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# Antioxidant and antifungal activity of selected plant species used in traditional medicine

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***In vitro*** **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*) was determined using a serial dilution assay against *Aspergillus fumigatus*. These plant species were selected from 600 evaluated, inter alia, against two animal fungal pathogens. All plant extracts were screened for antifungal activity against *A. fumigatus*. Of the six plant species, *B. salicina* had the best antifungal activity against the test microorganism with a minimum inhibitory concentration (MIC) value of 0.08 mg/ml. Some of the extracts had moderate to low activity against the tested microorganism. Antioxidant activity of the aforementioned plant extracts were investigated using a qualitative assay (2, 2-diphenyl-1-picrylhydrazyl (DPPH)). The plant extracts of five of the plant species did not have strong antioxidant activity. The methanol extract of *X. kraussiana* was the most active radical scavenger in the DPPH assay amongst the six medicinal plants screened. No quantitative assay was conducted since the plant extracts did not possess strong antioxidant activity. A bioautography assay was used to determine the number of active compounds in the plant extracts. No antifungal compounds were observed in some of the plant extracts with good antifungal activity shown in the microdilution assay, so synergistic effects of more than one active compound are possible. *B. salicina* was the most promising plant species with at least three distinct antifungal compounds. Plant extracts with low MIC values could be a good source of bioactive components with antimicrobial potency.**

**Key words:** Antioxidant activity, antifungal activity, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), minimum inhibitory concentration, bioautography assay, *Breonadia salicina*.

## INTRODUCTION

Plants contain a wide variety of free radical scavenging molecules such as flavonoids, anthocyanins, carotenoids, vitamins and endogenous metabolites. More importantly, these natural products are rich in antioxidant activities (Hertzog et al., 1992). Antioxidants are effective free radical scavengers which tend to retard or prevent the oxidation of other molecules by capturing free radicals (Breton, 2008). Plant compounds have various biological activities such as anti-inflammatory, anticarcinogenic and anti-atherosclerotic, antibacterial, antifungal, antivira antimutagenic and antiallergic activities (Ikken et al., 1999; Noguchi et al., 1999; Mishra et al., 2009). These

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biological activities may be associated with their antioxidant activity (Chung et al., 1998).

Antioxidant compounds help delay and inhibit lipid oxidation, and they play an important role in the maintenance of health and prevention of several diseases. The best way to help prevent diseases is consumption of an optimal diet containing natural antioxidants. When these constituents are added to foods they tend to minimize rancidity, retard the formation of toxic oxidation products, help maintain the nutritional quality and increase shelf life (Fukumoto and Mazza, 2000). The consumption of food such as fruit, vegetables, red wines and juices helps protect the body from being afflicted with diseases such as cancer and coronary heart disease. This protection is due to the capacity of antioxidants in the plant foods to scavenge free radicals, which are responsible for the oxidative damage of lipids, proteins, and nucleic acids.

Several medicinal plants contain large amounts of anti-oxidants such as polyphenols, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Djeridane et al., 2006; Kumar et al., 2013). A polyphenol antioxidant is a type of antioxidant containing a polyphenolic substructure found in a wide array of phytonutrient-bearing foods. For example, most legumes, fruits (such as apples, grapes, pears, plums, raspberries and strawberries), vegetables (such as broccoli, cabbage and onion) are rich in polyphenol antioxidants (Breton, 2008). Previously, it has been reported that polyphenolic compounds have antioxidant activity, free-radical scavenging capacity, coronary heart disease prevention ability, and anticarcinogenic properties (Satora et al., 2008).

Two free radicals, 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) are commonly used to determine antioxidant activity in plant extracts and isolated compounds. The ABTS assay measures the relative ability of antioxidants to scavenge free radicals generated in aqueous phase, compared with Trolox, a water soluble vitamin E analogue standard (Miller and Evans, 1997). The method is rapid and can be used over a wide range of pH values (Arnao, 1999; Lemanska et al., 2001), in both aqueous and organic solvent systems. It is also preferred since it has good reproducibility and is easy to perform.

In this paper, the antioxidant and antifungal activities of the leaf extracts of the following South African tree species were investigate: *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) using the qualitative antioxidant method (DPPH) and serial dilution assay. These plant species were selected based on good preliminary activity of leaf extracts against human and animal fungal pathogens. In recent studies, various plant extracts had good antifungal activity against *Aspergillus fumigatus* (Sulieman et al., 2010; Shai et al., 2008). It has also been reported that plant extracts possess biological activities such as antimicrobial, antifungal and antioxidant activities (Iscan et al., 2002; Soković et al., 2009; Kanatt et al., 2008). However, there are few different classes of effective antifungal drugs available for the treatment of fungal diseases of plants, animals and humans.

Further development of antifungal compounds is necessary, because there has been an alarming increase in the incidence of new and re-emerging infectious diseases.

## MATERIALS AND METHODS

### 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 had 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.

### 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 to 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.

### 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:ammonium hydroxide: 90:10:1 [BEA] (nonpolar/basic). Development of the chromatograms was under eluent-saturated conditions. Plant extract 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.

**Assay for free radical scavenging (DPPH)**

The antioxidant activities of plant extracts were determined using the qualitative DPPH method. This assay is preferred, because it provides stable free radicals (Fatimi et al., 1993). The effect of antioxidants on DPPH radical scavenging is due to their hydrogen donating ability.

The prepared TLC chromatograms were visualized under UV light and the compounds were identified and highlighted by light pencil circles. A solution of 0.2% DPPH in methanol was prepared and then sprayed on the plates (until they became wet) and allowed to dry in a fume cupboard. The presence of antioxidant compounds was indicated by yellow bands which showed radical scavenger capacity against a purple background. The intensity of the yellow band depends on the quantity and nature of the radical scavenger present in the plant extracts.

**Fungal strain and inoculums quantification**

*A. fumigatus* was obtained from the culture collection of the Department of Veterinary Tropical Diseases 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. Fungal culture was subcultured (1% inoculums) in Potato Dextrose (PD) broth at 35°C for at least 4 to 5 days before being used in the screening procedure.

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 SD agar slants for seven days at 35°C. The culture on the slant was rubbed carefully with a sterile cotton swab and transferred to a sterile tube containing fresh SD 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 SA). The final inoculum concentration was adjusted to approximately $1.0 \times 10^6$ cells/ml. To confirm the inoculum adjustment, 100 µl of serial dilutions of the conidial suspensions were spread onto SD 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.

**Determining antifungal activity**

***Microdilution assay***

The microplate method of Eloff (1998a), 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 up to 25% 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 3 to 5 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 plant species have the highest potential for further development, not only the MIC value is important, but also the quantity extracted from the plant material. According to Eloff (1999), the MIC value is inversely related to the quantity of antifungal compounds present, and the quantity of antifungal compounds present (total activity) may be 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).

**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: EMW, CEF and BEA. The chromatograms were dried at room temperature under a stream of air overnight to remove the remaining solvent. Fungal cultures were grown on SD agar for 3 to 5 days. Cultures were transferred into SD broth from agar with sterile swabs. The developed TLC plates were sprayed with a concentrated suspension containing c. $1.0 \times 10^6$ 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.

## RESULTS AND DISCUSSION

The DPPH assay is a common method used for the screening of antioxidant activity in various plant extracts. DPPH produces a violet colour in methanol solution and is reduced to a yellow coloured product, diphenylpicryl hydrazine. Antioxidants react with DPPH, a stable nitrogen-centered free radical, and convert it to α,α-diphenyl-β-picryl hydrazine. The degree of discoloration indicates the scavenging potential of the antioxidant extracts (Kumar et al., 2012).

The chromatograms developed in BEA did not possess strong antioxidant activity in most of the plant extracts, probably because antioxidant compounds are usually too polar to be separated well with the BEA solvent system.

**Table 1.** Minimum inhibitory concentration (MIC) of six plant species against *Aspergillus fumigatus* using different extractants (A = acetone, H = hexane, D = dichloromethane, M = methanol).

| Plant species | Time (h) | MIC (mg/ml) | | | | Mean |
|---|---|---|---|---|---|---|
| | | A | H | D | M | |
| *B. buceras* | 24 | 0.04 | 0.32 | 0.16 | 0.16 | 4.94 |
| | 48 | 1.25 | 1.25 | 2.5 | 2.5 | 11.1 |
| *B. salicina* | 24 | 0.08 | 0.08 | 0.08 | 0.08 | 0.32 |
| | 48 | 1.25 | 1.25 | 2.5 | 2.5 | 11.1 |
| *H. caffrum* | 24 | 0.16 | 0.32 | 0.16 | 0.32 | 0.96 |
| | 48 | 1.25 | 2.5 | 2.5 | 2.5 | 11.4 |
| *O. ventosa* | 24 | 0.16 | 0.32 | 0.16 | 0.32 | 0.96 |
| | 48 | 1.25 | 2.5 | 2.5 | 2.5 | 11.4 |
| *V. infausta* | 24 | 0.08 | 0.16 | 0.16 | 0.32 | 0.72 |
| | 48 | 2.5 | 2.5 | 1.25 | 1.25 | 11.1 |
| *X. kraussianna* | 24 | 0.02 | 0.04 | 0.04 | 0.08 | 0.18 |
| | 48 | 0.63 | 0.63 | 0.63 | 2.5 | 10.5 |
| Average | - | 0.72 | 0.99 | 1.09 | 1.28 | 1.02 |

The results are the mean of three replicates and the standard deviation was zero (0).

Only the methanol extract of *X. kraussiana* showed a yellow band with an $R_f$ value of 0.14. However, the activity was not strong since the yellow band is not very clear. Most of the yellow bands are visible at the spotted area and these indicate that the compounds were not separated.

Methanol extracts had a higher antioxidant activity than the acetone, hexane and dichloromethane (DCM) extracts. This is again due to the polar nature of most antioxidant compounds, as methanol extracts are largely polar compounds. In comparison to members of the Combretaceae (Masoko and Eloff, 2007), the extracts of the species tested in this study contain very few antioxidant compounds and inhibition of microbial infections by stimulating the immune system of the host does not appear to be a realistic mechanism for their activity and traditional use.

Amongst all of the extracts tested for antifungal activity against *A. fumigatus*, 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 mg/ml (Table 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. The 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, respectively. *H. 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 values were observed in the MeOH extracts (Table 1). Based on the MIC results, acetone was the best extractant, and additional positive features include its volatility, miscibility with polar and non-polar solvents and its relative low toxicity to test organisms (Eloff, 1998a). These results are consistent with those obtained for plant pathogens (Mahlo et al., 2010).

In an earlier study, the crude acetone, hexane and MeOH extracts of *B. buceras* had the highest antifungal activity against four plant pathogenic fungi, *Penicillium janthinellum*, *Penicillium expansum, Trichoderma harzianum* and *Fusarium oxysporum* with MIC values ranging between 0.02 and 0.08 mg/ml (Mahlo et al., 2010). Moreover, 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 1). Surprisingly, the extracts of *X. kraussiana* possess strong antifungal

**Table 2.** Total activity in ml/g of six plant species extracted with acetone (A), hexane (H), dichloromethane (D) and methanol (M).

| Plant species | Time (h) | MIC (mg/ml) | | | | Average |
|---|---|---|---|---|---|---|
| | | A | H | D | M | |
| *B. buceras* | 24 | 875 | 159 | 314 | 797 | 434 |
| | 48 | 28 | 41 | 20 | 51 | 434 |
| *B. salicina* | 24 | 1769 | 507 | 1250 | 2781 | 1266 |
| | 48 | 57 | 32 | 40 | 89 | 53 |
| *H. caffrum* | 24 | 407 | 78 | 68 | 282 | 172 |
| | 48 | 52 | 10 | 17 | 71 | 40 |
| *O. ventosa* | 24 | 567 | 127 | 255 | 1219 | 438 |
| | 48 | 73 | 16 | 16 | 156 | 62 |
| *V. infausta* | 24 | 1134 | 252 | 316 | 283 | 402 |
| | 48 | 36 | 16 | 40 | 72 | 42 |
| *X. kraussianna* | 24 | 1250 | 500 | 750 | 2344 | 974 |
| | 48 | 40 | 32 | 48 | 75 | 49 |

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 only moderate activity with MIC ranging between 0.16 and 2.50 mg/ml (Mahlo et al., 2010). This indicates that all plant extracts were active against plant and animal pathogens. In the current study, extracts of *H. caffrum* were particularly active against *A. fumigatus*. Some of the plant extracts did not show the best antifungal activity against *Aspergillus* species (Table 1). 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 positive control (amphotericin B) had the lowest MIC with value of <0.02 mg/ml against the tested animal fungal pathogen. This confirms the results of plant extracts active against plant fungal pathogens (Mahlo et al., 2010).

The total activity values of the six plant species extracted with acetone, hexane, dichloromethane and methanol are given in Table 2. 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 of *H. caffrum* after 24 h (Table 2). This total activity value means that the methanol extract from 1 g of *B. salicina* leaves diluted to 2 781 ml will still inhibit the growth of the fungus. All of the extracts did not possess strong antifungal activity after 48 h indicating that the fungal pathogen was able to overcome the antifungal activity to some degree after time.

The bioautography assay was used to determine the number of antifungal compounds in the different plant extracts. Figure 1 shows the chromatograms of TLC plates separated in CEF. Most of the antifungal compounds were visible when eluted with CEF, where at least three compounds were observed in the acetone, one in the 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. In BEA, one antifungal compound was observed in all the extracts ($R_f$ 0.08) of *B. salicina*, while the extractants of *X. kraussiana* had 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 1). In the CEF solvent system, 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 the 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*. 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*.

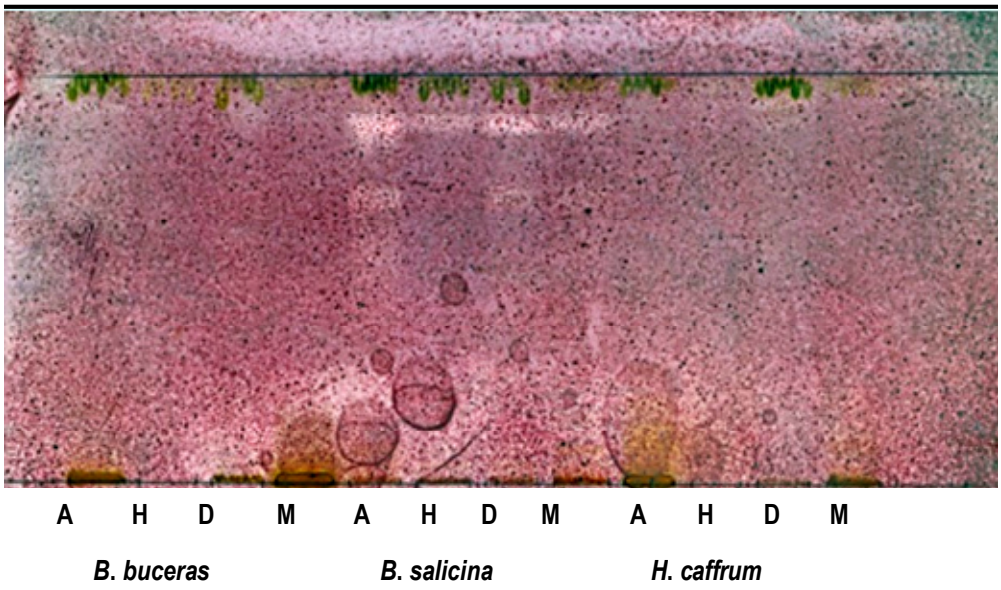


**Figure 1.** Bioautograms of extracts of *Bucida buceras*, *Breonadia salicina* and *Harpephyllum caffrum*. TLC plates developed in CEF sprayed with *Aspergillus fumigatus*. White areas indicate inhibition of fungal growth. Lanes from left to right: A = acetone, H= hexane, D = dichloromethane, M = methanol.

## Conclusion

There are two ways in which a plant extract can protect the host against infections. In the first place it could directly inhibit the growth of pathogens. It is however also possible that the extract could stimulate the immune system of the host so that it is able to withstand the infection. Antioxidant activity was determined qualitatively by spraying TLC plates with 0.2% DPPH. The plant extracts of the five plant species did not possess strong antioxidant activity. As expected, the polar methanol extracts of *X. kraussiana* showed antioxidant activity. Due to the limited activity visualised in the qualitative assays, it was deemed unnecessary to include quantitative antioxidant assays in the screening procedure.

Acetone was the best extractant since it extracted active antifungal compounds from most of the test plant species and is low in toxicity to the test organism at the concentrations used in the assay. *B. salicina* extracts had the highest antifungal activity against the animal fungal pathogen *A. fumigatus*. Amongst the six plant species used in the screening, all four extracts of *O. ventosa* had moderate to low antifungal activity against *A. fumigatus*. This aspect of the study was initiated since other researchers obtained good results using plant extracts against *A. fumigatus* to protect poultry against aspergillosis. 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.

It appears that the compounds with antifungal activity, detected using bioautography techniques, did not have any antioxidant activity. If they did have such activity, it would have been easier to isolate the active compounds by using the DPPH assay rather than the more complicated and time consuming antifungal bioautography assay for bioassay-guided fractionation to isolate the antifungal compounds.

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