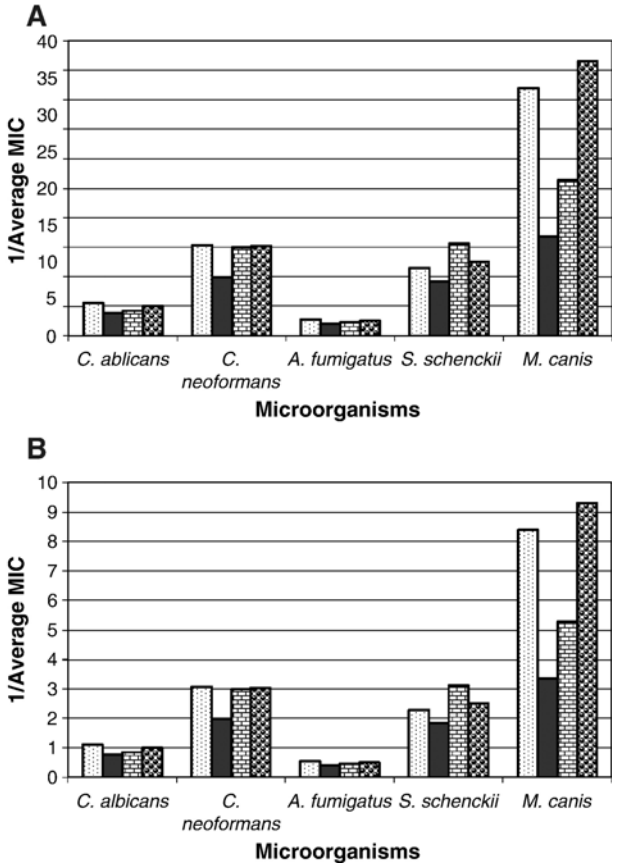

Fig. 2. The sensitivity of different fungal pathogens in ml/mg to acetone, hexane, dichloromethane, and methanol extracts of 24 *Combretum* species (antifungal activity expressed as inverse of MIC in mg/ml) after 24 h (A) and 48 h (B).

active compound present in 1 g of the dried plant material can be diluted and still kill the fungi (Eloff, 1999).

Extracts with higher activity were considered the best to work with, from Table 3 *M. canis* was the most sensitive of all the organisms tested and all 96 extracts inhibited the growth at low concentrations 0.02 to 0.04 mg/ml. *A fumigatus* had a higher resistance against all plant extracts with the exception of *C. kraussii* (methanol), *C. apiculatum* ssp. *apiculatum* (acetone and methanol), *C. edwardsii* (acetone), *C. moggii* (methanol) and *C. acutifolium* (acetone, dichloromethane and methanol) extracts. After 48 h of incubation the situation changed. Only *C. kraussii* extracts had a better activity after 48 h. Average MIC values of all *Combretum* species were calculated (Table 4) after 24 and 48 h incubation with and without *A. fumigatus*, because results with this organism differed much compared to that with other fungi (Tables 2 and 3).

The average MIC values of all *Combretum* species using all pathogens after 24 h was 0.34 mg/ml and after the results against *A. fumigatus* were excluded, it was 0.21 mg/ml (Table 4). After 48 h there was a difference in the average MIC values of 0.33 mg/ml. Although there was inhibition of the *A. fumigatus* after 24 h of incubation, this inhibition was overcame after 48 h of incubation. This could be due to a breakdown of the active antifungal compounds allowing the inhibited fungus to grow, or the fungus may have been able to overcome the inhibitory effects of the antifungal compound(s) with time.

Amphotericin B was used as a positive control to ensure that the test was functioning properly. This was the case as all the fungi tested had MIC's of <0.02 mg/ml. In subsequent experiments with lower concentrations of amphotericin B, the MIC's for *C. albicans*, *C. neoformans* variety *gattii*, *S. schenckii* and *M. canis* were 0.4, 0.3, 0.4, and 0.2 μg/ml respectively after 48 h incubation and for *A. fumigatus* it was 0.2 μg/ml after 24 h. All the tested pathogens were sensitive to different *Combretum* species extracted with different solvent systems (Fig. 2). The methanol and acetone extracts (Fig. 3) were generally the most active, followed by dichloromethane. Hexane extracts were the least active.

C. nelsonii was selected for fractionation and bioassay-guided isolation of the antifungal compounds because the crude extracts had low average MIC values in acetone, hexane, DCM and methanol i.e. (0.12, 0.38, 0.21 and 0.16 mg/ml respectively) and high total activities (i.e. 1456, 1257, 594 and 1680 ml/g) respectively. The next best species was *C. albopuntactum* with average MIC values of 0.44, 0.88, 0.50 and 0.32 mg/ml respectively and total activities of 582, 384, 499 and 660 ml respectively. The third best was *C. imberbe* with average MIC values of 0.95, 0.79, 0.49 and 0.71 mg/ml and total activities of

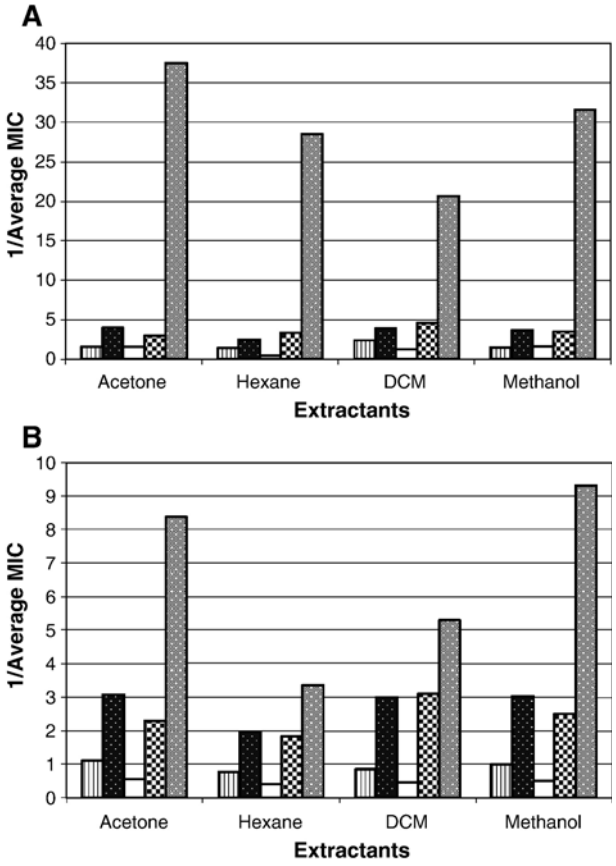

Fig. 3. The average antifungal activity of different leaf extracts of 24 *Combretum* species towards *C. albicans*, *C. neoformans*, *A. fumigatus*, *S. schenckii*, and *M. canis* after 24 h (A) and 48 h (B).

219, 128, 605 and 134 ml/g respectively. There was no correlation between subgeneric classification and antifungal activities (Fig. 1). These species were also selected because they have not previously been investigated for antifungal activity.

Baba-Moussa et al. (1999) investigated the antifungal activities of seven West African Combretaceae used in traditional medicine, (*Combretum glutinosum*, *C. hispidum*, *C. molle*, *C. nigricans* and some *Terminalias* (*T. avicennioides* and *T. mollis*) on five pathogenic fungi, which were *Epidermophyton floccosum*, *Microsporum gypseum*, *Trichophyton mentagrophytes*, *Trichophyton rubrum* and *C. albicans*. Their MIC values were in the range of 0.25–4 mg/ml, *T. mentagrophytes* was the most sensitive fungus on average. Fyhrquist et al. (2002) have found evidence that some of the 12 different *Combretum* species tested had antifungal activities. Motsei et al. (2003) have tested number of plants on *C. albicans* but their results of MIC values were very high (>25 mg/ml).

Some authors have determined antifungal activities and MIC using different plant species. Their MIC values were generally high. Delaporte et al. (2004) used *Tillandsia streptocarpa* (Bromeliaceae) to test antimicrobial activity on *C. albicans* (MIC >0.5 mg/ml), Chandrasekaran and Venkatesalu (2004) have found that seed extracts of *Syzgium jambolanum* were effective against different pathogens, *C. albicans*, *C. neoformans*, *A. fumigatus* and *M. gypseum* with the MIC values of 0.62, 0.25, 0.125 and 0.25 mg/ml respectively, and Chamundeeswari et al. (2004) found antifungal activity of *Trewia polycarpa* root extracts on *C. albicans*, *A. niger*, *C. neoformans* and *Penicillum* sp. Alcoholic extracts had mild antifungal activity with MIC values of 0.25, 0.25, 0.125 and 0.313 mg/ml respectively. When comparing the MIC values with our data it is clear that extracts of *Combretum* species have substantial activity against fungal pathogens.

We have also shown that there are a number of active compounds against fungi present in *Terminalia* species (Masoko and Eloff, 2005). From the R_f values in bioautography data and activity in non-polar and intermediate polarity extracts it appears that antifungal activity may not only be attributable to tannins found in *Combretum* spp. 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 of *Terminalia* spp. (Masoko et al., 2005).

4. Conclusion

The results of the present work indicate that the *Combretum* species assayed possess substantial antifungal properties. If there are no synergistic effects and the antifungal compounds comprise 0.1% of the mass [in our experience this is a reasonable rule of thumb for compounds in some members of the Combretaceae], the antifungal compound may have an MIC of 0.02 to 0.2 μg/ml compared to MIC's of 0.2–0.4 μg/ml of amphotericin B for these pathogens. The results of this study support several of the traditional medicinal uses of *Combretum* species all over southern Africa and in the whole continent. The isolation and characterization of three compounds with excellent antifungal activities from *C. nelsonii* as well as the *in vivo* activity of isolated compounds and several extracts on rats are reported elsewhere (Masoko, 2006).

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