Antifungal activity of leaf extracts from South African trees against plant pathogens

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Abstract

The antifungal activity of acetone, methanol, hexane and dichloromethane leaf extracts of six plant species (*Bucida buceras, Breonadia salicina, Harpephyllum caffrum, Olinia ventosa, Vangueria infausta* and *Xylotheca kraussiana*) were evaluated for antifungal activity against seven plant pathogenic fungal species (*Aspergillus niger, Aspergillus parasiticus, Colletotricum gloeosporioides, Penicillium janthinellum, Penicillium expansum, Trichoderma harzianum* and *Fusarium oxysporum*). These plant species were selected from 600 evaluated inter alia, against two animal fungal pathogens. All plant extracts were active against the selected plant pathogenic fungi. Of the six plant species, *B. buceras* had the best antifungal activity against four of the fungi, with minimum inhibitory concentration (MIC) values as low as 0.02 mg/ml and 0.08 mg/ml against *P. expansum, P. janthinellum, T. harzianum and F. oxysporum*. Some of the plant extracts had moderate to low activity against other fungi, indicating that the activity is not based on a general metabolic toxicity. *P. janthinellum, T. harzianum and F. oxysporum* were the most sensitive fungal species, with a mean MIC of 0.28 mg/ml, while the remaining four fungi were more resistant to the extracts tested, with mean MICs above 1 mg/ml. The number of active compounds in the plant extracts was determined using bioautography with the listed plant pathogens. No active compounds were observed in some plant extracts with good antifungal activity as a mixture against the fungal plant pathogens, indicating possible synergism between the separated metabolites, *B. salicina and O. ventosa* were the most promising plant species, with at least three antifungal compounds. Leaf extracts of different plant species using different methods (acetone, hexane, DCM and methanol) had antifungal compounds with the same Rf values. The same compounds may be responsible for activity in extracts of different plant species. Based on the antifungal activity, crude plant extracts may be a cost effective way of protecting crops against fungal pathogens. Because plant extracts contain several antifungal compounds, the development of resistant pathogens may be delayed.

Keywords: Antifungal activity; Plant pathogens; Minimum inhibitory concentration; Bioautography; *Breonadia salicina*

1. Introduction

Fungi attacking plants cause major losses in agricultural production. *Aspergillus* spp. cause spoilage of mangoes while Fusarium spp. cause spoilage during food production. *Aspergillus flavus* and *A. parasiticus* also produce highly toxic aflatoxins that contaminate cotton seed, corn, peanuts and tree nuts during harvesting or storage (Wilson and Payne, 1994). *Fusarium oxysporum* causes vascular wilt disease in tomato crops. Fusarium head blight (FHB) is the most common fungal disease affecting wheat and barley worldwide. This results in direct economic losses including reduction of grain yield and quality. Contamination caused by mycotoxins leads to the rejection of the seed in the market place (Parry et al., 1995). Moreover, many fungi are capable of producing mycotoxins, for example deoxynivalenol, nivalenol, and moniliform nivalenol, which have a range of toxicity to animals (Rotter et al., 1996, Desjardin, 2006 and Leslie and Summerell, 2006). Another fungus, *P. expansum* produces a mycotoxin patulin, which causes immunological, neurological and gastrointestinal toxicity in animals (Pitt, 1997). *P. expansum* also causes post harvest losses especially in fruit such as apples, peaches and cherries. Fungal diseases in plants can be controlled by using fungicides. Use of fungicides may have adverse effects including toxicity to humans and organisms in the environment to this practice. Alternatively, biological control using microorganisms may be used against plant pathogens.

Owing to the increase of fungal disease in humans, animals and plants caused by pathogens that are becoming more resistant to currently available drugs, it is important that novel antifungal agents be identified and developed. Recent research has focused on screening medicinal plants to develop new drugs that can

be used to combat fungal infectious diseases (Eloff et al., 2005 and Aliero and Afolayan, 2006). In an ongoing project, leaf extracts of approximately 600 randomly selected South African trees have been screened for activity against human bacterial and fungal pathogens in the Phytomedicine Programme [www.phyto.up.ac.za/phyto] (Pauw and Eloff unpublished data). Some plant extracts have excellent activities against plant fungal pathogens (Masoko et al., 2005 and Masoko et al., 2007).

In this paper, we investigate the antifungal activity of leaf extracts of the following South African tree species: *Bucida buceras* L. (Combretaceae), *Breonadia salicina* (Vahl) Hepper and J.R.I Wood (Rubiaceae), *Harpephyllum caffrum* Bernh. ex Krauss (Anacardiaceae), *Olinia ventosa* (L.) Cufod (Oliniaceae), *Vangueria infausta* Burch. (Rubiaceae) and *Xylotheca kraussiana* Hochst (Flacourtiaceae) against seven plant pathogens (*Aspergillus niger, Aspergillus parasiticus, Colletotricum gloeosporioides, Penicillium janthinellum, P. expansum, Trichoderma harzianum* and *F. oxysporum*). These trees were selected based on the good activity of leaf extracts against two human and animal fungal pathogens. We found no publication on the antifungal activity of the selected plant species.

The motivation for investigating tree leaves is that it can become a sustainable resource if a product is developed from the extract. In some cases leaves have higher activity than bark even though only bark had been used traditionally to combat animal infections (Shai et al., 2009). The use of plant extracts may also be useful to poor rural farmers as they cannot afford commercial fungicides. The use of plant extracts in protecting crops against fungal pathogens may inhibit the development of resistance in the pathogen population due to the different antifungal compounds it contains. The use of a crude plant extract to protect crops against fungal attack may be acceptable in the organic production of crops.

2. Materials and methods

2.1. Plant collection

Plant leaves were collected from labelled trees growing in the Lowveld National Botanical Garden in Nelspruit, Mpumalanga during the summer on a sunny day after all traces of moisture has evaporated. The tree labels indicated the year of planting and contained a reference number. From this the origin of the seed collection could be traced using the herbarium database. To ensure efficient drying, leaves were collected in open mesh orange bags and kept in the shade to minimize photo-oxidative changes.

Collected fresh plant material was examined and the old, insect- and fungus-infected leaves were removed. Leaves were dried at room temperature (c. 25 °C) for about a week in a forced air draught in a purpose-built drying machine until the leaves were brittle enough to break easily. The dried plant material was ground to a fine powder (diameter c. 0.1 mm) using a laboratory grinding mill (Telemecanique/MACSALAB model 200 LAB) and stored in airtight bottles in the dark until extraction.

2.2. Extraction procedure

Separate aliquots of finely ground plant material (4 g) were extracted with 40 ml of solvents of increasing polarities: hexane, dichloromethane, acetone and methanol (technical grade-Merck) in polyester plastic tubes, while shaking vigorously for 3–5 min on a Labotec model 20.2 shaking machine at high speed. The solvent polarity parameters of these extractants are 0.1, 3.1, 5.1 and 5.1, respectively. The solvent strength parameters for these extractants on alumina are 0.01, 0.42, 0.56 and 5.1, respectively. After centrifuging at 3500 rpm for 5 min, the supernatants were decanted into labelled, weighed glass vials. The process was repeated three times on the marc and the extracts were combined. The solvent was removed under a stream of cold air at room temperature.

2.3. Phytochemical analysis

Chemical constituents of the extracts were analyzed using aluminium-backed Thin Layer Chromatography (TLC) plates (ALIGRAM®SIL g/UV 254-MACHEREY-NAGEL, Merck), that were developed with either one of the three eluent systems developed in the Phytomedicine Programme (Kotze and Eloff, 2002):

- Ethyl acetate:methanol:water: 40:5:4:4 [EMW] (polar)

- Chloroform:ethyl acetate:formic acid: 5:4:1 [CEF] (intermediate polarity:acidic)
- Benzene:ethanol:ammonia hydroxide: 90:10:1 [BEA] (non-polar/basic)

Development of the chromatograms was under eluent saturated conditions. Samples (100 µg) were applied on the TLC plates in a c. 1 cm band and developed without delay to minimize the possibility of photo-oxidative change. The separated components were visualized under visible and ultraviolet light (254 and 360 nm, Camac Universal UV lamp TL-600). For the detection of chemical compounds not visible under UV light, vanillin-sulphuric acid spray reagent (Stahl, 1969) was used for detection.

2.4. Fungal strains and inoculum quantification

The seven test fungal species, *A. niger, A. parasiticus, C. gloeosporioides, T. harzianum, P. expansum, P. janthinellum* and *F. oxysporum,* were obtained from the Department of Microbiology and Plant Pathology at the University of Pretoria. These fungi are among the most important pathogenic fungi of economical significance to plants. Fungal strains were maintained on Potato Dextrose (PD) agar. Fungal cultures were subcultured (1% inoculum) in PD broth at 35 °C for at least two to four days before being used in the screening assays.

For quantification of fungi, the haemocytometer cell-counting method described by Aberkane et al., (2002) with some modifications was used for counting the number of cells for each fungal culture. The inoculum of each isolate was prepared by first growing the fungus on potato dextrose (PD) agar slants for seven days at 35 °C. The slant was rubbed carefully with a sterile cotton swab and transferred to a sterile tube with fresh PD broth (50 ml). The sterile tubes were then shaken for 5 min and appropriate dilutions were made in order to determine the number of cells by microscopic enumeration using a haemocytometer (Neubauer chamber; Merck S.A.). The final inoculum concentration was adjusted to approximately 1.0 × 106 cells/ml. To confirm the inoculum adjustment, 100 µl of serial dilutions of the conidial suspensions was spread onto PD agar plates. The plates were incubated at 35 °C and observation of the presence of fungal growth was done daily. The colonies were counted after the observation of visible growth and used to calculate the corresponding cells/ml.

2.5. Determining antifungal activity

2.5.1. Microdilution assay

The microplate method of Eloff (1998b), modified for antifungal activity testing by Masoko et al. (2005), was used to determine the minimum inhibitory concentration (MIC) values for plant extracts. The plant extracts were tested in triplicate in each assay, and the assays were repeated in their entirety to confirm results. Residues of different extracts were dissolved in acetone to a concentration of 10 mg/ml. The plant extracts (100 µl) were serially diluted 50% with water in 96 well microtitre plates (Eloff,1998b), and 100 µl of fungal culture was added to each well. Amphotericin B was used as the reference antibiotic and 100% acetone as the negative control. It was previously shown (Eloff et al., 2007) that the final concentration of acetone in the microplate well that the fungi are subjected to has no influence on the growth of fungi. As an indicator of growth, 40 µl of 0.2 mg/ml p-iodonitrotetrazolium violet (INT) dissolved in water was added to the microplate wells. The covered microplates were incubated for three to five days at 35 °C at 100% relative humidity after sealing in a plastic bag to minimize fungal contamination in the laboratory. The MIC was recorded as the lowest concentration of the extract that inhibited antifungal growth. The colourless tetrazolium salt acts as an electron acceptor and is reduced to a red-coloured formazan product by biologically active organisms (Eloff, 1998b). Where fungal growth is inhibited, the solution in the well remains clear or shows a marked reduction in intensity of colour after incubation with INT.

In order to determine which plants have the highest potential for further development, not only the MIC value is important, but also the quantity extracted from the plant material. Since the MIC value is inversely related to the quantity of antifungal compounds present, the quantity of antifungal compounds present (total activity) was calculated by dividing the quantity extracted in mg extracted from 1 g of plant material by the MIC in mg/ml. The total activity indicates the volume at which an extract from 1 g of plant material can be diluted

and still inhibit the growth of the test organism (Eloff, 1999). It can also be used to evaluate losses during isolation of active compounds and the presence of synergism (Eloff, 2004).

2.5.2. Bioautography

TLC plates (10 × 10 cm) were loaded with 100 µg of each of the extracts with a micropipette in a line c. 1 cm wide. The prepared plates were developed using different mobile systems of varying polarity: CEF, BEA and EMW. The chromatograms were dried at room temperature under a stream of air overnight to remove the remaining solvent. Fungal cultures were grown on potato dextrose (PD) agar for 3–5 days. Cultures were transferred into PD broth from agar with sterile swabs. The developed TLC plates were sprayed with a concentrated suspension containing c. 1.0 × 106 cells/ml of actively growing fungi. The plates were sprayed until wet, incubated overnight, sprayed with 2 mg/ml solution p-iodonitrotetrazolium violet and further incubated overnight or longer at 35 °C in a clean chamber at 100% relative humidity in the dark. White areas indicated where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of the test fungi. The plates were sealed in plastic to prevent the spreading of the fungi in the environment and to retain the humidity and then scanned to produce a record of the results.

3. Results and discussion

Acetone was the best solvent, extracting a larger quantity of material (between 8 and 12%) than the other three solvents (Table 1). This confirmed conclusions reached earlier (Eloff, 1998a). Acetone and methanol extracted large quantities from *B. buceras, H. caffrum, V. infausta* and *X. kraussiana* while methanol extracted the lowest quantity from *B. salicina* and *O. ventosa*.

Table 1. *Percentage of material extracted from powdered leaf material of different species by acetone [A], hexane [H], dichloromethane [D] and methanol [M].*

Plant species	Plant material extracted (%)			
	A	H	D	M
Bucida buceras	11.75	8.8	12.0	11.3
Breonadia salicina	8.5	8.5	7.8	6.5
Harpephyllum caffrum	11.0	8.5	9.8	11.5
Olinia ventosa	10.3	8.3	9.8	7.5
Vangueria infausta	11.7	12.3	10.3	12.0
Xylotheca kraussiana	11.7	8.8	8.5	11.5

P. janthinellum, T. harzianum and *F. oxysporum* had significant sensitivity to the plant extracts with the lowest average MIC values of 0.28 mg/ml (Table 2). The acetone extract had excellent activity with an MIC of 0.02 mg/ml against these organisms. Acetone and methanol extracts of several other plant species also had good antifungal activity against these three sensitive test organisms. *F. oxysporum* was sensitive. This fungus has been reported to cause vascular wilt and damping off in plants which could result in substantial stand reduction and yield (Kishi, 1974). The other four fungi (*A. niger, A. parasiticus, C. gloeosporioides* and *P. expansum*) were more resistant to all of the extracts with a mean MIC of 1.06, 1.63, 1.54 and 1.27 mg/ml, respectively. *A. niger* was also more resistant to DCM, aqueous and methanolic extracts of 14 plants used traditionally in Paraguay (Portillo et al., 2001).

Table 2. *Minimum inhibitory concentration (MIC) of six plant species against plant pathogenic fungi using different extractants (A = acetone, H = hexane, D = dichloromethane, M = methanol). The results are the mean of three replicates and the standard deviation was zero.*

Microorganism[a]	Bucida buceras				Breonadia salicina				Harpephyllum caffrum				Olinia ventosa				Vangueria infausta				Xylotheca kraussiana				Mean
	A	H	D	M	A	H	D	M	A	H	D	M	A	H	D	M	A	H	D	M	A	H	D	M	
A. p.	0.63	1.25	1.25	1.25	0.63	2.50	0.63	1.25	1.25	2.50	1.25	0.63	1.25	1.25	0.63	1.25	1.25	2.50	0.63	0.63	0.16	0.63	0.32	0.32	1.06
A. n.[b]	0.63	2.50	2.50	1.25	2.50	1.25	1.25	2.50	1.25	2.50	1.25	0.63	1.25	2.50	1.25	2.50	1.25	2.50	2.50	2.50	0.63	2.50	1.25	1.25	1.63
C. g.[b]	0.63	2.50	2.50	0.63	1.25	2.50	1.25	1.25	1.25	0.63	1.25	2.50	1.25	2.50	1.25	2.50	1.25	2.50	2.50	2.50	1.25	2.50	0.63	0.63	1.54
P. e.	0.08	0.63	0.32	0.32	1.25	2.50	2.50	2.50	2.50	1.25	2.50	0.63	0.32	1.25	1.25	0.63	2.50	1.25	1.25	1.25	0.63	2.50	1.25	1.25	1.27
P. j.[b]	0.02	0.08	0.32	0.02	0.08	0.08	0.08	0.08	0.04	0.32	0.16	0.08	0.08	0.32	0.16	0.32	0.63	0.63	0.63	0.32	0.32	0.63	0.63	0.63	0.28
T. h.[b]	0.02	0.08	0.63	0.02	0.63	0.32	0.63	0.63	0.08	0.63	0.63	0.08	0.04	0.04	0.04	0.08	0.32	0.32	0.32	0.32	0.16	0.32	0.16	0.32	0.28
F. o.	0.02	0.63	0.32	0.04	0.32	0.08	0.16	0.16	0.02	0.32	0.16	0.04	0.08	0.63	0.32	0.16	0.32	0.63	0.32	0.32	0.63	0.32	0.32	0.32	0.28
Mean	0.29	1.02	1.05	0.5	0.92	1.25	0.89	1.12	0.88	1.09	0.99	0.62	0.61	1.14	0.70	0.99	1.04	1.37	1.09	1.05	0.54	1.23	0.65	0.67	0.91

Positive control: Amphotericin B was used as a positive control. MIC for *A. parasiticus* and *A. niger* was 0.02 mg/ml after 24 and 48 incubation and for the other fungi was <0.02 mg/ml.

[a] Microorganisms: *A. p. = Aspergillus parasiticus, A. n. = Aspergillus niger, C. g. = Colletotricum gloeosporioides, P. e. = Penicillium expansum, P. j. = Penicillium janthinellum, T h = Trichoderma harzianum and F. s. = Fusarium oxysporum.*

[b] With these organisms little reaction was observed after 24 h and values were measured after 48 h.

Table 3. *Total activity in ml/g of six plant species extracted with acetone (A), hexane (H), dichloromethane (D) and methanol (M) tested against seven fungi.*

Microorganism[a]	Bucida buceras				Breonadia salicina				Harpephyllum caffrum				Olinia ventosa				Vangueria infausta				Xylotheca kraussiana				Mean
	A[b]	H[b]	D[b]	M[b]	A	H	D	M	A	H	D	M	A	H	D	M	A	H	D	M	A	H	D	M	
A. p	746	140	762	360	540	272	248	208	352	151	312	730	328	264	619	240	376	218	651	762	2938	556	1063	1438	595
A. n	209	280	384	200	540	151	138	208	352	151	312	730	328	147	312	133	376	218	182	213	746	156	272	368	296
C g	376	155	384	360	540	151	138	413	352	540	312	204	328	147	312	133	376	218	182	213	376	156	540	730	318
P. e	376	156	213	200	4250	540	969	813	196	272	173	730	1281	264	312	476	209	392	328	384	746	156	272	368	587
P. j	5875	4375	6000	5625	17000	4250	969	130	11000	1063	2438	5750	5125	1031	2438	938	746	777	651	1500	1469	556	540	730	3910
T. h	746	1094	762	714	17000	4250	492	130	5500	540	619	5750	10250	8250	9750	3750	1469	1531	1281	1500	2938	1094	2125	1438	3993
F. o	1467	4375	3000	2813	17000	540	969	6500	220	106	243	11500	5125	524	1219	1875	1469	778	1281	1500	746	1094	1063	1438	4222
Average	1400	1510	1644	1467	8124	1451	560	4877	5679	1906	943	3628	3252	1518	2137	1078	717	590	651	868	1423	538	839	930	1989

[a] Microorganisms: *A. p. = Aspergillus parasiticus, A. n. = Aspergillus niger, C. g. = Colletotricum gloeosporioides, P. e. = Penicillium expansum, P. j. = Penicillium janthinellum, T h = Trichoderma harzianum and F. s. = Fusarium oxysporum*.

[b] Extractant: A = acetone, H = hexane, D = dichloromethane, M = methanol.

Acetone and methanol extracts of *H. caffrum* had good antifungal activity against the three sensitive fungi, with mean MIC values ranging from 0.02 to 0.08 mg/ml, except for the DCM extract against *T. harzianum* with MIC = 0.63 mg/ml. The acetone and DCM extracts of *V. infausta* had lowest activity against the tested fungi (MIC values of 0. 32 mg/ml). All of the *O. ventosa* extracts had the highest activity against *T. harzianum.*

Acetone extracts had the lowest mean MIC value of 0.71 mg/ml against all the tested fungi, followed by DCM (0.83 mg/ml), methanol (0.89 mg/ml) and hexane (1.19 mg/ml). This indicates that compounds with intermediate polarity have the highest activity. This may be related to the uptake of compounds by fungal cells. Plant extracts with low MIC values could be a good source of bioactive components with antimicrobial potency. In particular, acetone extracts have shown potentially interesting activity compared to the other extracts. In our experience acetone was the best extractant for extracting and isolating antibacterial compounds from many plant species. Based on the MIC results obtained in this study with different extractants, it confirmed the results obtained using bacteria that acetone is the best extractant (Eloff, 1998a) to isolate antimicrobial compounds. Other reasons for its suitability include its volatility, miscibility with polar and non-polar solvents and its relatively low toxicity against test microorganisms (Eloff, 1998a). In the current study acetone was not harmful to the plant pathogens at the chosen concentrations, as shown by lack of antifungal effect in the solvent controls in the assay. This confirmed results found with human and animal fungal pathogens (Eloff et al., 2007).

The total activity values of six plant species extracted with acetone, hexane, dichloromethane and methanol are given in Table 3. The lowest total activity was observed in methanol extracts of *O. ventosa* (109 ml/g) against *T. harzianum* and *F. oxysporum*. The highest total activity was found in acetone extract of *O. ventosa* (5125 ml/g) against *F. oxysporum*. This implies that if an acetone extract from 1 g of plant material was diluted to 5 L it would still kill *F. oxysporum*, indicating the possible commercial value of such an extract if the mammal and environmental toxicity is within an acceptable level.

Bioautography was used to determine the number of active compounds of different plant extracts. A representative bioautogram is shown in Fig. 1. The active compounds separated with CEF had similar Rf values of 0.70, 0.85 and 0.95 in acetone, hexane, DCM and methanol extracts, respectively, of *B. salicina* against *A. parasiticus*. Antifungal compounds with Rf values of 0.54, 0.72 and 0.95 were visible in the acetone, DCM and methanol extracts, respectively, of *O. ventosa*. Acetone and hexane extracts of *B. salicina* had similar active compounds against *P. janthinellum* and *A. niger* at Rf value 0.17 in EMW (non-polar). Active compounds with the same Rf value of 0.13 were observed in the acetone and hexane extracts of *B. salicina* against *C. gloeosporioides* and *A. parasiticus*. *B. salicina* and *O. ventosa* were the most promising species since the four extracts (acetone, hexane, DCM and methanol) displayed several compounds inhibiting fungal growth and can be used for further isolation. The separated compounds in the extracts of the four remaining plant species (*B. buceras, H. caffrum, V. infausta* and *X. kraussiana*) had no active zones on the bioautograms screening against the test organisms but the microplate assay gave good activity. Possible reasons may be that some of the active compounds were volatile and evaporated or inactivated by photo-oxidation during the drying period of the TLC plates. If there were compounds with synergistic effects present in the crude extract and these compounds were separated on the bioautograms it would also explain the discrepancy.

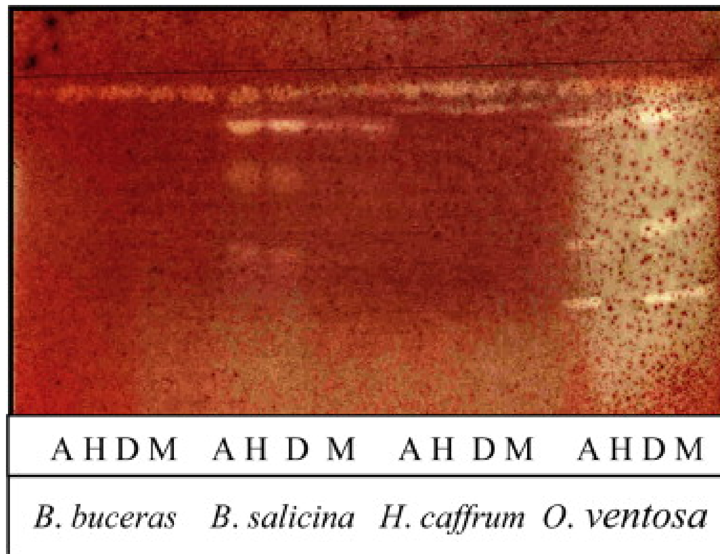

Fig. 1. :Bioautogram of extracts of *Bucida buceras*, *Breonadia salicina, Harpephyllum caffrum* and *Olinia* v*entosa*. TLC plates developed in CEF sprayed with *Aspergillus parasiticus*. White areas indicate inhibition of fungal growth. Lanes from left to right: acetone (A), hexane (H), DCM (D) and methanol (M). Some tree leaf extracts have good activity against plant fungal pathogens.

4. Conclusion

Acetone was the best extractant, since it extracted active antifungal compounds from many of the test plants, and is low in toxicity to the test organisms at the concentrations used in the assay. The current findings showed that leaf material of some of these six selected plant species have promising antifungal activity. B. buceras had the highest antifungal activity against a range of plant pathogenic fungal organisms.

Based on both MIC and bioautography results, *B. salicina* and *O. ventosa* appear to be the best plant species for isolation of antifungal compound(s). Compounds active against several of the fungal pathogens have been isolated and characterised from *B. salicina* and had promising activity in a trial to protect oranges against infection (Mahlo, 2009). This study demonstrates the potential of plant species as sources of extracts or pure compounds with activity against plant pathogenic fungi.

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