CHAPTER 5

Activity of crude leaf extracts of plant species against *Aspergillus fumigatus*

5.1 Introduction

We have recently shown that a crude plant extract can be as effective in treating animals infected with *Aspergillus fumigatus* as the commercially used fungicide (Suleiman 2009). Aspergillosis is a very important disease especially affecting birds. Because the plant extracts had good activity against *Aspergillus niger* a plant pathogen (see Chapter 3), the activity against the animal pathogen *Aspergillus fumigatus* will be determined in this chapter.

Aspergillus fumigatus is a mold that causes various infectious diseases in humans and animals. Molds are fungi that form a threadlike filament and they are produced by spore formation. The spores of molds are usually coloured and can be seen on the surface of the substrate as a sign of food growth. Molds prefer dark, moist, aerobic environments and organic matter in order to grow (Todar 2006).

Aspergillus fumigatus is an asexual fungus that propagates via highly dispersible conidia. This fungus has adapted to survive and grow under a broad range of environmental conditions, contributing to ubiquity of the species. One of the most important distinguishing characteristics of *A. fumigatus* from other *Aspergillus* species is its ability to survive and grow at higher temperatures of 52 to 55°C. Since *A. fumigatus* can survive at higher temperatures, they are considered to be thermo-tolerant fungi (Chang et al. 2004).

Aspergillus fumigatus causes various diseases such as aspergillosis, for example allergic aspergillosis and invasive pulmonary aspergillosis in humans. Aspergillosis is acquired by inhalation of air-borne conidia and invasive pulmonary aspergillosis is one of the leading causes of life-threatening fungal diseases among immunosuppressed patients (Denning, 1998). The treatment of aspergillosis diseases with Western medicine is limited due to a lack of information on the toxicity of the drugs and in some instances, the medication is very expensive. More importantly, the percentage mortality rate is very high (80-90%) despite the

current available antifungal drugs such as amphotericin B, to which most diseases are resistant, and triazole drugs (Denning 1996, Gigolashvili 1999).

Invasive aspergillosis (IA) is the leading cause of infectious death in bone marrow transplant recipients and patients with hematologic malignancies (Kontoyiannis and Bodey 2002). Two commonly known antifungal agents have been used, itraconazole and caspofungin, which is a novel echinocandin that inhibits fungal cell wall biosynthesis. Previously, it has been reported that the drug has antifungal activity against *Aspergillus* species and it can be used for the treatment of invasive aspergillosis (Groll et al. 1998).

5.2 Materials and methods

5.2.1 Fungal strain

Aspergillus fumigatus was obtained from the culture collection of the Department of Microbiology at the University of Pretoria. The fungus was isolated from a chicken which suffered from aspergillosis. Fungal strains were maintained on Sabouraud Dextrose (SD) agar at 4ºC and incubated at 37 ºC for four to five days before use.

5.2.2 Quantification of fungal inoculum

The method is described in detail in chapter 3, section **3.2.1**.

5.2.3 Bioassays for antifungal activity

The methods are described in detail in chapter 3, section **3.2.2.1** and **3.2.2.2**.

5.3 Results and discussion

5.3.1 Dilution method

Amongst all of the extracts tested, only acetone extracts of *B. buceras*, *B. salicina*, *V. infausta* and *X. kraussiana* had good antifungal activity against the animal pathogen. Their MIC values ranged between 0.02 and 0.08 (Table 5-1). Similarly, the hexane, DCM and MeOH extracts of the two plant species, *B. buceras* and *V. infausta*, had activity with the same MIC value of 0.16 mg/ml. It is interesting to note that all of the extracts of *B. salicina* possess a very strong antifungal activity (MIC = 0.08 mg/ml) against the tested fungus. Four extracts of *X. kraussiana* had the best antifungal activity with MIC values ranging between 0.02 and 0.08 mg/ml. Of the four extracts, acetone and hexane extracts of *H. caffrum* and *O. ventosa* were active against the tested microorganism with MIC values of 0.16 and 0.32 mg/ml for the acetone and hexane extracts respectively. *Harpephyllum caffrum* is reported to contain phenolic compounds which may be responsible for its biological activity (El Sherbeiny and El Ansari 1976).

The acetone extracts had the lowest average MIC value (0.72 mg/ml) while the highest were observed in the MeOH extracts (Table 5-1). This confirms that acetone was the best extractant and is also not toxic to the tested animal pathogen. These results are consistent with those obtained for plant pathogens, as discussed earlier in Chapter 3 (Table 3-1).

The crude acetone, hexane and MeOH extracts of *B. buceras* had the highest antifungal activity against the four plant pathogenic fungi, *P. janthinellum, P. expansum, Trichoderma harzianum* and *Fusarium oxysporum* with MIC values ranging between 0.02 and 0.08 mg/ml. When the four extracts were tested against *A. fumigatus* it was discovered that only the acetone extract had a strong antifungal activity with MIC = 0.04 mg/ml. However, in the case of *B. salicina*, all of the four extracts had a strong antifungal activity against the animal pathogen with MIC ranging between 0.04 and 0.08 mg/ml. More importantly, hexane, DCM and MeOH extracts had the same MIC value of 0.08 mg/ml that was observed against *P. janthinellum*. On the other hand, the acetone extract of *V. infausta* had activity with MIC = 0.08 mg/ml while the extracts of *O. ventosa* had a moderate antifungal activity against *A.*

fumigatus with MIC ranging between 0.16 and 0.32 mg/ml (Table 5-1). More surprisingly, the extracts of *X. kraussiana* possess strong antifungal activity (MIC between 0.02 and 0.08 mg/ml) against *A. fumigatus*, in contrast to extracts tested against plant pathogens, where all of the four extracts had a moderate activity with MIC ranging between 0.16 and 2.50 mg/ml. In the current study, extracts of *H. caffrum* were particularly active against *A. fumigatus*. All of the extracts did not show the best antifungal activity against *Aspergillus* species. Previously, the water, ethanol and ethyl acetate extracts of *H. caffrum* were tested against the yeast, *Candida albicans* (Buwa and Van Staden 2006). Their findings revealed that the extracts were not active against the animal pathogenic fungus *C. albicans* since their MIC value was very high (6.25 mg/ml).

The highest total activity was observed in the MeOH extract of *B. salicina* (2781 ml/g) and the lowest was found in the hexane extract after 24 hours (Table 5-1). More importantly, all of the extracts did not possess a strong antifungal activity after 48 hours. When we compared the total activity obtained from the plant and animal pathogens it was found that the highest total activity was obtained in the acetone extract of *H. caffrum* (22 000 ml/g) against *F. oxysporum* while the lowest was observed in the methanol extract of *O. ventosa* against *A. niger* (133 mg/l) (Table 3-2). However, in the case of the animal pathogen, it was discovered that the highest total activity was found in MeOH extract of *B. salicina* (2781 ml/g) and the lowest was observed in hexane extract of *H. caffrum*. This total activity value means that the methanol extract from 1 g of *B. salicina* leaves diluted to 22 000 ml will still inhibit the growth of the fungus.

Table 5-1 Minimum inhibitory concentration (MIC) of six plant species against *Aspergillus fumigatus* using different extractants (A = acetone, H = hexane, D = dichloromethane, M = methanol). The results are the average of three replicates and the standard deviation was zero (0).

Plant species	Time	Extractants									
		A		H		D		M		Average	
		MIC	TA	MIC	TA	MIC	TA	MIC	TA	MIC	TA
Bucida buceras	24	0.04	875	0.32	159	0.16	314	0.16	797	4.94	434
	48	1.25	28	1.25	41	2.5	20	2.5	51	11.1	38
Breonadia salicina	24	0.08	1769	0.08	507	0.08	1250	0.08	2781	4.86	1266
	48	1.25	57	1.25	32	2.5	40	2.5	89	11.1	53
Harpephyllum caffrum	24	0.16	407	0.32	78	0.63	68	0.63	282	5.15	172
	48	1.25	52	2.5	10	2.5	17	2.5	71	11.4	40
Olinia ventosa	24	0.16	567	0.32	127	0.16	255	0.32	1219	5.0	438
	48	1.25	73	2.5	16	2.5	16	2.5	156	11.4	62
Vangueria infausta	24	0.08	1134	0.16	252	0.16	316	0.32	283	4.94	402
	48	2.5	36	2.5	16	1.25	40	1.25	72	11.1	42
Xylotheca kraussiana	24	0.02	1250	0.04	500	0.04	750	0.08	2344	4.84	974
	48	0.63	40	0.63	32	0.63	48	2.5	75	10.5	49
Average		0.72	524	0.99	148	1.09	261	1.28	685	1.02	404

5.3.2 Bioautography assay

In BEA, one antifungal compound was observed in all extracts (R_f 0.08) of *B. salicina*, while the extractants of *X. kraussiana* had three antifungal compounds with R_f 0.02, 0.04, 0.04 and 0.08 in acetone, hexane, dichloromethane and methanol extracts, respectively. Similarly the acetone extract of *V. infausta* also had an antifungal compound with an R_f value of 0.08 (Figure 5-1). The active compound (with R_f 0.08) observed in the above plant extracts is the same since the chromatograms were developed in the same solvent system, BEA. In CEF, three antifungal compounds were found in acetone, DCM and methanol extracts of *B. salicina* with R_f values of 0.70, 0.85 and 0.90. On the other hand, one active compound with the R_f value of 0.70 was visible in hexane extract. The DCM extract of *O. ventosa* had active compounds while no clear bands were observed in acetone, hexane and DCM extracts of *V. infausta* against *A. fumigatus*. Most of the antifungal compounds were visible in CEF, where at least three compounds were observed in acetone, one in hexane, and two in each of the DCM and MeOH extracts. In general, acetone extracts showed more of the active compounds (total of 9) in CEF (Figure 5-1). However, no antifungal compounds were observed in chromatograms developed in EMW in any of the plant extracts. The non-activity of some of the plant extracts used in the current study could be due to the disruption of synergism between active compounds or a very low concentration of the compounds present in the crude extracts that are active against *A. fumigatus*.

In the current study, it was found that for chromatograms separated using CEF, three antifungal compounds with R_f values 0.70, 0.85 and 0.90 in acetone extracts of *B. salicina* were active against three plant pathogens, *P. janthinellum*, *A. parasiticus* and *T. harzianum* (Chapter 3, Figure 3-1). Three antifungal compounds were also observed in acetone extract of *B. salicina* against *A. fumigatus*. There was a very distinct clear active band with R_f value of 0.90. Although there were three active compounds in hexane extracts against the three plant pathogens above (*P. janthinellum*, *A. parasiticus* and *T. harzianum*), only one compound was observed against *A. fumigatus*. In the case of DCM and MeOH extracts, only one antifungal compound was found against *P. janthinellum* and *A. parasiticus*. However, it was different in the case of the animal pathogen, where only two active compounds were visible in DCM and MeOH. In general, all extracts of *B. salicina* showed several antifungal compounds compared

with extracts of the remaining five plant species. In bioautography using BEA, three extracts (acetone, DCM and MeOH) of *O. ventosa*, *V. infausta* and *X. kraussiana* showed one antifungal compound on bioautograms screening against *A. fumigatus*. This was not observed in all extracts against plant pathogens (Figure 3-1). In CEF, acetone, DCM and MeOH extracts of *O. ventosa* showed antifungal compounds with R_f values of 0.54, 0.72 and 0.95 against *A. parasiticus*. Surprisingly, these compounds were not observed in extracts tested against *A. fumigatus* (Figure 5-1). The total number of antifungal compounds found in extracts against plant pathogens was 35 while 19 were visible against the animal pathogenic *A. fumigatus*.

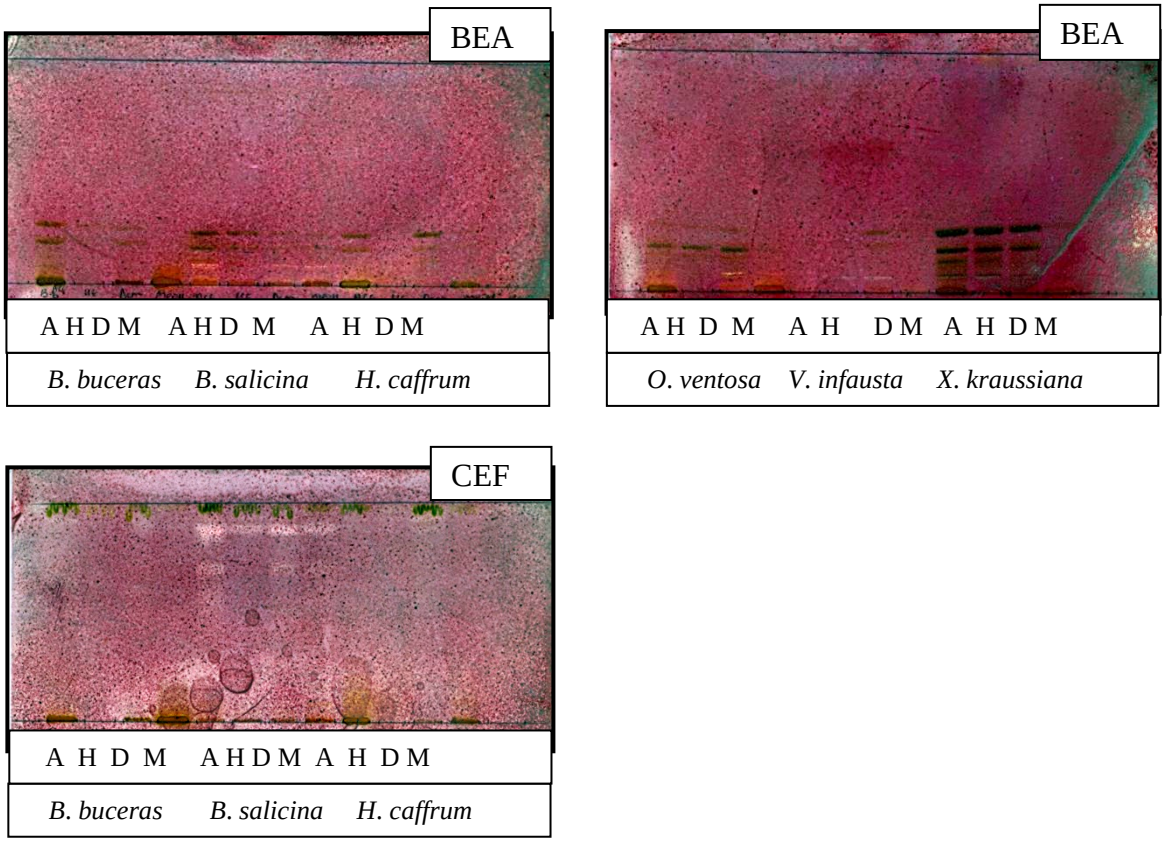

Figure 5-1 Bioautograms of six plant species (left to right: *Bucida buceras*, *Breonadia salicina*, *Harpephyllum caffrum*, *Olinia ventosa*, *Vangueria infausta* and *Xylotheca kraussiana*) extracted with acetone, hexane, DCM and MeOH (left to right), developed in BEA and CEF, and sprayed with *Aspergillus parasiticus*. Clear zones on the bioautograms indicate inhibition of fungal growth.

5.4 Conclusion

Antifungal compounds were observed in all extracts of *B. salicina* while in some of the other plant species (*B. buceras*, *O. ventosa*, *V. infausta* and *X. Kraussiana*), the active compounds were visible in varying extracts. *B. salicina* extracts had the highest antifungal activity against plant and animal pathogens. Amongst the six plant species used in this current study, all four extracts of *O. ventosa* were very active against the plant pathogens but when tested against animal pathogenic *A. fumigatus* they had moderate to low antifungal activity. Not all plant extracts active against plant pathogenic fungi are also active against animal pathogens. This aspect of the study was initiated since other researchers obtained good results using plant extracts against *A. fumigatus* to protect poultry against aspergillosis. The promising activity of plant extracts found in the present research study against plant pathogenic fungi prompted a continued investigation on their potential antifungal activity against *A. fumigatus*. Leaf extracts of *B. salicina* showed strong antifungal activity against *A. fumigatus* and the plant may therefore be a good candidate for further research into a treatment for systemic fungal infections. In the next two chapters, further targeted investigation of the antifungal nature of *B. salicina* leaf extracts and isolation of the antifungal compounds from the plant will be discussed.

CHAPTER 6

Antifungal activity of *Breonadia salicina* leaf extracts

6.1 Introduction

Breonadia salicina (Vahl) Hepper and J.R.I Wood belongs to the family Rubiaceae and is found in Limpopo, Mpumalanga and KwaZulu-Natal provinces (Furness and Breen 1980). The Rubiaceae family is one of the largest of the angiosperms with 10 700 species distributed in 637 genera. It is subdivided into four subfamilies, namely Cinchonoideae, Ixoroideae, Antirheoideae and Rubioideae (Mongrand et al. 2005, Robbrecht 1988, 1993b). Members of the Rubiaceae are mainly tropical woody plants and consist mostly of trees and shrubs, less often of perennial to annual herbs, as in the subfamily Rubioideae, which are found in temperate regions (Mongrand et al. 2005).

Breonadia salicina, commonly known as motumi (Sepedi), is a small to large tree up to 40 m in height. It usually grows in riverine fringes, forest, and usually near the banks or in the water of permanent streams and rivers. The bark is grey to grey-brown and rather rough, with longitudinal ridges. The leaves are usually in whorls of 4 and crowded at the ends of the branches (Palgrave 2002). Leaves are without hair; the veins are pale yellowish-green and the thickset petiole is up to 20 mm long. Flowers are small, pale mauve, sweetly scented, and are present in compact, round axillary heads up to 40 mm in diameter on long slender stalks up to 60 mm long, with 2 leaf-like bracts along their length. They are bisexual, all floral parts are in fives, widening into a funnel-shaped throat and 5-lobed cup-shaped disc. The stamens are inserted in the throat of the tube protruding from the mouth. The 2-chambered ovary with light yellow balls grows in the leaf origin from November to March. The fruit is a small, brown, 2-lobed capsule and is densely clustered into round heads which grow in the leaf origin, giving a rough, crusty, wart-like appearance. The diameter of the fruit is 2 - 3 mm and they are visible during January and February (Palgrave 2002).

Many plant species from the Rubiaceae family are used traditionally for the treatment of various diseases. In particular, *B. salicina* is used to treat wounds, ulcers, fevers, headaches, gastrointestinal illness, cancer, arthritis, diabetes, inflammation and bacterial and fungal

infections (Chang et al. 1989). In South Africa, Zulu people use the bark for stomach complaints and the Vhavenda use root decoctions for the treatment of tachycardia (Arnold and Gulumian 1984). The bark of *B. salicina* is reported to be astringent (Doke and Vilakazi 1972).

Previous work indicates that anthraquinones have been isolated from species in the family Rubiaceae, and these compounds have *in vivo* activities such as antimicrobial, antifungal and antimalarial activity (Sittie et al. 1999, Rath et al. 1995). On the other hand, alkaloids, terpenes, quinonic acid glycosides, flavonoids, and coumarins, have also been isolated from the Rubiaceae (Heitzman et al. 2005). No chemical isolation and characterization of constituents of *B. salicina* have been reported in the literature surveyed.

(a) (b)

Figure 6-1 A photograph of (a) small and large tree and (b) leaves of *Breonadia salicina* taken from Lowveld National Botanical Garden in Nelspruit**.**

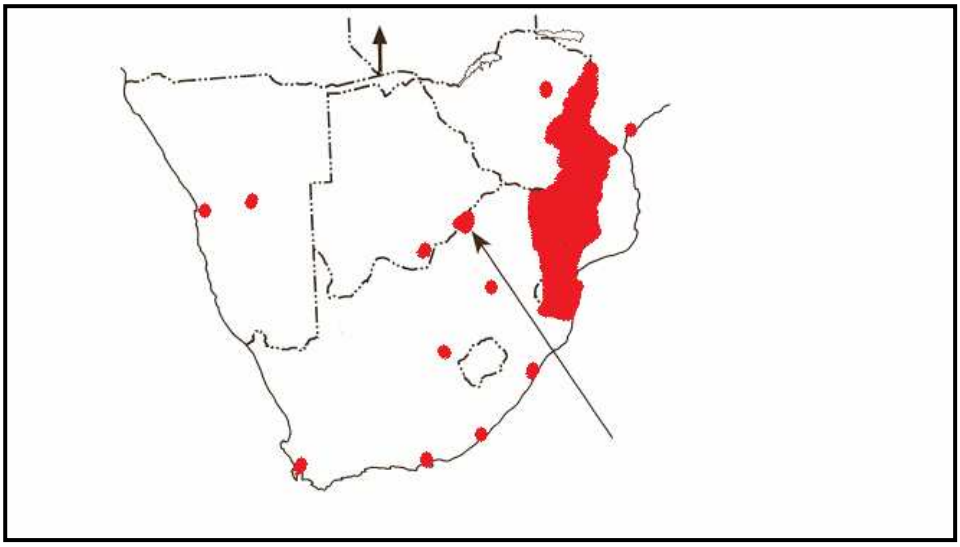

Figure 6-2 Map showing geographic distribution of *Breonadia salicina* (Palgrave 2002). The red shaded areas indicate the places where the plant species grows and the arrows show other places/countries where the plant species is found.

6.2 Materials and methods

6.2.1 Exhaustive sequential extraction

The plant material was collected in February 2007 and prepared as explained in section **2.2.3**. Finely ground leaf material (500 g) was serially extracted with 1500 ml of solvents of increasing polarities, namely hexane, chloroform, acetone and methanol. In each step, the solvent was allowed to extract the ground plant material for three hours on a Labotec Model 20.2 shaking apparatus. The extract was filtered through Whatman No.1 filter paper using a Büchner funnel. With each solvent, the plant material was extracted four times using fresh solvent (1500 ml) to exhaustively extract the material, and the process was repeated with chloroform, acetone, and methanol in sequence. The resulting filtrates were dried under reduced pressure at 40ºC in a rotavapor (Büchi rotary evaporator) and the reduced extracts were transferred into vials and allowed to dry. The masses of the extract yields were determined.

6.2.2 Solvent-solvent fractionation

The chloroform extract (10 g) was dissolved in 500 ml chloroform and transferred into a 1 L separatory funnel before being mixed with water (500 ml)). When separation of the two layers occurred, the bottom layer was collected to yield the chloroform fraction, and the process was repeated three times by extracting the water fraction with fresh chloroform. Following this, one litre of butanol was added to the water fraction and the top layer was collected, yielding the butanol fraction. The chloroform and butanol fractions were evaporated to dryness at 45ºC under reduced pressure using a Büchi Rotavapor R-114. The water fraction was evaporated using a Specht Scientific freeze dryer.

6.2.3 Microplate dilution assay

The fractions and extracts were tested for antifungal activity against seven plant pathogenic fungi. The method is described in section **3.3.2.** The total activity of each fraction was also calculated as described in section **3.2.2.1**.

6. 2.4 TLC fingerprinting

TLC fingerprinting was done on the fractions according to the method described in section **2.2.5**.

6.2.5 Bioautography assay

Bioautography was used to determine the number of active compounds in the fractions after each stage of serial extraction and solvent-solvent fractionation. The fractions were tested against seven plant pathogenic fungi. The method is described in section **3.2.2.2.**

6.3 Results and discussion

6.3.1 Serial extraction with different solvents

Almost a quarter of the plant material (a total of 112.4 g) was extracted from 500 g of *B. salicina* dried leaves with four different extractants, namely hexane, chloroform, acetone and methanol, as shown in Figure 6-3. Methanol extracted the largest quantity of plant material 12.3% (61.5g), followed by acetone 5.6% (27.8 g), hexane 2.6% (12.8 g) and chloroform 2.1% (10.3 g).

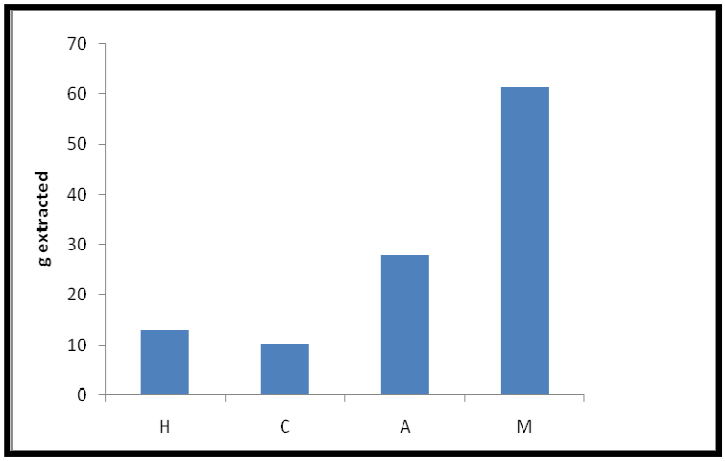

Figure 6-3 Quantity of plant material sequentially extracted from 500 g of *B. salicina*, with different extractants. **Lanes from left to right:** hexane (H), chloroform (C), acetone (A), and methanol (M).

The minimum inhibitory concentration (MIC) values and total activity of the four extracts from the serial extraction process against seven plant pathogenic fungi were determined in triplicate (Table 6.1). The standard deviation was zero for all experiments for all the tables. The highest total activity was observed in the methanol extract (141 ml/g) and the lowest was shown by the hexane extract (25.0 ml/g). The results confirm that where the average MIC value was low (0.54 mg/ml) the total activity was high (102.8 ml/g), consistent with the results in Chapter Two. The chloroform extract had good antifungal activity with an MIC value of 0.16 mg/ml against three fungi, namely: *P. expansum*, *P. janthinellum* and *F.*

oxysporum. The methanol extract also showed good activity with MIC = 0.16 mg/ml against *P. janthinellum*. However, the hexane and acetone extracts were not active against the tested microorganisms, with high MIC values ranging between 0.32 and 1.25 mg/ml. *Aspergillus parasiticus* was relatively resistant to the acetone, hexane, chloroform and methanol extracts with high MIC values between 1.25 and 2.5 mg/ml.

Table 6-1 Minimum inhibitory concentration (MIC) and total activity of four serial extracts against seven plant pathogenic fungi. The results show the average of three replicates and the standard deviation was 0 in all cases

Plant pathogens	Time (hr)	Extractants MIC (mg/ml)				
		Hexane	$CHCl_3$	Acetone	MeOH	AmpB
Aspergillus parasiticus	24	1.25	1.25	1.25	2.5	2.5
Aspergillus niger	48	0.63	0.63	0.32	1.25	1.25
Colletotrichum gloeosporioides	48	1.25	0.63	0.32	0.63	<0.02
Penicillium expansum	48	0.63	0.16	0.32	0.32	<0.02
Penicillium janthinellum	48	2.5	0.16	0.32	0.16	<0.02
Trichoderma harzianum	48	0.63	1.25	0.63	0.63	0.63
Fusarium oxysporum	48	0.32	0.16	0.63	0.63	2.5
Quantity of fraction in mg		12800	10300	27800	61500	112400
Average		1.03	0.61	0.54	0.87	-
Total activity (ml/fraction)		25	34	103	141	303
% of total activity		42.24	34.0	91.75	202.97	370.95

The total activity values of the acetone extract of *B. salicina* against seven plant pathogenic fungi are given in Table 6-2. The highest total activity was found in the acetone leaf extracts of *B. salicina* (174 ml/g) against *A. niger*, *C. gloeosporioides*, *P. expansum* and *P. janthinellum* whilst the lowest activity (45 ml/g) was observed against *A. parasiticus*. These values were in the same range of values found in the antibacterial activity of different *Combretum* spp (Eloff 1999).

Table 6-2 Total activity of the crude acetone extract of *B. salicina* leaves tested against seven plant pathogenic fungi

Plant pathogens	Total activity (ml/g)
Aspergillus parasiticus	45
Aspergillus niger	174
Colletotrichum gloeosporioides	174
Penicillium expansum	174
Penicillium janthinellum	174
Trichoderma harzianum	88
Fusarium oxysporum	88

The chloroform solvent-solvent fraction had reasonable antifungal activity against *A. niger*, *C. gloeosporioides*, *P. janthinellum* and *T. harzianum* with MIC values ranging between 0.16 and 1.25 mg/ml (Table 6-3). The aqueous fraction was less active with MIC values ranging between 0.32 and 2.5 mg/ml against A. *parasiticus*, *A. niger*, *C. gloeosporioides* and *P. janthinellum*. However, the butanol fraction had the lowest antifungal activity against *A. parasiticus*, *A. niger*, *T. harzianum* and *F. oxysporum*, with MIC values ranging between 1.25 and 2.5 mg/ml. The highest average MIC value (1.25 ml/g) was observed in the aqueous fraction, while the lowest average MIC value (0.43 ml/g) was obtained in the chloroform fraction. Furthermore, the lowest total activity of 7 ml/g was recorded for the butanol fraction, while the highest total activity (48.2 ml/g) was observed in the chloroform fraction.

The crude acetone extracts had the best activity against *P. janthinellum* with an MIC value of 0.08 mg/ml. It appears that serial extraction and solvent-solvent fractionation removed some of the compounds with synergism since the aqueous, butanol and chloroform fractions were relatively inactive against the tested plant pathogenic fungi.

Table 6-3 Minimum inhibitory concentration (MIC) and total activity of solvent-solvent fractions against plant pathogenic fungi. The results show the average of three replicates with standard deviation 0 in all cases

Plant pathogens	Time (hr)	Fractions MIC (mg/ml)			
		Aqueous	Butanol	$CHCl_3$	AmpB
Aspergillus parasiticus	24	2.5	1.25	0.63	2.5
Aspergillus niger	48	2.5	2.5	0.16	1.25
Colletotrichum gloeosporioides	48	0.32	0.32	0.16	<0.02
Penicillium expansum	48	0.63	0.63	0.32	<0.02
Penicillium janthinellum	48	0.32	0.63	0.16	<0.02
Trichoderma harzianum	48	1.25	1.25	1.25	0.63
Fusarium oxysporum	48	1.25	1.25	0.32	2.5
Average		1.25	1.12	0.43	-
Total activity (ml/g)		21	7	48	-

6.3.2 TLC analysis

6.3.2.1 Separation of compounds in the serial extraction fractions

The BEA solvent system separated more compounds from the serial extraction fractions than CEF and EMW, after chromatograms were sprayed with vanillin-sulphuric acid (Figure 6-3). With the BEA eluent, some separation of compounds was observed in the acetone and chloroform fractions, but separation of constituents was seen in the hexane and methanol fractions at the base of chromatograms. Addition of a more polar solvent can enhance the separation, moving the compounds further up the TLC chromatograms. In contrast to BEA, a different separation was observed in the CEF solvent system, since the relatively polar compounds moved to the top of the TLC chromatograms. Only one separated compound was visible in the acetone, hexane and $CHCl_3$ fractions, while no movement from the origin was observed in the MeOH fraction. However, using the EMW eluent, most of the compounds moved to just below the solvent front. Only one compound (R_f= 0.41) was visible under UV-

light in both the acetone and $CHCl_3$ fractions, and two compounds (R_f = 0.11 and 0.41) were also visible in methanol fraction (circled in pencil in Figure 6-3). Furthermore, no compounds were visible under UV-light in the hexane fraction.

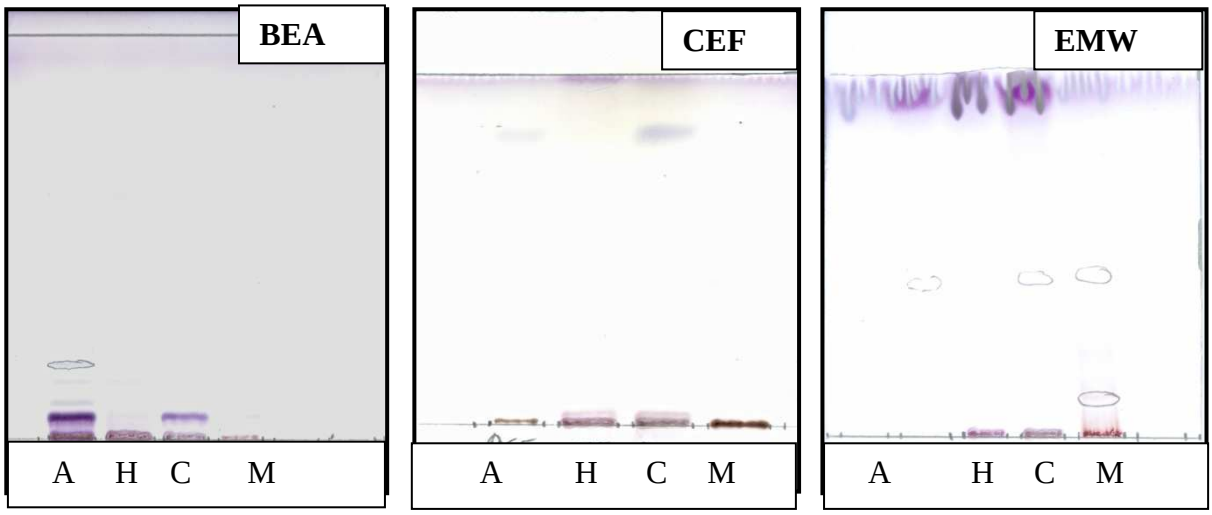

Figure 6-3 Chromatograms separated in BEA (left), CEF (centre) and EMW (right) solvent systems, sprayed with vanillin-sulphuric acid. **Lanes from left to right:** (A) = Acetone, (H) = Hexane, (C) = Chloroform and (M) = Methanol.

6.3.2.2 Separation of compounds in the solvent-solvent fractions

Figure 6-4 shows chromatograms of the fractions from solvent-solvent fractionation developed with BEA (left), CEF (centre) and EMW (right) solvent systems, sprayed with vanillin-sulphuric acid. With the BEA eluent, more compounds were separated in the $CHCl_3$ fraction at the base of the chromatograms, while no compounds were observed in the aqueous and butanol fractions. However, one compound in the aqueous and two compounds in the $CHCl_3$ fraction were visible in the CEF solvent system, indicating better separation with CEF than BEA. In EMW, the separation was no improvement in the aqueous fraction, since the compounds were observed near the base of the chromatograms under UV-light (R_f = 0.08, 0.15, 0.21 and 0.32). No compounds were visible in the butanol fractions using the three solvent systems.

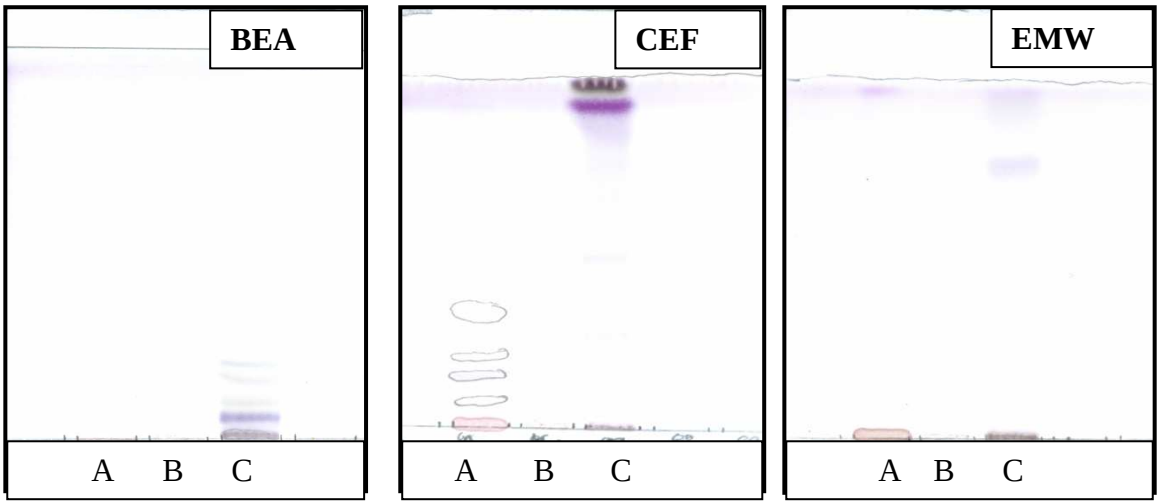

Figure 6-4 Chromatograms of *Breonadia salicina* fractions, developed in BEA (left), CEF (centre) and EMW (right), left to right: Aqueous (A), Butanol (B) and Chloroform (C) and sprayed with vanillin-sulphuric acid (0.1% in vanillin in sulphuric acid).

6.3.3 Bioautography assay

Figure 6-5 shows the chromatograms of the extracts developed in BEA and EMW and sprayed with *A. parasiticus*. The TLC chromatograms developed in the CEF solvent system showed no antifungal compounds and were not included in the Figure. In the BEA eluent system, one antifungal compound was visible in the acetone and chloroform fractions, with R_f value of 0.15. However, no compounds were observed in the hexane and methanol fractions. For extracts separated using EMW, only one compound ($R_f = 0.90$) was observed in the hexane and chloroform extracts. No antifungal compounds were observed in the fractions against the other six plant pathogenic fungi.

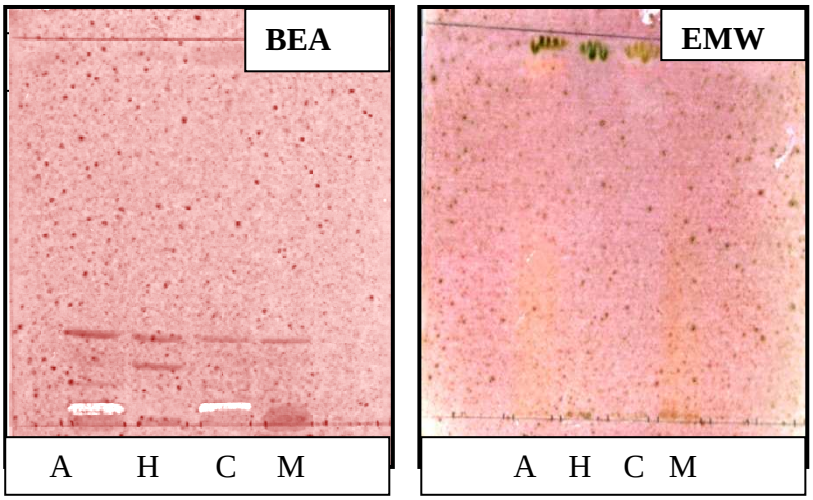

Figure 6-5 Bioautograms of *Breonadia salicina* extracts, serially extracted with A= Acetone (A), Hexane (H), Chloroform (C) and Methanol (M), developed in BEA, and EMW, and sprayed with *A. parasiticus*. White areas indicate inhibition of fungal growth.

6.4 Conclusion

The four serial extraction fractions were not very active against the tested plant pathogenic fungi since they had high MIC values. The methanol fraction had the lowest MIC value (0.16 mg/ml) against *P. expansum*, *P. janthinellum* and *F. oxysporum*. The average MIC values of the fractions varied, with the acetone fraction displaying the lowest average MIC value (0.54 mg/ml). The highest total activity was observed in methanol fraction (141 ml/g) while the hexane fraction had the lowest total activity (25 ml/g).

Of the three fractions resulting from solvent-solvent fractionation only the chloroform fraction had reasonable activity with an MIC of 0.43 mg/ml. This may suggest that there were some inactive compounds still present in the fractions that are associated with high MIC values or separation affected the antifungal activity by disrupting synergy. As could be expected based on the best MIC values, the highest total activity was observed in the chloroform fraction (48 ml/g), while the butanol fraction had the lowest total activity (7 ml/g).

The chloroform serial extraction fraction had highly visible compounds in the chromatograms prepared using BEA, CEF and EMW, and was used for solvent-solvent fractionation to yield aqueous, butanol and chloroform fractions (Figure 6-4). Only the chloroform fraction showed

visible compounds after spraying with vanillin sulphuric acid and no compounds were visible in the aqueous and butanol fractions. However, in the bioautography assay, no antifungal compounds were observed in the fractions (aqueous and butanol) in BEA, CEF and EMW solvent systems. This may suggest, firstly, that some of the compounds may have been volatile and evaporated during the drying period of the TLC chromatograms after developing using three solvent systems. Secondly, some of the residues of formic acid or ammonia following evaporation could have inhibited growth of the plant pathogenic fungi.

To summarise, in serial extraction procedure, the four fractions showed varying degrees of activity against seven plant pathogenic fungi. The chloroform fraction showed the lowest MIC values. After solvent-solvent fractionation of the chloroform fraction, the aqueous, butanol and chloroform fractions had the lowest activity against the tested microorganism. This may suggest that the antifungal activity of *B. salicina* may involve synergistic effects of several compounds. The crude acetone extract had the best antifungal activity when tested against *P. janthinellum* and *F. oxysporum* (MIC value of 0.08 mg/ml, Table 3-1). For further investigation, it therefore appears to be best to focus on the crude extract without preliminary serial extraction. For quality control purposes it is important to know the identity of the active compounds even if they have much lower activity than the crude extract. In the next chapter, isolation of antifungal compounds from leaves of *Breonadia salicina* and their activity against seven plant pathogenic fungi will be discussed.