Chapter 3
Evaluation of several tree species for activity against the animal fungal pathogen *Aspergillus fumigatus*

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PREFACE

The plant species that were selected in the previous chapter had to be evaluated to determine which species should be used for in depth investigation. The minimum inhibitory concentration (MIC) of the extract was determined to confirmed earlier investigation. We evaluate the effects of the extract further on other important animal fungal pathogens. The text in this chapter was submitted and accepted as manuscript for publication to South African Journal of Botany, Vol. 76, pages 64-71 to enable wider readership of the results obtained.

Abstract

Aspergillus fumigatus causes severe problems in poultry production systems. Seven South African tree species were selected from the database of the Phytomedicine Programme based on its antifungal activity against the fungus *Cryptococcus neoformans*. The acetone leaf extracts of the plants had minimum inhibitory concentrations (MICs) of 0.1 mg/ml and lower in the preliminary screening. The antibacterial and antifungal activities of hexane, dichloromethane, acetone and methanol extracts of the leaves were determined using a two-fold serial microdilution method against a range of commonly encountered animal pathogenic fungi (*Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Microsporum canis* and *Sporothrix schenckii*) and four nosocomial bacteria (*Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*). The plant species investigated were *Combretum vendae* (A.E. van Wyk) (Combretaceae), *Commiphora harveyi* (Engl.) Engl. (Burseraceae), *Khaya anthotheca* (Welm.) C.DC (Meliaceae), *Kirkia wilmsii* Engl. (Kirkiaceae), *Loxostylis alata* A. Spreng. ex Rchb. (Anacardiaceae), *Ochna natalitia* (Meisn.) Walp. (Ochnaceae) and *Protorhus longifolia* (Bernh.) Engl. (Anacardiaceae). All the extracts had activity against at least one of the test organisms over an incubation period of 24 or 48 h. The MIC values of the hexane extracts of *Ochna natalitia*, *Khaya anthotheca* and *Combretum vendae* against one or more of the tested pathogens were as low as 0.08 mg/ml. Similarly the hexane and acetone extracts of *Commiphora harveyi*, acetone extract of *Protorhus longifolia* and dichloromethane extract of *Combretum vendae* depicted MICs as low as 0.08 mg/ml against at least one of the tested bacteria. Furthermore, the acetone extracts of *Loxostylis alata*, *Kirkia wilmsii*, *Ochna natalitia* and *Combretum vendae* had antifungal activities with MIC values ranging from 0.04-0.08 mg/ml against at least one of the tested fungi. The average MIC values of the plant extracts against bacteria ranged from 0.17-

2.11 mg/ml, while the range was 0.23-1.98 mg/ml for fungi. The Gram-positive organisms (*S. aureus* and *E. faecalis*) were more susceptible to the plant extracts than the Gram-negative organisms (*E. coli and P. aeruginosa*). *E. faecalis* was the most susceptible microbe and *Combretum vendae* extracts were the most active against nearly all the bacteria tested. The acetone extract of *Loxostylis alata* was the most active against fungal pathogens, with activity against at least 3 fungal organisms. *Loxostylis alata* was selected for further work to isolate compounds active against *Aspergillus fumigatus* and other fungal pathogens.

Keywords: Antibacterial; Antifungal; Medicinal plants; Minimum inhibitory concentration; Microdilution assay

3.1. Introduction

With the development of relatively effective and safe antibiotics in the 1940's, medical treatment had been revolutionalised leading to a drastic drop in morbidity and mortality previously induced by microbial diseases (Rang et al., 2003). Unfortunately, this development was rapidly hampered by the emergence of drug-resistant microbes (Walsh, 2000). This resistance has resulted in an increased incidence of infectious diseases with some pathogens (Kunin, 1993; Archibald et al., 1997; Sahm et al., 1999). With the evolutionary process that enables microbes to adapt genetically to changes in their environment, the unwise use of antibiotics inevitably selects for resistant microbes (Clardy et al., 2006). As a result new drugs have to be consistently developed to counteract the development of resistance and to possibly reduce the cost of controlling the disease. (Cowan, 1999).

In addition to the pathogenic bacteria, opportunistic fungal infections are becoming more important especially due to the immune deficiency induced by HIV-AIDS (Groll et al., 1996). Invasive pulmonary aspergillosis (IPA) is a serious fungal infection of immunocompromised patients usually caused by *Aspergillus fumigatus* with ever increasing incidence (Stevens, 1990; Denning, 1998). In contrast, however, there are only a limited number of antimicrobial drugs which are active against fungal pathogens (Denning, 1998). Although conventional antifungals remain the standard therapy for many invasive or life-threatening mycoses, these drugs are associated with significant toxicity (Dismukes, 2000). Against this backdrop, there is the need to develop cheaper, safer and effective antifungal drugs that could be used to control opportunistic fungal infections.

Plants have an almost limitless ability to synthesize secondary chemical substances, which play a pivotal role in their ecophysiology (Briskin, 2000). Accordingly, secondary products may have both a defensive role against herbivores, pathogen attack, and interplay competition and an attractant role toward beneficial organisms such as pollinators or symbionts (Wink and Schimmer, 1999). Some of the secondary-derived compounds may therefore have beneficial effects in the treatment of microbial infections in animals and humans (Cowan, 1999; Kuete et al., 2007). Recently, the interest in these metabolites has increased following searches for new antimicrobial agents from plant sources (Hostettmann et al., 2000).

Southern Africa is exceptionally rich in plant and animal diversity. It has the richest temperate flora in the world, with a floristic diversity of about 24 000 species and intraspecific taxa in 368 families. Only 2.5% of the world's land surface area and contains more than 10% of the world's vascular plant flora (Germishuizen and Meyer, 2003). Southern Africa also contains a major proportion of the 50 500 taxa present in sub-Saharan Africa (Klopper et al., 2006).

From data of approximately 350 plant species tested for biological activity obtained in an ongoing tree screening project of the Phytomedicine Programme, seven plant species (Table 3.1) with minimum inhibitory concentrations of 0.16 mg/ml and lower against *Cryptococcus neoformans* were selected for evaluation of their potential action against other important pathogenic bacteria and fungi. Although the selection was based on previously reported activity against the fungus *Cryptococcus neoformans* (L. Pauw and J.N. Eloff, unpublished data), these plant species are also used by indigenous healers for different disease conditions (van Wyk et al., 2000) and a summary of their traditional usage is presented in Table 3.1. Leaves of the selected tree species were screened for activity against five important fungal pathogens (*Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Microsporum canis* and *Sporothrix schenckii*). Additionally, we also investigated the antibacterial activity of against four important nosocomial bacteria (*Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*). The focus was however, on Aspergillus *fumigatus* one of the most common pathogenic fungal species in humans and animals (Rippon, 1982). It also plays role in the economically important disease aspergillosis in poultry. The aim of this publication was therefore to select the tree species with the best potential for developing a commercially useful antifungal product. To promote the sustainable use of plants only tree leaves were considered in this study.

Table 3.1. Botanical names and traditional use of the plants studied

Botanical name	Family	Voucher specimen number	Traditional medicinal uses	Reference
Combretum vendae A.E. van Wyk	Combretaceae	PRU96507	Leprosy, ophthalmic remedy, and blood purification	Watt and Breyer-Brandwijk, 1962
Commiphora harveyi (Engl.) Engl.	Burseraceae	PRU96506	Used as disinfectant for wounds, anthelmintic and treatment of snake bite	Watt and Breyer-Brandwijk, 1962
Khaya anthotheca (Welm.) C.DC	Meliaceae	PRU96509	Skin diseases, black quarter, helminthosis	Watt and Breyer-Brandwijk, 1962, Nfi et al., 2001
Kirkia wilmsii Engl.	Kirkiaceae	PRU96503	Treatment of malaria and feverish conditions.	Clarkson et al., 2004
Loxostylis alata A. Spreng. ex Rchb.	Anacardiaceae	PRU96508	Stimulation of immune system, relief of pain during child birth	Pooley, 1993; Pell, 2004
Ochna natalitia (Meisn.) Walp.	Ochnaceae	PRU96504	Infusions and decoctions for headache, and respiratory diseases	Watt and Breyer-Brandwijk, 1962
Protorhus longifolia (Bernh. ex C.krauss) Engl.	Anacardiaceae	PRU96505	Treatment of diarrhoea and heartwater	Dold and Cocks, 2001

3.2. Materials and methods

3.2.1. Plant collection

Commiphora harveyi (Engl.) Engl. (Burseraceae), *Combretum vendae* (A.E. van Wyk) (Combretaceae), *Khaya anthotheca* (Welm.) C.DC (Meliaceae), *Loxostylis alata* A. Spreng. ex Rchb. (Anacardiaceae) and *Protorhus longifolia* (Bernh.) Engl. (Anacardiaceae) leaves were collected at the Manie van der Schijff Botanical Garden of the University of Pretoria, South Africa. *Kirkia wilmsii* Engl. (Kirkiaceae) and *Ochna natalitia* (Meisn.) Walp. (Ochnaceae) were collected at the Lowveld National Botanical Garden in Nelspruit, South Africa. All plant leaves were collected in summer (November 2006) between 9:30 am and 12:30 pm. Samples of the plants were identified and authenticated by Lorraine Middleton, the herbarium curator, and Magda Nel at the Botanical Garden of the University of Pretoria. Voucher specimens of the plants were deposited at the Schweikert Herbarium of the Department of Plant Sciences, University of Pretoria, South Africa. The botanical names of the plants, tree reference numbers, and the plant parts used are presented in Table 3.1.

3.2.2. Plant storage

Immediately after collection and transportation to the laboratory, leaves were separated from stems and dried at room temperature under natural ventilation. The dried plant leaves 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.

3.2.3. Plant extraction

Plant samples from each species were individually extracted by weighing four aliquots of 1 g of finely ground plant material and extracting each aliquot with 10 ml of acetone, hexane, dichloromethane (DCM) or methanol (technical grade-Merck) respectively in centrifuge tubes. Tubes were vigorously shaken for 1 h using a Labotec model 20.2 shaking machine at a moderate speed. Extracting at lower speed for a longer period allows the solvent to penetrate more into the plant tissues, allowing the extraction of more of the compounds contained in the plant species (Silva et al., 1998). After centrifuging at 3500 x *g* for 10 min, the supernatant was decanted into pre-weighed labelled glass vials. The whole process was repeated three times on the marc to exhaustively extract the plant material. The solvent was removed under a stream of air in a fume cupboard at room temperature to quantify the extraction.

3.2.4. Microorganisms and medium

The bacterial organisms used in this study were obtained from the Department of Microbiology at the Medical Campus, University of Pretoria. They included the Gram-positive bacteria, *Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212), and the Gram-negative bacteria, *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 27853). All bacterial cultures were maintained on Mueller Hinton (MH) agar and subcultured before use in MH broth (Oxoid, Basingstoke, UK). The five fungal organisms that were used included *Aspergillus fumigatus, Microsporum canis*, *Candida albicans*, *Cryptococcus neoformans* and *Sporothrix schenckii*. All fungal organisms were isolated from animal clinical cases prior to treatment, by the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. These fungi are important disease-causing pathogens of animals and man. Sabouraud dextrose (SD) agar (Oxoid, Basingstoke, UK) was used as the maintenance growth medium for the fungi.

3.2.5. Antimicrobial sensitivity test

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of extract that inhibits visible growth of the micro-organism. For the different microbial species, the MIC was determined using the serial microdilution assay (Eloff, 1998a).

3.2.5.1. Bacterial organisms

Different plant extracts (hexane, acetone, dichloromethane and methanol) were dissolved in acetone to a concentration of 10 mg/ml. Acetone was non-toxic to the micro-organisms at the concentrations used in this assay (Eloff, 1998b; Masoko et al, 2005). One hundred µl of each plant extract were serially diluted 2-fold with sterile distilled water in 96-well microtitre plates. One millilitre of concentrated bacterial culture grown at 37 ºC for 3 days was transferred to 100 ml of fresh MH broth and 100 µl of the resultant culture was added to each well. Densities of bacterial cultures used for the screening were as follows: *S. aureus*, 2.6×10^{12} cfu/ml; *E. faecalis*, 1.5×10^{10} cfu/ml; *P. aeruginosa*, 5.2×10^{13} cfu/ml; *E. coli*, 3.0×10^{11} cfu/ml. Gentamicin at 0.1 mg/ml (Virbac®) and acetone were used as positive and negative control agents, respectively. After incubation overnight at 37 °C, *p*-iodonitrotetrazolium violet (INT, Sigma) at a concentration of 0.2 mg/ml was used as an indicator of bacterial growth. Forty µl of INT was added to each of the microtitre wells. Thereafter, the plates were incubated at 37 °C and the MIC was assessed 1 and 2 h after the addition of INT. Bacterial cultures react with INT and give red or purple colouration within 10-60 min (Eloff, 1998a).

3.2.5.2. Fungal organisms

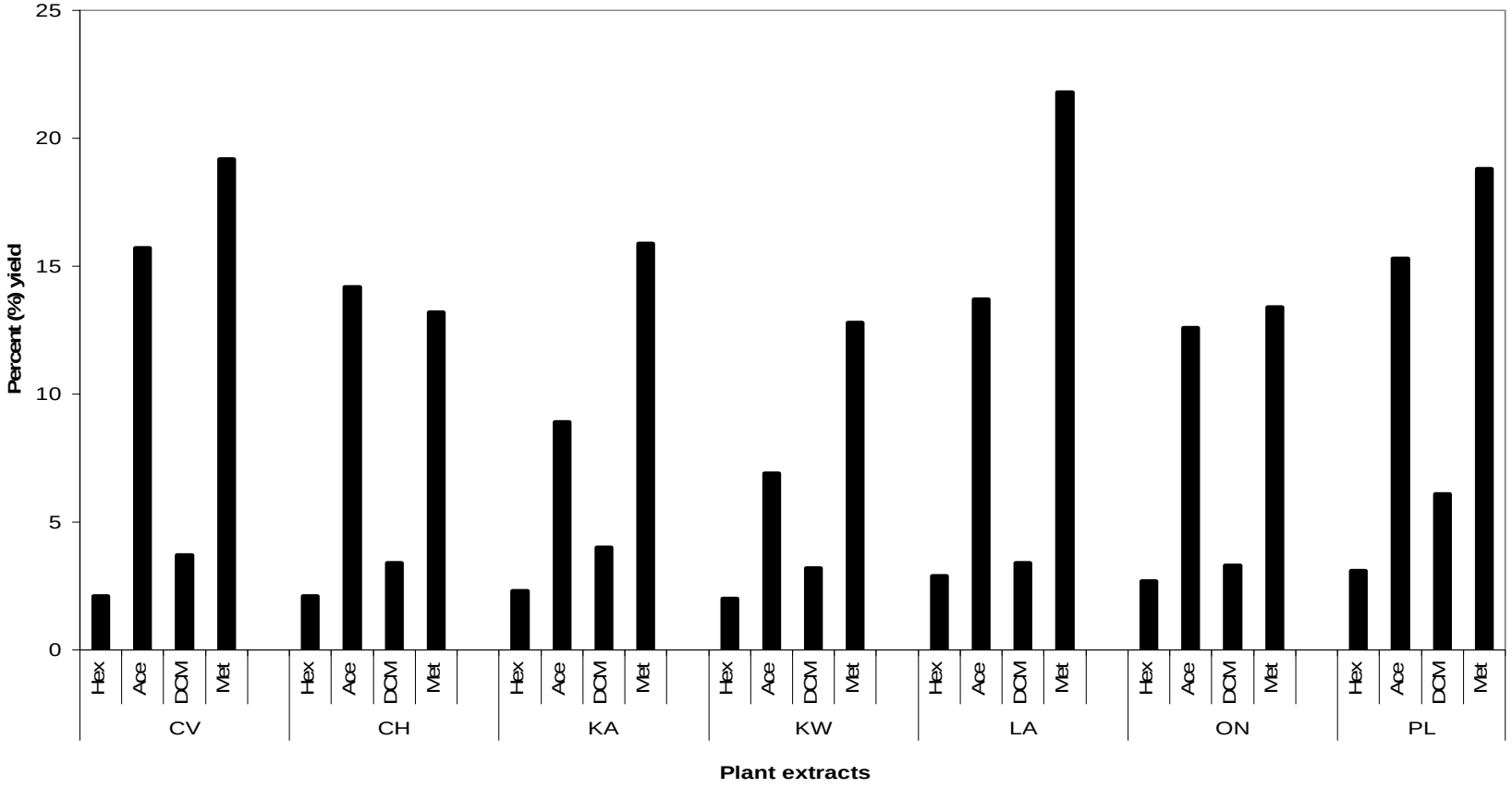
Fungal cultures were transferred from agar culture plates to fresh SD broth and 100 µl of the broth was added to each well. Densities of fungal cultures used for the screening were as follows: *A. fumigatus*, 8.1 × 10^4 cfu/ml; *C. albicans*, 2.5 × 10^4 cfu/ml; *C. neoformans*, 2.6 × 10^4 cfu/ml; *M. canis*, 1.5 × 10^5 cfu/ml; *S. schenckii*, 1.4 × 10^5 cfu/ml. Amphotericin B and acetone were used as positive and negative control substances, respectively. Forty µl of *p*-iodonitrotetrazolium violet INT (0.2 mg/ml) was added to each of the microtitre wells to serve as an indicator of fungal growth. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of extract to inhibit bacterial growth in the microtitre plate as indicated by a reduction in the red colour of the INT formazan (Masoko et al., 2005) and was assessed after 24 and 48 h incubation period.

3.3. Results and discussion

3.3.1. Mass extracted

The amounts of dried plant material extracted by each of the solvents (hexane, acetone, dichloromethane and methanol) used in this study are presented in Figure 3.1. Methanol extracted the highest quantity of plant material. The highest quantity was extracted from *Loxostylis alata* (218 mg) representing 21.8%. The masses extracted by acetone and dichloromethane extracts were generally second and third highest, respectively, in all the plants except *Commiphora harveyi*. The result was comparable to what was reported by Kotze and Eloff (2002) where methanol was the best extractant for *Combretum erythrophyllum* found in South Africa. In contrast, acetone extracted more plant material from *Commiphora harveyi* (142 mg) representing 14.2%. In a study with 27 members of the Combretaceae family, acetone extracted more plant material than the other solvents used (Eloff, 1999). The lowest amount of extract was obtained from the hexane extraction of *Kirkia wilmsii* (20 mg) representing 2% of extractable material. In traditional medical practice, water is used as the major extractant. The implication of this is that potential active compounds that are not hydrophilic may not be extracted and a plant disregarded as not being active biologically, especially if the polar extracts are not active in the bioassay. Such a problem was circumvented in our study by extracting the plant leaves in parallel with solvents of low to high polarity. Acetone was used to re-dissolve the extracts of hexane, acetone, dichloromethane and methanol prior to bioassay.

Figure 3.1. Percentage yield extracted by different solvents from leaves of different South African plant species



Hexane (Hex); acetone (Ace), dichloromethane (DCM) and methanol (Met).
Loxostylis alata (LA), *Kirkia wilmsii* (KW), *Ochna natalitia* (ON), *Khaya anthotheca* (KA), *Combretum vendae* (CV), *Commiphora harveyi* (CH) and *Protorhus longifolia* (PL)

3.3.2. Minimum inhibitory concentration

3.3.2.1. Bacterial species

The plant extracts differed greatly in their activity against the test bacteria and the best bacterial inhibition was observed with MIC = 0.04 mg/ml by the hexane extract of *Khaya anthotheca* against *S. aureus*. There are no validated criteria for the MIC end points for *in vitro* testing of plant extracts. However, an attempt was made to grade MIC of plant extracts/compounds by Holetz et al (2002). He proposed: good antimicrobial activity = MIC less than 0.1 mg/ml; moderate antimicrobial activity = MIC of 0.1 to 0.5 mg/ml; weak antimicrobial activity = MIC of 0.5 to 1 mg/ml; MIC of greater than 1 mg/ml was considered inactive.

Among the tested extracts, the hexane extracts of *Combretum vendae*, *Commiphora harveyi*, *Khaya anthotheca*, *Ochna natalitia* and *Loxostylis alata*, the acetone extracts of *Commiphora harveyi*, *Loxostylis alata* and *Protorhus longifolia*, and the dichloromethane extracts of *Combretum vendae*, *Commiphora harveyi* and *Loxostylis alata* had the best antibacterial activity against at least one of the tested pathogens. The MIC values of these extracts were the lowest, ranging from 0.04 to 0.01 mg/ml (Table 3.2). The extracts of *Loxostylis alata* had very promising results with good antibacterial activity in 3 out of 4 of the extracts tested. The hexane, acetone and dichloromethane extracts of *Loxostylis alata* had MIC values as low as 0.08 mg/ml against *S. aureus*, *E. faecalis* and *E. coli*. The reference antibiotic (gentamicin) had an MIC of 0.025 mg/ml against the mentioned pathogen. Perhaps when the active compound(s) are isolated in pure forms from the crude extracts they might have increased antimicrobial action. The action of most of the extracts appeared to be bacteriostatic, as growth of the bacteria and resulting red colour formation appeared to resume after the 24-h incubation period with INT (Table 3.2).

The Gram-negative organisms (*E. coli* and *P. aeruginosa*) were more resistant to the extracts than the Gram-positive organisms (*S. aureus* and *E. faecalis*) as indicated by their high MIC values. Gram-negative bacteria are relatively resistant to plant extracts owing to the presence of an outer membrane which is known to present a barrier to penetration of numerous antimicrobial molecules, and the periplasmic space contains enzymes which are capable of breaking down foreign molecules introduced from outside (Nikaido, 1996). *S. aureus* exhibited the highest susceptibility to the plant extracts used in other studies conducted (Stickler and King, 1992; Martínez et al., 1996; Chariandy et al., 1999). Similar results were obtained in this study.

Table 3.2. Minimum inhibitory concentrations (average of triplicate determinations) of four different extracts from seven South African plants tested against bacteria. MIC assessment was done 1 and 2 h after INT (indicator of bacterial growth) was added to the bacterial cultures.

Microorganism	Time (h)	MIC (mg/ml)															
		Combretum vendae				*Commiphora harveyi*				*Khaya anthotheca*				*Kirkia wilmsii*			
		H	A	D	M	H	A	D	M	H	A	D	M	H	A	D	M
P. aeruginosa	1	0.31	0.16	0.16	1.25	0.31	0.63	0.63	2.50	0.31	0.16	0.16	1.25	0.16	0.24	0.31	0.63
	2	0.63	0.31	0.16	1.25	0.63	1.25	0.63	2.50	0.63	0.31	0.16	1.25	0.31	0.47	0.63	2.50
S. aureus	1	0.31	0.63	0.31	1.25	0.31	0.63	0.08	2.50	0.04	0.16	0.08	1.25	0.63	0.31	0.63	1.25
	2	0.31	1.25	0.63	2.50	0.31	0.63	0.16	2.50	0.08	0.16	0.08	1.25	1.25	0.31	0.63	1.25
E. coli	1	0.78	0.63	0.31	1.25	2.50	1.25	1.25	2.50	2.50	1.25	1.25	2.50	0.16	0.31	0.63	1.25
	2	1.25	0.63	0.63	2.50	2.50	2.50	1.25	2.50	2.50	2.50	0.63	2.50	0.16	0.31	1.25	2.50
E. faecalis	1	0.08	0.12	0.08	0.16	0.06	0.08	0.08	0.63	0.16	0.16	0.16	1.25	0.31	0.31	0.31	1.25
	2	0.16	0.12	0.08	0.31	0.31	0.24	0.16	1.25	0.16	0.31	0.16	2.50	0.31	0.31	0.31	1.25
Average		0.48	0.48	0.30	1.31	0.87	0.90	0.53	2.11	0.80	0.63	0.34	1.72	0.41	0.32	0.59	1.49

H= hexane extract; A= acetone extract; D= dichloromethane extract; M= methanol extract

Table 3.2. cont...

Microorganism	Time (h)	MIC (mg/ml)												Gentamicin
		Loxostylis alata				*Ochna natalitia*				*Protorhus longifolia*				
		H	A	D	M	H	A	D	M	H	A	D	M	
P. aeruginosa	1	0.31	0.31	0.47	0.31	0.16	0.31	0.31	1.25	0.16	0.08	0.16	2.50	0.015
	2	0.63	0.31	0.47	0.31	0.31	1.25	0.63	1.25	0.63	0.16	0.31	2.50	0.06
S. aureus	1	0.08	0.06	0.63	2.50	0.16	0.31	0.16	1.25	0.63	0.63	0.63	1.25	0.007
	2	0.1	0.06	1.25	2.50	0.16	1.25	0.63	2.50	0.63	0.63	0.31	1.25	0.025
E. coli	1	0.08	0.06	0.63	1.25	0.31	0.63	2.50	2.50	0.63	0.31	0.31	1.25	0.025
	2	0.16	0.08	0.08	1.25	0.63	0.63	1.25	2.50	0.63	0.31	0.63	2.50	0.05
E. faecalis	1	1.25	0.16	0.16	0.16	0.08	0.16	0.24	0.63	0.31	0.63	0.63	1.25	0.003
	2	2.5	0.31	0.63	0.63	0.08	0.16	0.31	1.25	1.25	1.25	0.63	2.50	0.006
Average		0.64	0.17	0.54	1.11	0.24	0.59	0.75	1.64	0.61	0.50	0.45	1.88	

H= hexane extract; A= acetone extract; D= dichloromethane extract; M= methanol extract

3.3.2.2. Fungal species

In the present investigation, the 28 extracts screened had activity against at least one of the test organisms (Table 3.3). The acetone extracts of *Loxostylis alata*, *Kirkia wilmsii*, *Ochna natalitia*, and *Combretum vendae* had high antifungal activity with MIC values ranging from 0.04-0.8 mg/ml against one or more of the tested micro-organisms. In a similar study, a member of the Anacardiaceae family, *Sclerocarya birrea* exhibited very good antifungal activity against some selected fungal pathogens (Hamza et al., 2006). The hexane extracts of *Combretum vendae*, *Khaya anthotheca* and *Ochna natalitia* had MIC values ranging from 0.05-0.09 mg/ml. The hexane extract of *Combretum vendae* had the lowest average MIC of 0.23 mg/ml against all the tested pathogens. As in the bacterial assays, most of the methanol extracts were relatively inactive against all the tested pathogens. However, the methanol extract of the stem bark of *Khaya anthotheca* was reported by Hamza et al (2006) to be very active against *Candida krusei* but inactive against other pathogenic yeast namely *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* and *Cryptococcus neoformans*. The highest average MIC value of 1.73 mg/ml was obtained with the methanol extract of *Ochna natalitia*. Plant extracts with MIC of 0.78 mg/ml against *Candida albicans* are regarded to have good activity (Buwa and van Staden, 2006). Similarly, Hamza et al (2006) reported that extracts having MIC of 0.5 mg/ml or less as being strong inhibitors of fungal growth. Their report was based on classification of MIC earlier reported by Aligiannis et al. (2001) who proposed that plant extracts having MIC of 0.5 mg/ml as strong inhibitors; moderate inhibitors have MIC between 0.6 and 1.5 mg/ml. Extracts having MIC above 1.6 mg/ml are considered weak inhibitors.

M. canis had the highest susceptibility to the extracts, being sensitive to 5 of the tested extracts at concentrations as low as 0.05-0.08 mg/ml, while *C. albicans* had the lowest sensitivity to the plant extracts. *C. neoformans*, *S. schenckii* and *A. fumigatus* were sensitive to 3, 2 and 1 of the tested extracts respectively, with MIC values lower than 0.1 mg/ml. No growth inhibition was detected in the negative control wells. The antifungal activities of the plant extracts screened were not as effective as that of amphotericin B which is the reference compound (Table 3.3). The positive control (amphotericin B) had MIC values of 0.01-0.0003 mg/ml against the tested fungi. Similarly, as was mentioned for antibacterial screening, the action of most of the extracts on fungi appears to be fungistatic, as growth of the organisms and resulting red colour formation appeared to resume after the 48-h incubation period with INT (Table 3.3).

Table 3.3. Minimum inhibitory concentrations (average of triplicate determinations) of four different extracts from seven South African plants tested against some animal pathogenic fungi.

Microorganism	Time (h)	MIC (mg/ml)															
		Combretum vendae				*Commiphora harveyi*				*Khaya anthotheca*				*Kirkia wilmsii*			
		H	A	D	M	H	A	D	M	H	A	D	M	H	A	D	M
A. fumigatus	24	0.31	0.26	0.31	0.63	1.88	2.5	1.25	2.50	0.31	0.31	0.31	0.13	0.16	0.13	0.21	1.04
	48	0.42	0.37	0.31	0.63	2.5	2.5	1.67	2.50	1.25	1.67	1.25	2.08	1.25	1.25	2.50	2.50
C. albicans	24	0.21	0.26	0.26	0.63	0.52	0.16	0.32	1.25	0.63	0.83	0.63	1.67	0.31	0.83	1.25	1.25
	48	0.31	0.31	0.63	1.25	0.52	0.13	0.37	2.08	2.08	1.46	1.67	2.50	0.83	1.25	1.67	2.50
C. neoformans	24	0.08	0.31	0.31	1.25	2.08	0.21	1.25	0.84	0.05	0.16	0.31	1.04	1.25	0.07	0.63	0.31
	48	0.16	0.63	1.67	2.50	2.08	0.63	2.50	0.84	0.11	0.31	0.63	2.50	2.50	0.13	2.08	1.25
M. canis	24	0.16	0.08	0.11	0.31	0.11	0.16	0.63	1.25	0.21	0.26	0.63	1.04	1.04	0.08	0.84	1.25
	48	0.31	0.21	0.11	1.25	0.16	0.16	1.67	2.50	0.31	0.37	1.88	2.50	2.50	0.37	1.04	2.50
S. schenckii	24	0.13	0.31	0.63	1.11	0.63	0.31	1.04	2.50	0.16	0.11	0.63	0.94	1.25	0.26	1.25	0.31
	48	0.16	0.63	0.63	2.5	1.67	0.84	2.08	2.50	0.26	0.16	1.25	1.04	2.50	0.63	2.50	2.50
Average		0.23	0.34	0.50	1.21	1.22	0.76	1.28	1.88	0.54	0.56	0.92	1.54	1.50	0.50	1.40	1.54

H= hexane extract; A= acetone extract; D= dichloromethane extract; M= methanol extract

Table 3.3 cont...

Microorganism	Time (h)	MIC (mg/ml)												Amphotericin B (mg/ml)
		Loxostylis alata				*Ochna natalitia*				*Protorhus longifolia*				
		H	A	D	M	H	A	D	M	H	A	D	M	
A. fumigatus	24	1.25	0.05	0.52	0.42	2.08	1.25	1.25	2.50	1.67	0.31	0.63	1.25	0.005
	48	2.08	0.16	1.25	2.50	2.50	2.50	2.08	2.50	2.50	2.50	1.67	2.50	0.01
C. albicans	24	2.50	0.31	1.25	1.67	0.31	1.25	1.25	1.25	2.50	1.67	0.52	1.25	0.025
	48	2.50	1.04	2.50	1.67	0.31	1.25	2.08	2.50	2.08	1.67	0.52	1.25	0.005
C. neoformans	24	1.67	0.21	0.63	1.67	0.84	0.31	0.42	1.67	2.08	0.21	0.52	0.31	0.00063
	48	2.50	0.52	1.25	2.50	1.25	1.25	0.62	2.50	2.50	0.31	0.63	0.63	0.025
M. canis	24	1.04	0.07	1.04	0.63	0.08	0.07	0.11	0.13	0.31	0.63	0.94	0.31	0.00031
	48	2.50	0.07	1.25	1.25	0.52	0.09	0.52	0.52	1.04	1.67	1.67	2.50	0.00063
S. schenckii	24	1.25	0.04	0.13	1.25	0.08	0.26	0.42	1.25	1.04	1.25	0.84	1.25	0.00063
	48	2.50	0.08	0.31	2.5	0.21	0.31	1.25	2.50	2.50	1.25	0.84	2.50	0.005
Average		1.98	0.26	1.01	1.61	0.82	0.85	1.00	1.73	1.82	1.15	0.88	1.38	

H= hexane extract; A= acetone extract; D= dichloromethane extract; M= methanol extract

3.3.3. Total activity

For ethnopharmacological research to be locally relevant, not only the MIC is important, but also the quantity extracted from each plant species. Total activity is calculated by dividing the quantity extracted from one gram of plant material with the MIC value in mg/ml (Eloff, 2001). This value indicates the volume to which the active constituent present in one gram of the plant material can be diluted and still inhibit the growth of the test organism.

For bacterial organisms, the total activity of the plants ranged from 5 to 2283 ml/g (Table 3.4). The highest total activity of 2283 mg/ml was produced by the acetone extract of *Loxostylis alata* against *S. aureus* and *E. faecalis*. It therefore means that 1 gram of *Loxostylis alata* acetone extract can be diluted in 2283 ml of the solvent used and still inhibit the growth of the organisms. Similarly, the total activities of the plant extracts against fungi ranged from 5-3425 ml/g (Table 3.5). *Loxostylis alata* was the most active, with the acetone extract having a total activity of 3425 ml/g against *Microsporum canis* over an incubation period of 24 h. This is a step towards the rational use of plants in traditional primary health care and could be of benefit in enabling rural use of the plants as information regarding the usefulness of the plant could be handed to rural people. Higher values of total activity indicate increased usefulness and potential economic value.

This study investigated the *in vitro* antimicrobial activity of selected plant species, and has supplied preliminary evidence of the efficacy of these plant species for the traditional treatment of various bacterially-related diseases. However, *in vivo* data is necessary in determining the potential usefulness of these plants for treatment of infectious diseases. One of the inherent problems associated with *in vitro* testing is the absence of body metabolic processes. More importantly, factors such as absorption and metabolism may be responsible for discrepancies between *in vitro* and *in vivo* activity (Houghton et al., 2007). However, *in vitro* activity may serve as a lead towards the discovery of plant chemical agents that are potentially active *in vivo*.

In terms of conservation, the results revealed that leaf material of these plants is useful for antimicrobial uses because this material can be used without any detrimental effect on the plant (Holetz et al., 2002).

Table 3.4. Total activity in ml/g of seven South African plants screened for antibacterial activity.

Microorganism	Time (h)	Total activity (ml/g)															
		Combretum vendae				*Commiphora harveyi*				*Khaya anthotheca*				*Kirkia wilmsii*			
		H	A	D	M	H	A	D	M	H	A	D	M	H	A	D	M
P. aeruginosa	1	68	981	231	154	68	225	54	5	74	556	250	127	125	288	103	203
	2	33	506	231	154	33	114	54	5	37	287	250	127	65	147	51	51
S. aureus	1	68	249	119	154	68	225	425	5	575	556	500	127	32	223	51	102
	2	68	126	30	77	68	225	213	5	288	556	500	127	16	223	51	102
E. coli	1	27	249	119	154	8	114	27	5	9	71	32	64	125	223	51	102
	2	17	249	59	77	8	57	27	5	9	36	63	64	125	223	26	51
E. faecalis	1	263	1308	463	1200	350	1775	425	21	144	556	250	127	65	223	103	102
	2	131	1308	463	619	68	592	213	11	144	287	250	64	65	223	103	102
Average		84	622	214	323	84	416	180	8	160	363	262	103	77	221	67	102

H= hexane extract; A= acetone extract; D= dichloromethane extract; M= methanol extract

Table 3.4 cont...

Microorganism	Time (h)	Total activity (ml/g)											
		Loxostylis alata				*Ochna natalitia*				*Protorhus longifolia*			
		H	A	D	M	H	A	D	M	H	A	D	M
P. aeruginosa	1	94	442	72	703	169	406	106	107	194	1913	381	75
	2	46	442	72	703	87	101	52	107	49	956	197	75
S. aureus	1	363	2283	54	87	169	406	206	107	49	243	97	150
	2	290	2283	27	87	169	101	52	54	49	243	197	150
E. coli	1	363	2283	54	174	87	200	13	54	49	494	197	150
	2	181	1713	425	174	43	200	26	54	49	494	97	75
E. faecalis	1	23	856	213	1363	338	788	138	213	100	243	97	150
	2	12	442	54	346	338	788	106	107	25	122	97	75
Average		171	1343	121	455	175	374	88	100	71	588	170	113

H= hexane extract; A= acetone extract; D= dichloromethane extract; M= methanol extract

Table 3.5. Total activity in ml/g of seven South African plants screened for antifungal activity.

Microorganism	Time (h)	Total activity (ml/g)															
		Combretum vendae				*Commiphora harveyi*				*Khaya anthotheca*				*Kirkia wilmsii*			
		H	A	D	M	H	A	D	M	H	A	D	M	H	A	D	M
A. fumigatus	24	68	604	119	305	11	57	27	5	74	287	129	1223	125	531	152	123
	48	50	424	119	305	8	57	20	5	18	53	32	76	16	55	13	51
C. albicans	24	100	604	142	305	40	888	106	11	37	107	63	95	65	83	26	102
	48	68	506	59	154	40	1092	92	6	11	61	24	64	24	55	19	51
C. neoformans	24	263	506	119	154	10	676	27	16	460	556	129	153	16	986	51	413
	48	131	249	15	77	10	225	14	16	209	287	63	64	8	531	15	102
M. canis	24	131	1963	119	619	191	888	54	11	110	342	63	153	19	863	38	102
	48	68	748	30	154	131	888	20	5	74	241	21	64	8	186	31	51
S. schenckii	24	162	506	33	173	33	458	33	5	144	809	63	169	16	265	26	413
	48	131	249	15	77	13	169	16	5	88	556	32	153	8	110	13	51
Average		117	636	77	232	49	540	41	9	123	330	62	221	30	366	38	146

H= hexane extract; A= acetone extract; D= dichloromethane extract; M= methanol extract

Table 3.5. cont...

Microorganism	Time (h)	Total activity (ml/g)											
		Loxostylis alata				*Ochna natalitia*				*Protorhus longifolia*			
		H	A	D	M	H	A	D	M	H	A	D	M
A. fumigatus	24	23	2740	65	519	13	101	26	54	19	494	97	150
	48	14	856	27	87	11	50	16	54	12	61	37	75
C. albicans	24	12	442	27	131	87	101	26	107	12	92	117	150
	48	12	132	14	131	87	101	16	54	15	92	117	150
C. neoformans	24	17	652	54	131	32	406	79	80	15	729	117	606
	48	12	263	27	87	22	101	53	54	12	494	97	298
M. canis	24	28	1957	33	346	338	1800	300	1031	100	243	65	606
	48	12	1957	27	174	52	1400	63	258	30	92	37	75
S. schenckii	24	23	3425	27	174	338	485	79	107	30	122	73	150
	48	12	1713	14	87	129	406	26	54	12	122	73	75
Average		16	1414	32	187	111	495	68	185	26	254	83	234

H= hexane extract; A= acetone extract; D= dichloromethane extract; M= methanol extract

3.4. Conclusion

The plant extracts tested had varying levels of activity against bacteria. MIC data revealed that the hexane extract of *Khaya anthotheca* had the highest antibacterial activity with lowest MIC against *Staphylococcus aureus*, while the acetone extract of *Loxostylis alata* exhibited the most antifungal activity with lowest MIC against *Sporothrix schenckii*. From this study, we can infer that the South African flora offers a good potential as a source of antimicrobial agents. The bioassay-guided fractionation procedure to isolate and characterise active compounds from *Loxostylis alata* and other active plants is currently being undertaken.

Postscript

All the plant species possess varying degree of antibacterial and antifungal activities. We therefore went further to determine the number of antimicrobial compounds present in each extract. That will assist in further designing a strategy for isolating the active plant components.

Chapter 4
Detection of antimicrobial compounds by direct bioautography of different extracts of leaves of selected South African plant species

M.M. Suleiman, L.J. McGaw, V. Naidoo and J.N. Eloff

Preface

In addition to the antifungal activity one of the most important aspects to consider in selecting the species to work on is how many antifungal compounds are present in extracts that have promising. In some cases the antifungal activity is due to the presence of a mixture of compounds because no activity is found after the compounds are separated and evaluated by bioautography. This makes it impossible to isolated antifungal compounds. Bioautography is therefore a very important step in selecting the best species to work on. The text in this chapter was submitted and accepted as manuscript for publication to African Journal of Traditional, Complementary and Alternative Medicines, Vol. 7, pages 64-78.

Abstract

The hexane, acetone, dichloromethane and methanol extracts of *Combretum vendae* A.E. van Wyk (Combretaceae), *Commiphora harveyi* (Engl.) Engl. (Burseraceae), *Khaya anthotheca* (Welm.) C.DC (Meliaceae), *Kirkia wilmsii* Engl. (Kirkiaceae), *Loxostylis alata* A. Spreng. ex Rchb. (Anacardiaceae), *Ochna natalitia* (Meisn.) Walp. (Ochnaceae) and *Protorhus longifolia* (Bernh. Ex C. Krauss) Engl. (Anacardiaceae) were screened for their antimicrobial activity. The test organisms included bacteria (*Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*), and fungi (*Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Microsporum canis* and *Sporothrix schenckii*). A simple bioautographic procedure, involving spraying suspensions of the bacteria or fungi on thin layer chromatography (TLC) plates developed in solvents of varying polarities was used to detect antibacterial and antifungal activity of the extracts. All the extracts had varying levels of antimicrobial activity against at least one of the test microorganisms. This activity was denoted by white spots against a red-purple background on the TLC plates after spraying with a tetrazolium salt indicator. Twenty seven TLC plates; 9 for each solvent system and 3 different solvent systems per organism were tested in the bioautographic procedure. Of the bacteria tested, *S. aureus* also appeared to be the most susceptible organism, being inhibited by almost all the compounds separated on the TLC plates from all the tested plants. Similarly, *C. neoformans* depicted the highest susceptibility among fungal organisms.
Loxostylis alata appeared to be the most active plant with the highest number of inhibition zones when compared with other plants tested against both bacteria and fungi.

Keywords: Bioautography; Medicinal plants; South Africa; Plant extracts; R_f values

4.1. Introduction

Despite the existence of conventional antimicrobial agents, resistant or multi-resistant strains of pathogenic microorganisms are continuously appearing, imposing the need for a thorough search for and development of new drugs (Silver and Bostian, 1993). Fungi and bacteria cause important human and animal diseases, especially in tropical and subtropical regions, and commonly occur in immunocompromised or immunodeficient patients. Over the last decade, there has been a renewed interest in plants; and the pharmaceutical industry considers plants as a viable option for the discovery of new leads (Soejarto, 1996). In fact, it is also estimated that natural products are implicated in the development of 44% of all new drugs, generally as leads for the preparation of semi-synthetic derivatives (Hostettmann et al., 2000 and 2001).

In an effort to discover new lead compounds, many research groups screen plant extracts to detect secondary metabolites with relevant biological activities. In this regard, several bioassays were developed for screening purposes (Hostettmann 1991).

Once the technique has been mastered, bioautography can be considered a highly efficacious assay for the detection of antimicrobial compounds because it allows localization of activity even in a complex matrix, and therefore permits a target-directed isolation of the active constituents (Rahalison et al., 1991). Bioautography has enabled rapid progress for quick detection of new antimicrobial compounds from plants and other natural products. This technique allows the localization of antimicrobial activity directly on a chromatographic plate where the organism is applied (Navarro et al 1998). The method is fast, cheap, and permits a better bioassay-directed fractionation of bioactive compounds (Hamburger and Cordell, 1987). Bioautography is particularly important to avoid the time-consuming isolation of known substances or inactive ones. TLC bioautographic methods combine chromatographic separation and *in situ* activity determination facilitating the localization and target-directed isolation of active constituents in a mixture (Shahverdi et al., 2007). A number of bioautographic assays have been developed, which can be divided into three groups (Rios et al., 1988). These include direct bioautography; where the microorganisms grow directly on thin-layer chromatography (TLC) plates, contact bioautography; where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact, and agar overlay or immersion bioautography; where a seeded agar medium is applied onto the TLC plate. The latter technique can be considered as a hybrid of direct and contact bioautography (Islam et al., 2003).

The purpose of our research was to determine the antimicrobial activity of hexane, dichloromethane, acetone and methanol extracts of seven South African tree leaves by direct bioautography. In the ongoing tree screening project of the Phytomedicine Programme, University of Pretoria (www.up.ac.za/phyto), plant species that had excellent activity against *Candida albicans* depicted by low minimum inhibitory concentrations (≤ 0.08 mg/ml) in a broth microdilution assay (Eloff, 1998a) were selected for evaluation of their potential antimicrobial activity against other animal fungal and bacterial organisms. The localization of active compounds contained in these extracts will assist in directing methods for their isolation.

4.2. Materials and methods

4.2.1. Plant collection

Combretum vendae, *Commiphora harveyi*, *Khaya anthotheca* and *Loxostylis alata* leaves were collected at the Manie van der Schijff Botanical Garden of the University of Pretoria, South Africa. *Kirkia wilmsii* and *Ochna natalitia* were collected at the Lowveld National Botanical Garden in Nelspruit, South Africa. All plant leaves were collected in summer (November 2006) between 9:30 am and 12:30 pm. Samples of the plants were identified and authenticated by Ms Lorraine Middleton and Magda Nel of the University of Pretoria. Voucher specimens of the plants were deposited at the Schweikert Herbarium of the Department of Plant Sciences, University of Pretoria, South Africa.

4.2.2. Plant storage

Immediately after collection and transportation to our laboratory, leaves were separated from stems and dried at room temperature with good ventilation. 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 use.

4.2.3. Plant extraction

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 either acetone, hexane, dichloromethane (DCM) or methanol (technical grade-Merck) in centrifuge tubes. Tubes were vigorously shaken for 1 hour in a Labotec model 20.2 shaking machine at a moderate speed. Extracting plant powdered material at low speed for a longer period allows greater penetration of the solvent into the plant tissues which allows more of the plant compounds to be extracted (Silva et al., 1998). After centrifuging at 3500 x g for 10 min, the supernatant was decanted into pre-weighed labelled containers. The whole process was repeated three

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.

4.2.4. Microorganisms and medium

The fungal organisms used in this study were moulds (*Aspergillus fumigatus* and *Microsporum canis*), yeast (*Candida albicans* and *Cryptococcus neoformans*) and a thermally dimorphic fungus (*Sporothrix schenckii*). All fungal organisms were isolated from clinical cases that were not treated prior to sampling in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. *A. fumigatus* was isolated from a chicken, *C. albicans* from a Goldian finch, *C. neoformans* from a cheetah, *M. canis* from a cat suffering from dermatophytosis and *Sporothrix schenckii* from a horse with cutaneous lymphangitis. These fungi represent the most common and important disease-causing fungi of animals (Masoko et al., 2005). Sabouraud dextrose (SD) agar (Oxoid, Basingstoke, UK) was used as the maintenance growth medium for all the fungal strains used, and the fungi were cultured in SD broth.

The bacteria used were the Gram-positive bacteria: *Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212), and the Gram-negative bacteria: *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 27853). All bacterial cultures were maintained on Mueller Hinton (MH) agar (Oxoid, Basingstoke, UK) at 4°C and cultured in MH broth at 37°C.

4.2.5. Phytochemical analysis

Chemical constituents of the extracts were separated on aluminium-backed thin layer chromatography (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, 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/ammonia hydroxide (18:2:0.2): [BEA] (non-polar/basic) (Kotze and Eloff, 2002). Separated chemical compounds were detected using acidified vanillin (0.1 g vanillin: 28 ml methanol:1ml sulphuric acid) as a spray. After spraying, the chromatograms were heated at 110°C in an incubator to allow for optimal colour development.

4.2.6. Bioautography

Ten µl (10 mg/ml) of each extract were loaded onto TLC plates and eluted using the three different mobile solvent systems (CEF, BEA and EMW). The developed plates were dried under a stream of fast moving air for 5 days to remove traces of solvent on the plates. One week old cultures of fungal organisms grown on SD agar were each transferred into 250 ml of freshly prepared SD broth using a sterile swab. Densities of fungal cultures used for *A. fumigatus*, *C. albicans*, *C. neoformans*, *M. canis* and *S. schenckii* were approximately 8 $\times 10^6$, 3 $\times 10^6$, 3 $\times 10^6$, 2 $\times 10^5$ and 1 $\times 10^5$ cells/ml respectively, In the case of bacteria, overnight cultures grown on MH broth were used and the densities of bacterial organism used for *E. faecalis*, *E. coli*, *P. aeruginosa* and *S. aureus* were approximately 2 $\times 10^{10}$, 3 $\times 10^{11}$, 5 $\times 10^{13}$ and 3 $\times 10^{12}$ cfu/ml, respectively. The prepared chromatograms were sprayed with the fungal or bacterial suspension until wet. This process was carried out in a biosafety Class II cabinet (Labotec, SA) for fungi, and Laminar flow cabinet (Labotec, SA) for bacteria. Thereafter, the plates were incubated overnight at 35°C and 100% relative humidity in the dark and then sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet (Sigma®) (INT) (Begue and Klein, 1972) and further incubated overnight or longer in the case of *S. schenckii* and *M. canis*. White areas or spots 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 organisms.

4.3. Results and discussions

Phytochemical screening revealed the presence of varied chemical components in the different extracts of the plants. This is notable from the different colour changes depicted by individual compounds due to their reaction with the spray reagent used (vanillin/sulphuric acid) (Figure 4.1).

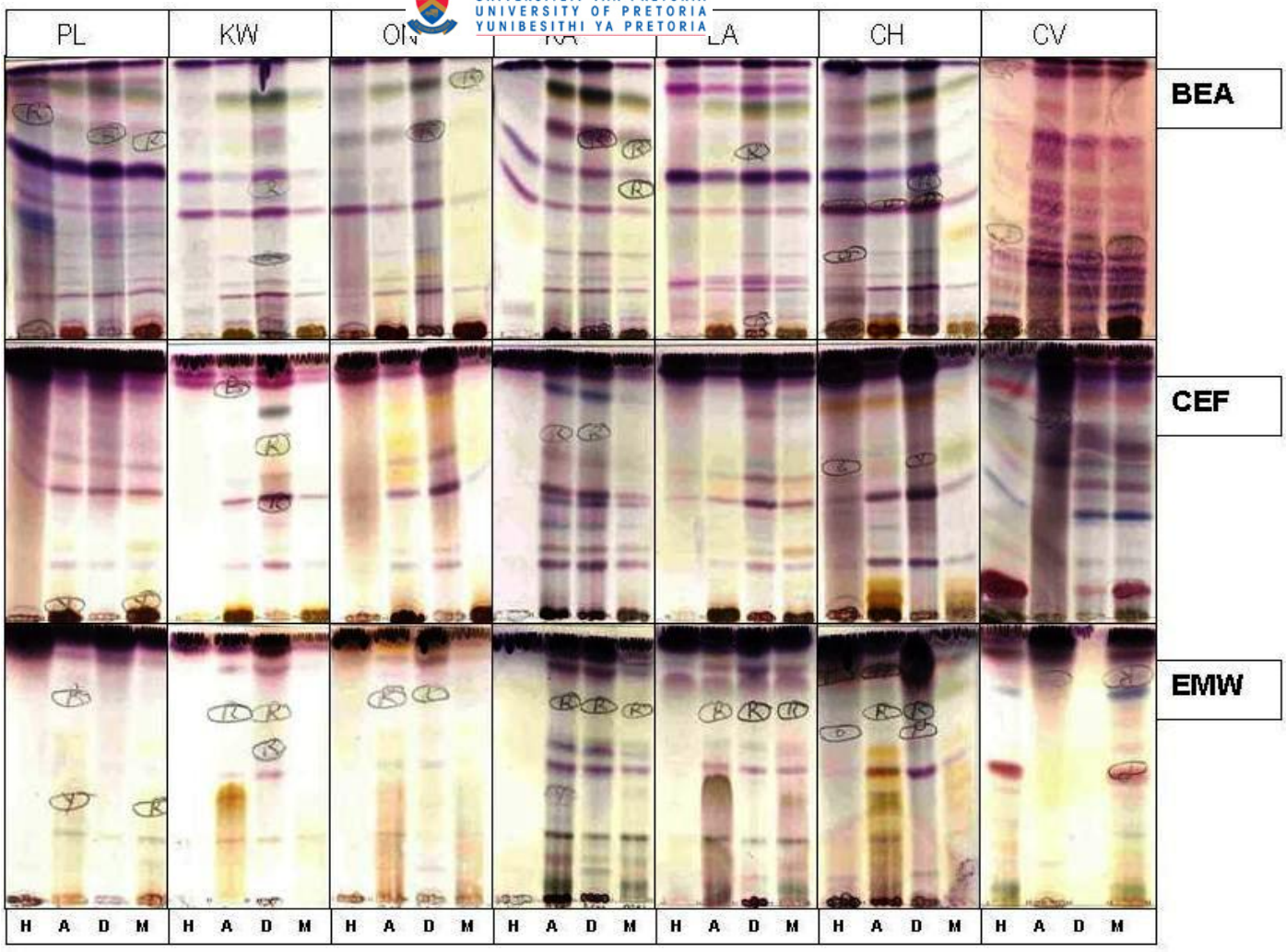


Figure 4.1. Chromatograms of extracts of hexane (H), acetone (A), dichloromethane (D) and methanol (M) developed in Benzene/Ethanol/Ammonia hydroxide: 90:10:1 [BEA] (non-polar/basic), Chloroform/Ethyl acetate/Formic acid: 5:4:1 [CEF] (intermediate polar/acidic) and Ethyl acetate/Methanol/Water: 40:5.4:4 [EMW] (polar/neutral) and sprayed with vanillin in concentrated sulphuric acid. PL = *P. longifolia*, KW = *Kirkia wilmsii*, ON = *Ochna natalitia*, KA= *Khaya anthotheca*, LA = *Loxostylis alata*, CH = *Commiphora harveyi*, CV = *Combretum vendae*.

For example, terpenes exhibit red or blue colouration on the chromatograms when sprayed with vanillin/sulphuric (Gibbons and Gray, 1998). Similarities exist between chemical compositions of the components of extracts separated using the same solvent system (Figure 4.1). Dellar et al. (1994) reported the isolation of antifungal sesquiterpenes aristolen-2-one and prostatherol from 2 species of *Prostanthera* (Labiatae). Studies into the effects of terpenoids on isolated bacterial membranes revealed their site of action to be at the phospholipid bilayer. They affect bacterial processes that include the inhibition of electron transport, protein translocation, phosphorylation steps and other enzyme-dependent reactions (Knobloch *et al*. 1986). Perhaps similar mechanisms of action were responsible for the antimicrobial actions of the plant extracts under study.

The appearance of white areas against a purple-red background on the chromatograms denotes inhibition of growth of the bacteria (Figure 3.2) or fungi (Figure 4.3) due to presence of compound(s) that inhibit their growth. Actively growing microorganisms have the ability to reduce INT to a purple-red colour (Begue and Klein, 1972). In the presence of active plant compounds on the chromatograms, the growth of the organism is inhibited.

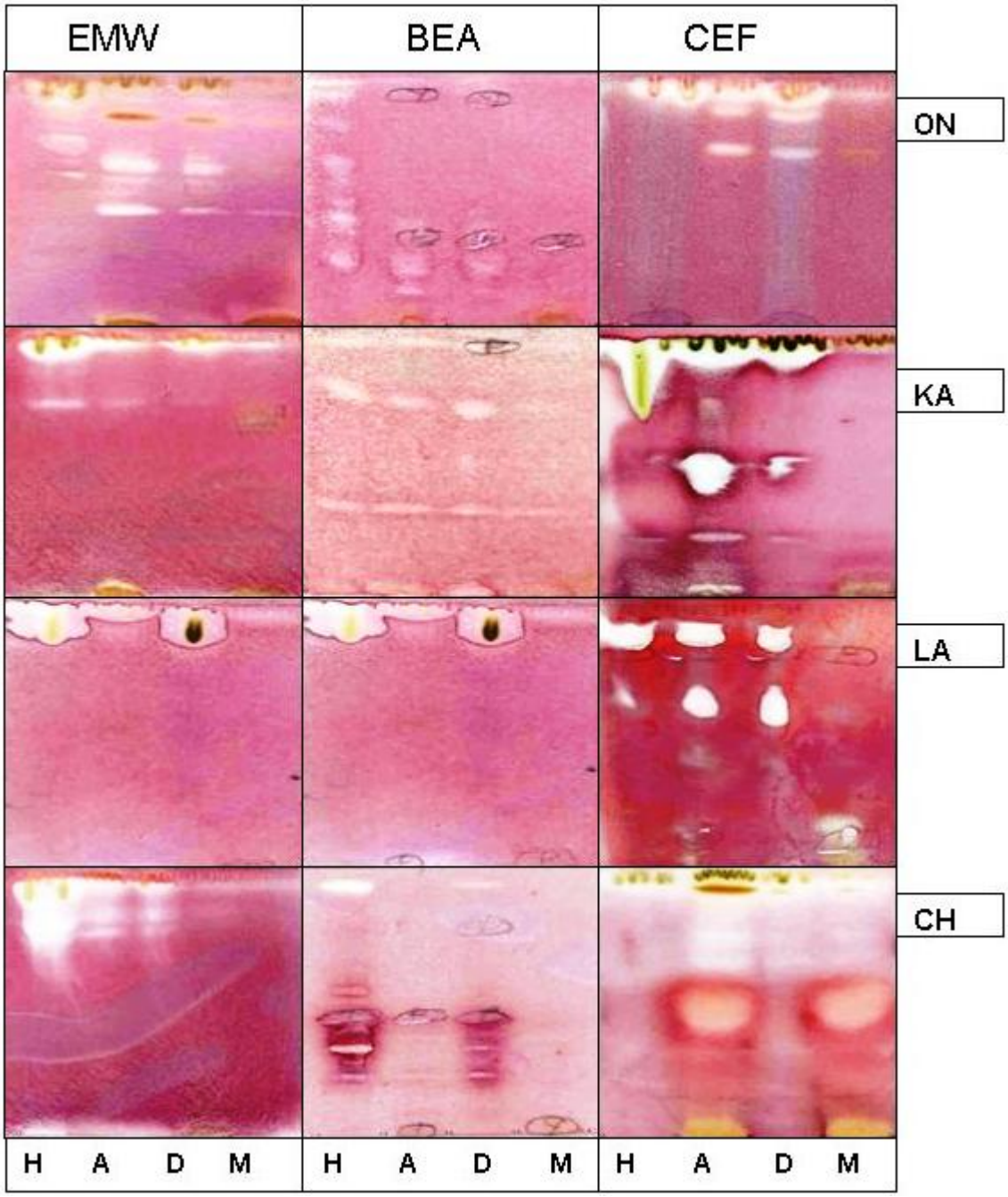


Figure 4.2. Hexane (H), acetone (A), dichloromethane (D), and methanol (M) extracts of *Ochna natalitia* (ON), *Khaya anthotheca* (KA) *Loxostylis alata* (LA) and *Commiphora harveyi* (CH) separated on TLC plates using EMW, BEA and CEF, sprayed with bacterial organisms and 24 hrs later by INT. White areas indicate inhibition of bacterial growth by compounds of the plant extract after 60 minutes of incubation at 37°C. ON and KA were sprayed with *E. coli* while LA and CH were sprayed with *S. aureus*.

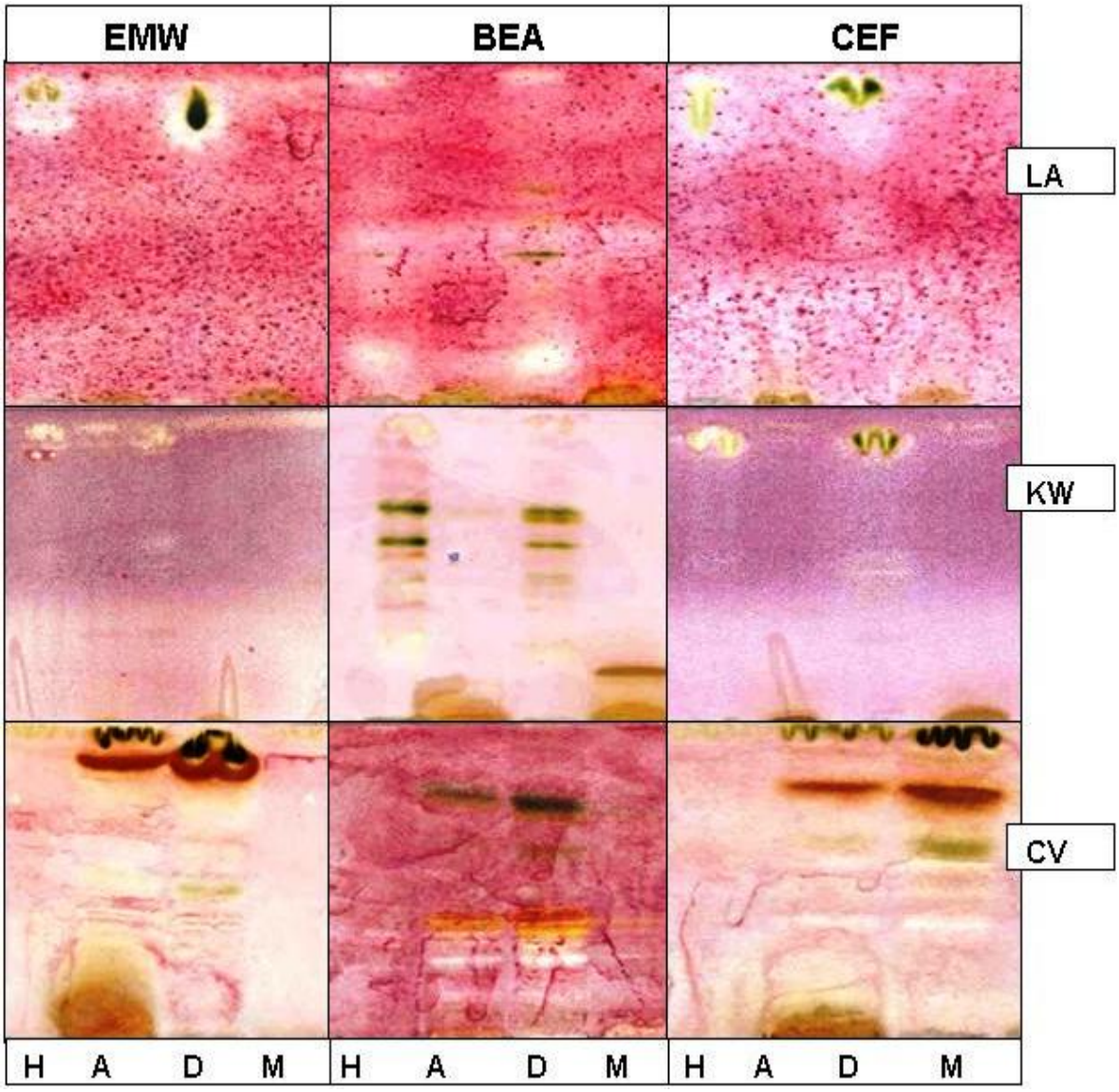


Figure 4.3. Hexane (H), acetone (A), dichloromethane (D), and methanol (M) extracts of *Loxostylis alata* (LA), *Kirkia wilmsii* (KW) and *Combretum vendae* (CV) separated on TLC plates using EMW, BEA and CEF, sprayed with fungal organisms and 24 hours later by INT. White areas indicate inhibition of fungal growth by compounds of the plant extract after 24 hrs of incubation at 37 °C. LA, KW and CV were sprayed with *A. fumigatus*, *C. albicans* and *S.schenkii*, respectively.

An important factor in quantifying the movement of a compound on a stationary phase e.g. silica with a certain solvent system is the R_f (relative front) value and is the ratio of the distance moved by the compound from its origin to the movement of the solvent from the origin. R_f values are less than 1 as they are ratios (Gibbons and Gray, 1998). As expected, compounds that had relatively high R_f values in polar solvents e.g. EMW, depicted low R_f values in non-polar solvents like BEA. As a consequence of development, compounds of a mixture will separate according to their relative polarities on TLC. Generally, based on the R_f values, the same compounds may be active against both fungi and bacteria. This suggests that the antimicrobial activity of compounds is not selective. The R_f values of the extracts eluted with

different solvents against *S. aureus*, *E. faecalis*, *E. coli*, *P. aeruginosa*, *A. fumigatus*, *S. schenckii*, *M. canis*, *C. neoformans* and *C. albicans* are presented on Tables 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8 and 4.9, respectively. In some cases, no inhibition of microbial growth was observed. The absence of activity could be due to evaporation of the active compounds, photo-oxidation or due to very little amount of the active compound (Masoko and Eloff, 2005).

Table 4.1. Inhibition of growth on bioautographic TLC plates by 24 extracts of 7 South African tree leaves against *S. aureus*.

Plant	Solvent system	Extracts	R_f values	Active bands	Total
L. alata	BEA	Hexane	0.92, 0.83, 0.47, 0.26	4	21
		Acetone	0.13	1	
		DCM	0.92, 0.83, 0.47, 0.26, 0.13	5	
	CEF	Hexane	0.53, 0.51	2	
		Acetone	0.1	1	
		DCM	0.53, 0.51, 0.2	3	
	EMW	Hexane	0.31, 0.2,	2	
		Acetone	0.83	1	
		DCM	0.31,0.2	2	
P. longifolia	BEA	Hexane	0.172	1	8
		Acetone	0.27	1	
		DCM	0.26	1	
	CEF	Hexane	0.31	1	
		Acetone	0.34	1	
	EMW	Hexane	0.72, 0.81	2	
		Acetone	0.63	1	
K. wilmsii	BEA	Acetone	0.23	1	5
		Methanol	0.62	1	
	CEF	Acetone	0.23	1	
		Acetone	0.54, 0.08	2	
		Methanol	0.08	1	
C. harveyi	BEA	Hexane	0.95, 0.32	2	11
		DCM	0.95, 0.32, 0.21	3	
	CEF	Hexane	0.41	1	
		DCM	0.41	1	
		Methanol	0.32	1	
	EMW	Hexane	0.31	1	
		DCM	0.31	1	
		Methanol	0.72	1	
O. natalitia	BEA	DCM	0.1	1	5
	CEF	Hexane	0.65	1	
		Acetone	0.76	1	
		DCM	0.97	1	
	EMW	Acetone	0.97	1	
K. anthotheca	BEA	Hexane	0.94	1	4
		DCM	0.94	1	
	CEF	Hexane	0.2	1	
		DCM	0.2	1	
C. vendae	BEA	Hexane	0.21	1	8
		Acetone	0.21	1	
		DCM	0.12	1	
		Methanol	0.32	1	
	CEF	Hexane	0.74	1	
		Acetone	0.41	1	
		DCM	0.65	1	
		Methanol	0.41	1	

Table 4.2. Inhibition of growth on bioautographic TLC plates by 24 extracts of 7 South African tree leaves against *E. faecalis*.

Plant	Solvent system	Extracts	R_f values	Active bands	Total
L. alata	BEA	Hexane	0.78, 0.82	2	6
		DCM	0.82	1	
		Acetone	0.16	1	
		DCM	0.67	1	
	EMW	Acetone	0.45	1	
	BEA	Acetone	0.56	1	
P. longifolia	CEF	Acetone	0.98	1	2
K. wilmsii	BEA	Hexane	0.34	1	3
		Acetone	0.56	1	
		Methanol	0.67	1	
O. natalitia	CEF	Hexane	0.91	1	3
		Acetone	0.91, 0.57	2	
K. anthotheca	BEA	Acetone	0.24	1	3
	CEF	Hexane	0.94	1	
		Acetone	0.76	1	
C. vendae	BEA	Hexane	0.56, 0.67	2	3
	CEF	Hexane	0.97	1	

Table 4.3. Inhibition of growth on bioautographic TLC plates by 24 extracts of 7 South African tree leaves against *E. coli*.

Plant	Solvent system	Extracts	R_f values	Active bands	Total
L. alata	BEA	Hexane	0.62	1	5
		DCM	0.62, 0.21	2	
	CEF	DCM	0.67	1	
	EMW	DCM	0.98	1	
P. longifolia	BEA	Acetone	0.76	1	6
		DCM	0.95	1	
	CEF	Acetone	0.33	1	
		DCM	0.33	1	
		Hexane	0.23	1	
	EMW	DCM	0.23	1	
K. wilmsii	CEF	Methanol	0.87	1	2
		Acetone	0.24	1	
O. natalitia	BEA	DCM	0.95	1	3
		Hexane	0.95	1	
	EMW	DCM	0.27	1	
K. anthotheca	CEF	Hexane	0.93	1	5
		Acetone	0.93, 0.67	2	
		DCM	0.93	1	
	EMW	Methanol	0.34	1	
C. vendae	BEA	Hexane	0.40, 0.2	2	8
		Acetone	0.13	1	
		DCM	0.26	1	
	CEF	Hexane	0.87	1	
		Acetone	0.98	1	
		DCM	0.98	1	
		Methanol	0.56	1	

Table 4.4. Inhibition of growth on bioautographic TLC plates by 24 extracts of 7 South African tree leaves against *P. aeruginosa*.

Plant	Solvent system	Extracts	R_f values	Active bands	Total
L. alata	EMW	Acetone	0.89	1	1
		Hexane	0.90	1	
P. longifolia	CEF	Acetone	0.90	1	3
		DCM	0.90	1	
		Hexane	0.1	1	
	BEA	DCM	0.1	1	
		Hexane	0.67	1	
K. wilmsii	CEF	Acetone	0.67	1	8
		DCM	0.67	1	
		Hexane	0.85	1	
	EMW	Acetone	0.85	1	
		DCM	0.85	1	
O. natalitia	CEF	Methanol	0.56	1	1
	BEA	DCM	0.27	1	
K. anthotheca		Hexane	0.93	1	3
	CEF	Acetone	0.42	1	
		Hexane	0.67	1	
C. vendae	CEF	Acetone	0.54	1	3
		DCM	0.45	1	

Table 4.5. Inhibition of growth on bioautographic TLC plates by 24 extracts of 7 South African tree leaves against *A. fumigatus*.

Plant	Solvent system	Extracts	R_f values	Active bands	Total
L. alata	BEA	Hexane	0.13, 0.54, 0.92	3	10
		DCM	0.13, 0.54, 0.92	3	
	CEF	Hexane	0.63	1	
		DCM	0.63	1	
	EMW	Hexane	0.81	1	
		DCM	0.81	1	
P. longifolia	BEA	DCM	0.2	1	3
	CEF	DCM	0.83	1	
	EMW	DCM	0.90	1	
C. harveyi	BEA	Hexane	0.33	1	3
		DCM	0.33, 0.46	2	
K. anthotheca	BEA	Hexane	0.26	1	8
		Acetone	0.21	1	
		DCM	0.21	1	
	CEF	Hexane	0.56	1	
		DCM	0.53	1	
	EMW	Hexane	0.93	1	
		Acetone	0.93	1	
		DCM	0.93	1	
C. vendae	CEF	Hexane	0.94	1	2
	EMW	Acetone	0.81	1	

Table 4.6. Inhibition of growth on bioautographic TLC plates by 24 extracts of 7 South African tree leaves against *S. schenckii*.

Plant	Solvent system	Extracts	R_f values	Active bands	Total
L. alata	BEA	DCM	0.89, 0.43	2	7
		Methanol	0.43	1	
	CEF	Hexane	0.58	1	
		Acetone	0.58	1	
		DCM	0.58	1	
	EMW	Hexane	0.92	1	
P. longifolia	BEA	DCM	0.62, 0.46	2	3
		Methanol	0.46	1	
		Acetone	0.31	1	
K. wilmsii	EMW	Methanol	0.31	1	2
		Hexane	0.96, 0.55, 0.44	3	
C. harveyi	BEA	Methanol	0.96	1	8
		Hexane	0.84	1	
	CEF	Acetone	0.68	1	
		Hexane	0.94	1	
	EMW	Methanol	0.12	1	
O. natalitia	EMW	Acetone	0.44	1	1
		Hexane	0.96	1	
K. anthotheca	CEF	Acetone	0.96	1	3
		DCM	0.96	1	
C. vendae	BEA	DCM	0.89	1	6
		Methanol	0.89	1	
		Acetone	0.58, 0.32	2	
	CEF	DCM	0.58, 0.32	2	

Table 4.7. Inhibition of growth on bioautographic TLC plates by 24 extracts of 7 South African tree leaves against *M. canis*.

Plant	Solvent system	Extracts	R_f values	Active bands	Total
L. alata	BEA	Acetone	0.31, 0.79	2	6
		DCM	0.31	1	
		Hexane	0.27, 0.42	2	
	EMW	Acetone	0.94	1	
		Hexane	0.87	1	
P. longifolia	CEF	Acetone	0.87, 0.41	2	5
		DCM	0.41	1	
	EMW	Acetone	0.91	1	
		DCM	0.77	1	
K. wilmsii	BEA				2
	CEF	Acetone	0.57	1	
O. natalitia	BEA	Acetone	0.73	1	1
K. anthotheca	BEA	Hexane	0.91	1	3
		Acetone	0.63	1	
	EMW	Hexane	0.29	1	
C. vendae	BEA	Hexane	0.33	1	6
		Acetone	0.29, 0.33	2	
		DCM	0.33	1	
	CEF	Acetone	0.67	1	
		DCM	0.67	1	

Table 4.8. Inhibition of growth on bioautographic TLC plates by 24 extracts of 7 South African tree leaves against *C. neoformans*.

Plant	Solvent system	Extracts	R_f values	Active bands	Total
L. alata	BEA	Hexane	0.23, 0.38	2	11
		Acetone	0.92	1	
		DCM	0.21	1	
	CEF	Hexane	0.90	1	
		Acetone	0.90	1	
		DCM	0.90	1	
		Methanol	0.90	1	
	EMW	Hexane	0.77	1	
		Acetone	0.26	1	
		DCM	0.85	1	
P. longifolia	CEF	Hexane	0.26, 0.41	2	8
		Acetone	0.26, 0.41, 0.52	3	
		DCM	0.26	1	
	EMW	Hexane	0.98	1	
		Acetone	0.98	1	
K. wilmsii	BEA	Hexane	0.84	1	5
		Acetone	0.14	1	
		DCM	0.96	1	
	CEF	Acetone	0.72	1	
	EMW	DCM	0.16	1	
C. harveyi	BEA	Hexane	0.93	1	5
		Acetone	0.18	1	
	CEF	Acetone	0.90	1	
		DCM	0.90	1	
		Methanol	0.90	1	
O. natalitia	BEA	Hexane	0.97	1	2
	EMW	Hexane	0.13	1	
K. anthotheca	BEA	Hexane	0.92	1	4
	CEF	Hexane	0.94	1	
		Acetone	0.94	1	
	EMW	Hexane	0.21	1	
C. vendae	BEA	Hexane	0.96	1	5
		Acetone	0.96	1	
		DCM	0.13	1	
	CEF	DCM	0.67	1	
	EMW	Acetone	0.12	1	

Table 4.9. Inhibition of growth on bioautographic TLC plates by 24 extracts of 7 South African tree leaves against *C. albicans*.

Plant	Solvent system	Extracts	R_f values	Active bands	Total
L. alata	BEA	Hexane	0.87, 0.21	2	6
		DCM	0.21	1	
	CEF	Hexane	0.93	1	
		Acetone	0.93	1	
	EMW	Hexane	0.26	1	
P. longifolia	BEA	Methanol	0.21	1	3
	CEF	Acetone	0.76	1	
	EMW	Hexane	0.42	1	
K. wilmsii	BEA	Hexane	0.93	1	2
	CEF	Hexane	0.74	1	
C. harveyi	BEA	Hexane	0.54	1	6
		Acetone	0.54	1	
		DCM	0.30	1	
	CEF	Hexane	0.93	1	
		Acetone	0.93	1	
		DCM	0.93	1	
O. natalitia	BEA	Methanol	0.34	1	3
	CEF	Acetone	0.94	1	
	EMW	Acetone	0.56	1	
K. anthotheca	BEA	Hexane	0.59	1	5
		Acetone	0.59	1	
		DCM	0.59	1	
	CEF	Hexane	0.93	1	
		Acetone	0.93	1	
C. vendae	BEA	Hexane	0.34	1	8
		Acetone	0.47	1	
		DCM	0.59	1	
	CEF	Hexane	0.71	1	
		Acetone	0.63	1	
		DCM	0.29	1	
		Methanol	0.88, 0.29	2	

Most of the antimicrobial agents detected in this study were present in extracts of non-polar solvents. These findings agreed with previously published results (Masoko and Eloff, 2005, 2006) that the substances responsible for the antimicrobial activity were strongly non-polar in nature. However, the acetone and methanol fractions of *Punica granatum* and *Delonix regia* in contrast to the benzene fraction had good activity against methicillin resistant *S. aureus* (Aqil et al., 2005). Bioautography therefore, allows the determination of the polarity of the active compounds.

Loxostylis alata appeared to be the most active plant; having the highest number of inhibition zones of 35 and 40 against bacteria and fungi, respectively, while *O. natalitia* was the less active plant with lowest number of inhibition zones of 18 and 11 against bacteria and fungi, respectively. *C. vendae*, *P. longifolia*, *K. anthotheca*, *C. harveyi, K. wilmsii* and *O. natalitia* had inhibition zones of 22, 19, 15, 11, 18 and 12, respectively, against bacteria, and 27, 22, 38, 22, 11 and 7, respectively, against fungi. In another study, the methanol extract of the stem bark of *Khaya anthotheca* was reported to be very active against the fungus *Candida krusei* Hamza et al., 2006. Some pentacyclic triterpenes isolated from *Combretum imberbe* and *Terminalia stuhlmannii* (Combretaceae) have antimicrobial properties against bacteria and fungi (Katerere et al., 2003). Perhaps similar chemical constituents might be responsible for the antimicrobial action of *Combretum vendae* a member of the same family. In a similar manner, some triterpenoid and phenolic compounds isolated from *Commiphora opobalsamum* (Burseraceae) possess antimicrobial activity (Abbas et al., 2007). Of the bacteria tested, *S. aureus* also appeared to be the most susceptible organism. The growth of *S. aureus* was inhibited most by the compounds of the tested plants. Similarly, *C. neoformans* depicted the highest susceptibility among fungal organisms. Some of the compounds are active against both bacteria and fungi, while others are selective in their activity. It is possible that compounds that have activity against all the tested organisms possess a broad antimicrobial action or they may likely be general metabolic toxins. That is actually a subject of another investigation and we are following it up.

The absence of bioactivity in some of the plant extracts does not preclude the fact that they are not active, as synergistic or additive interactions of plant extracts or phytocompounds is the basis of activity of several herbal formulations (Ahmad and Aqil, 2007). Moreover, the non-activity could also be explained by a weak selectivity of the extract components against the microorganisms chosen for this study, or the very low concentration of the active compounds in the crude extract under the tested conditions (Schmourlo et al., 2004).

4.4. Conclusion

The South African flora offers great potential in the search for natural compounds with antimicrobial activity. The bioassay-guided fractionation to isolate and characterise active compounds against bacteria and fungi from those plant extracts that had the highest activity is currently being undertaken in our laboratory. In addition, we are also carrying out toxicological and other pharmacological evaluations on the active plant extracts.

Postscript

All the plant species possess varying degree of antibacterial and antifungal activities and we therefore went further to examine their antioxidant and antiplatelet activities with a view of finding pharmacologically activity in the plant species under investigation.