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Short communication

The activity of extracts of seven common invasive plant species on fungal phytopathogens

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Abstract

Acetone extracts from different parts of seven common invasive plant species occurring in South Africa were studied as potential sources of antifungal agents for selected phytopathogenic fungi (*Penicillium janthinellum*, *Penicillium expansum*, *Aspergillus niger*, *Aspergillus parasiticus*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *Trichoderma harzianum*, *Phytophthora nicotiana*, *Pythium ultimum* and *Rhizoctonia solani*. The invasive plant species were *Cestrum laevigatum* (flowers and leaves), *Nicotiana glauca* (flowers, leaves and seeds), *Solanum mauritianum* (fruits and leaves), *Lantana camara* (fruits, flowers and leaves), *Datura stramonium* (seeds), *Ricinus communis* (leaves) and *Campuloclinium macrocephalum* (leaves and flowers). All extracts exhibited moderate to good activities on all tested fungi with minimum inhibitory concentrations (MICs) ranging from 0.08 mg/ml to 2.5 mg/ml. In all cases leaf extracts were more active than seed or flower extracts. The growth of *A. niger*, *P. expansum* and *R. solani* was the most sensitive to all the extracts tested, with average MICs of 0.81, 0.83 and 0.84 mg/ml respectively. *C. macrocephalum* leaf extract was the most active against *C. gloeosporioides* with an MIC of 0.05 mg/ml. If extracts of these species do not have deleterious effects against plants infected by the fungi or the environment, it may be useful to protect organically grown crops.
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Keywords: Antifungal activity; Fungal phytopathogens; Invasive plant species; Organic production

1. Introduction

More than 800 million people in developing countries do not have adequate food supplies and at least 10% of food is lost due to plant diseases (Strange and Scott, 2005). Plant diseases are caused by pathogens such as fungi, bacteria, nematodes and viruses. Compared to other plant parasites, fungi cause the greatest impact with regard to diseases and crop production losses. This includes considerable foliage and post harvest losses of fruits and vegetables which are brought about by decay due to fungal plant pathogens.

Common fungal diseases include powdery mildew, rust, leaf spot, blight, root and crown rots, damping-off, smut, anthracnose, and vascular wilts. Some notorious plant pathogenic fungi include *Pythium*, *Phytophthora*, *Fusarium* and *Rhizoctonia* spp, which cause root and crown rot, and seedling damping-off in many vegetables and ornamental plants. Apart from causing diseases in plants, many species of *Fusarium*, *Aspergillus*, *Penicillium* and *Alternaria* are also sources of important mycotoxins of concern in animal and human health (Robert and Richard, 1992; Eaton and Gallagher, 1994; Smith, 1997; Placinta et al., 1999). For example, aflatoxins produced by *Aspergillus flavus* and *A. parasiticus* may cause liver cancer.

The most important method of protecting plants against fungal attack is the use of fungicides. However, many fungicidal agents in the market are toxic and have undesirable effects on other organisms in the environment. Furthermore, halogenated hydrocarbons such as methyl bromide, widely used to control soil-borne pathogens, have ozone-depleting potential (Abritton and Watson, 1992). Some synthetic fungicides are non-biodegradable, and hence can accumulate in soil, plants and water, and consequently effect humans through the food chain.

The development of resistance of pathogenic fungi towards synthetic fungicides is of great concern. There is, therefore, a

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motivation to find safe, efficacious and environmentally friendly fungicides.

Plants have, and continue to be, sources of antifungal agents (Hostettmann et al., 2000). Random screening of the leaves of 350 tree species by the Phytomedicine Programme of the University of Pretoria for antimicrobial activities revealed that large percent of plant species investigated have antifungal activities with MICs values lower than 0.1 mg/ml against animal fungal pathogens (unpublished data).

Many plant species therefore contain antifungal compounds. To develop commercial products, a large quantity of the species has to be cultivated, raising an additional level of complication. If invasive and weedy species contain good antifungal activity they may be a useful source of antifungal compounds or extracts because large quantities of material are available.

Rejmanek and Richardson (1996) stated that there is general pessimism on the prospect of predicting which organisms are likely to become successful invaders. They investigated pine trees and concluded that three parameters can be used to predict invasive potential: small seed size, short juvenile period and a short interval between large seed crops. They speculated that these parameters may also be useful for other woody species. Vila and Weiner (2004) considered whether invasive plant species are better competitors than native plant species and concluded that this may be the case. There is another possibility that have not been addressed as far as we could ascertain. If fungal pathogens play an important role in the growth or establishment of plant species, invasive species may have better resistance against plant pathogens. We have found that a weedy species *Melianthus comosus* has excellent antifungal activity (Eloff et al., 2006).

In this work we investigate weeds and invasive plant species as a potential source of antifungal agents. Most invasive species have been introduced into an environment in which they did not evolve and thus frequently have no natural enemies to limit their reproduction and spread. They compete with agricultural crops for water, light and nutrients, causing enormous losses in food production. Some are also toxic to livestock. In South Africa, more than 120 species have been declared unwanted invasive plants (Bromilow, 2001). An estimated 8%, or 10 million hectares, of South Africa has been invaded by different alien species (Versveld et al., 1998). By using weeds and invasive plant species as raw material for plant-derived fungicides, we may protect indigenous South African plants, and at the same time may create economic uses for these unwanted species.

2. Material and methods

2.1. Plant collection

Leaves, flowers and fruits were collected, in summer, from labelled plant species in the University of Pretoria, Faculty of Veterinary Science toxic plant garden in the Onderstepoort Campus, South Africa. Voucher specimens were verified and easily identified by Prof. C.J. Botha because they are so well known before being deposited in the Herbarium of the Department of Paraclinical Sciences, Faculty of Veterinary Science, Onderstepoort, University of Pretoria. Samples collected were from the following seven species: *Cestrum laevigatum* Schtdl [inkberry] (flowers and leaves), *Nicotiana glauca* Graham [wild tobacco tree] (flowers, leaves and seeds), *Solanum mauritianum* Scop. [bug weed] (fruits and leaves) (Solanaceae), *Lantana camara* L. [tick berry] (fruits, flowers and leaves) (Verbenaceae), *Datura stramonium* L. [thorn apple] (seeds) (Solanaceae) and *Ricinus communis* L. [castor-oil plant] (leaves) (Euphorbiaceae). *Campuloclinium macrocephalum* (Less.) DC. [pom pom weed] (leaves and flowers) (Asteraceae) were collected along the roadside in Pretoria. Voucher specimen numbers are PM010 to PM016 respectively.

2.2. Plant storage

Leaves, flowers and fruits were separated from the stems and dried at room temperature. Most scientists have tended to use dried material because there are fewer problems associated with the large-scale extraction of dried rather than fresh plant material (Eloff, 1998a). The dried plants were milled to a fine powder in a Macsalab mill (Model 200 LAB), Eriez®, Bramley and stored at room temperature in closed containers in the dark until used.

2.3. Extraction procedure

Plant samples from each species were individually extracted by weighing 1.0 g of finely ground plant material and extracting with 10 ml of acetone (technical grade — Merck) in polyester centrifuge tubes. Acetone was elected based on its ability to extract compounds with a wide range of polarities (Eloff, 1998b) Tubes were vigorously shaken for 15 min on a Labotec model 20.2 shaking machine at a high speed. After centrifuging at 3500 rpm for 10 min the supernatant was decanted into pre-weighed labelled containers. The process was repeated two times on the marc to exhaustively extract the plant material and extracts were combined. The solvent was removed under a stream of air in a fume cupboard at room temperature. To quantify the subsequent processes (Eloff, 2004) extract were made up to 10 mg/ml in acetone.

2.4. Phytochemical analysis

Chemical constituents of the extracts were analysed by thin layer chromatography (TLC) using aluminium-backed 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] (intermediately polar/acidic); benzene/ethanol/ammonium hydroxide (90:10:1): [BEA] (non-polar/basic) (Kotze and Eloff, 2002).

To detect the separated compounds, vanillin-sulphuric acid (0.1 g vanillin (Sigma®): 28 ml methanol: 1 ml sulphuric acid) was sprayed on the chromatograms and heated at 110 °C to optimal colour development (Stahl, 1969).

2.5. Fungal test organisms

Ten microorganisms, namely *Penicillium janthinellum*, *Penicillium expansum*, *Aspergillus niger*, *Aspergillus parasiticus*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *Trichoderma harzianum*, *Phytophthora nicotiana*, *Pythium ultimum* and *Rhizoctonia solani* were obtained from Prof. Lise Korsten, Department of Microbiology and Plant Pathology, University of Pretoria. The fungi were chosen after discussion with Prof Korsten, because they are the most important plant pathogenic fungi of agricultural significance. All fungal strains were maintained on Potato dextrose agar (Oxoid, Basingstoke, UK).

3. Antifungal assays

3.1. Microdilution assay

A serial microdilution assay (Eloff, 1998c) was used to determine the minimum inhibitory concentration (MIC) values for plant extracts. To apply it to measuring antifungal activities, a slight modification was made to suit fungal growth conditions (Masoko et al., 2005). Briefly, dried extracts were dissolved in 100% acetone or dimethyl sulphoxide to a concentration of 10 mg/ml. The plant extracts (100 µl) were serially diluted 50% with water in 96 well microtitre plates. Fungal cultures were transferred into fresh Potato dextrose broth, and 100 µl of this was added to each well (containing approximately 10^9 cfu/ml). Positive controls and appropriate solvent blanks were included. As an indicator of growth, 40 µl of 0.2 mg/ml of *p*-iodonitrotetrazolium violet (Sigma®) (INT) dissolved in water was added to each of the microplate wells (Eloff, 1998c). The covered microplates were incubated for two to three days at 26 °C and 100% relative humidity. The MIC was recorded as the lowest concentration of the extract that inhibited fungal growth after 24 h. All determinations were carried out in triplicate. Amphoteracin B was used as a positive control.

3.2. Bioautography

A bioautography method developed in our laboratory (Masoko and Eloff, 2005) was used to determine active compounds. TLC plates (10×20 cm) were loaded with 100 µg (10 µl of 10 mg/ml) of each of the extracts. The plates were developed in the three different mobile systems used: CEF, BEA and EMW. Chromatograms were dried for up to a week at room temperature under a stream of air to remove the remaining solvent.

Cultures were grown on Potato dextrose agar for 3–7 days. Potato dextrose broth was prepared in 250 ml bottles. Cultures were transferred into broth from agar with sterile swabs. TLC plates developed were inoculated with a fine spray of the concentrated suspension containing approximately 10^9 organisms per ml of actively growing fungi e.g. conidia for filamentous fungi and yeast cells for other fungi in a Biosafety Class II cabinet (Labotec, SA). The plates were sprayed until they were just wet, incubated overnight and then sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet (Sigma®) (INT) in boiling water and further incubated overnight or longer at 26 °C in a clean chamber at 100% relative humidity in the dark. White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compound(s) that inhibited the growth of tested fungi. To minimize fungal spreading and infections in the laboratory, the bioautograms were sealed in clear plastic envelopes before scanning for a permanent record.

Table 1
Average MIC values in mg/ml of selected species after 24 h incubation at 26 °C.

Plant species	Yield (mg/g)	MIC values (mg/ml)									
		Fo	Pu	Rs	Ap	An	Pn	Cg	Th	Pe	Average
C. laevigatum leaves	31	0.27	1.67	0.27	0.37	0.21	0.64	0.08	0.16	0.32	0.44
C. laevigatum flowers	37	0.53	2.08	0.32	0.53	0.43	1.05	0.27	1.05	0.53	0.75
L. camara leaves	47	0.08	1.46	0.32	0.64	0.32	0.53	0.37	0.64	0.63	0.56
L. camara flowers	36	0.84	2.50	0.53	0.64	0.43	2.50	1.25	0.53	0.43	1.07
L. camara seeds	19	1.46	1.58	0.53	1.67	1.05	1.25	0.53	0.53	0.43	1.00
N. glauca leaves	50	0.53	0.63	0.64	0.32	0.21	0.64	0.32	0.16	0.43	0.43
N. glauca seed and flowers	98	1.46	1.67	2.08	2.50	1.46	2.08	0.16	1.05	0.84	1.48
S. mauritianum leaves	21	1.46	1.46	0.64	0.43	0.53	1.25	0.63	2.50	0.84	1.08
S. mauritianum fruits	104	2.50	2.50	2.50	2.50	2.50	2.50	0.53	2.50	1.67	2.19
D.. stramonium seeds	169	2.50	2.50	2.50	2.50	2.50	2.50	1.25	2.50	2.50	2.36
R. communis seeds	52	0.84	2.50	0.32	0.64	0.64	0.53	1.05	2.50	0.43	1.05
C. macrocephalum leaves	83	0.11	0.32	0.16	0.53	0.13	0.43	0.05	0.64	0.64	0.33
C. macrocephalum flowers	73	0.32	0.64	0.16	0.53	0.16	0.32	0.64	0.64	1.05	0.50
Average		0.99	1.66	0.84	1.06	0.81	1.25	0.55	1.18	0.83	

The results represent the average of three determinations. In most cases the standard deviation was 0.
Fo — *Fusarium oxysporum*, Pu — *Pythium ultimum*, Rs — *Rhizoctonia solani*, Ap — *Aspergillus parasiticus*.
An — *Aspergillus niger*, Pn — *Phytophthora nicotiana*, Cg — *Colletotrichum gloeosporioides*,
Th — *Trichoderma harzianum*, Pe — *Penicillium expansum*.
MIC for positive control amphotericin B was 0.02 mg/ml for *Aspergillus* spp and <0.02 mg/ml for other fungal spp.

4. Results and discussion

The growth of *C. gloeosporoides*, *A. niger*, *P. expansum* and *R. solani* (Table 1) was generally the most sensitive to the extracts tested, with an average minimum inhibitory concentration (MIC) of 0.55, 0.81, 0.83 and 0.84 mg/ml respectively. The extracts of *C. macrocephalum*, *N. glauca* and *C. laevigatum* leaves were the most active with average MIC values of 0.33, 0.43 and 0.44 mg/ml. *C. macrocephalum* leaves extract was the most active against *C. gloeosporioides* with an MIC value of 0.05 mg/ml. *L. camara* and *C. laevigatum* leaf extracts were most active against *F. oxysporium* and *C. gloeosporioides*, respectively, both with MIC values of 0.08 mg/ml. In all cases leaf extracts were more active than flower or seed extracts. This is not too surprising because leaves are the centres of intermediary metabolism leading to biologically active secondary metabolites. The MIC of the positive control was 0.02 mg/ml against the two *Aspergillus* spp and <0.02 mg/ml against the other fungi.

Acetone was selected as an extractant because it dissolves many hydrophilic and lipophilic components, it is miscible with water and has low toxicity to fungi, and hence is very useful in bioassays (Eloff, 1998b). Acetone extracted more material (see % yield, Table 1) from *D. stramonium* seeds and *S. mauritianum* fruits, and this is probably due to the presence of a high level of fatty acids and lipids in seeds.

Table 2
Total activity in ml/g of selected species extracted after 24 h incubation.

Plant species	Total activity (ml/g)									
	Fo	Pu	Rs	Ap	An	Pn	Cg	Th	Pe	Average
C. laevigatum leaves	115	19	115	84	148	48	388	194	97	134
C. laevigatum flowers	70	18	116	70	86	35	137	35	70	71
L. camara leaves	42	14	68	56	84	14	29	68	84	51
L. camara flowers	588	32	147	73	147	89	127	73	75	150
L. camara seeds	13	12	36	11	18	15	36	36	44	25
N. glauca leaves	94	79	78	156	238	78	156	313	116	145
N. glauca seed and flowers	67	59	47	39	67	47	613	93	117	128
S. mauritianum leaves	14	14	33	49	40	17	33	8	25	26
S. mauritianum fruits	42	42	42	42	42	42	196	42	62	61
D. stramonium seeds	68	68	68	68	68	68	135	68	68	75
R. communis seeds	62	21	163	81	81	98	50	21	121	77
C. macrocephalum leaves	755	259	519	157	638	193	1660	130	130	493
C. macrocephalum flowers	228	114	456	138	456	228	114	114	70	213
Average	166	58	145	79	162	75	283	92	83	

Fo — *Fusarium oxysporum*, Pu — *Pythium ultimum*, Rs — *Rhizoctonia solani*, Ap — *Aspergillus parasiticus*, An — *Aspergillus niger*, Pn — *Phytophthora nicotiana*, Cg — *Colletotrichum gloeosporioides*, Th — *Trichoderma harzianum*, Pe — *Penicillium expansum*.

Table 3
The bioautographic qualitative inhibition of fungal growth by extracts of selected species separated by TLC with BEA as eluent: R_f values of active components.

Rf. values	An	Ap	Pu	Fo	Pj	Pe	Th	Cg	Rs
Lantana camara leaves									
0.06		XXX	XXX		XXX				XXX
0.07	XXX	XXX	XXX	XXX	XXX				XXX
0.14				XX	XX				
0.16				XX	XX				
Lantana camara flowers									
0.06					XXX				
0.07				XXX	XXX				
0.14				XX	XX				
0.16				XX	XX				
Lantana camara seeds									
0.06					XXX				
0.07					XXX				
0.14					XX				
0.16					XX				
Cestrum laevigatum leaves									
0.13	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX
Cestrum laevigatum flowers									
0.13	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX

Fo — *Fusarium oxysporum*, Pu — *Pythium ultimum*, Rs — *Rhizoctonia solani*, Ap — *Aspergillus parasiticus*, An — *Aspergillus niger*, Pn — *Phytophthora nicotiana*, Cg — *Colletotrichum gloeosporioides*, Th — *Trichoderma harzianum*, Pe — *Penicillium expansum*, Pe — *Penicillium janthinellum*.
Relative degree of inhibition: X=slight inhibition, XXX=high inhibition.

The total activity of a plant is the quantity of material extracted from one gram of dried plant material divided by the minimum inhibitory concentration value (Eloff, 2000, 2004). The unit is ml/g and indicates the largest volume to which the biologically active compounds in one gram of plant material can be diluted and still inhibit the growth of the test organism. This parameter is important when evaluating the potential use of plant extracts for treating fungal infections. The extracts of *C. macrocephalum* exhibited the highest total activity compared to the others (Table 2). Thus one gram of *C. macrocephalum* acetone leaf extract can be diluted to 1.6 L with water and still inhibit the growth of *Colletotrichum gloeosporioides*.

Bioautography did not work well for most of the tested extracts except *C. laevigatum* and *L. camara*. In other cases there were growth, but no inhibition was observed even though MIC values indicated antifungal activity. The non-activity of these extracts in bioautography could be explained by volatilization or oxidation of antifungal compounds during the extended removal of the TLC eluents or by the disruption of synergism between active constituents caused by TLC (Masoko and Eloff, 2005).

Bioautography results (Table 3) of the TLC plates developed with the solvent system BEA showed clear zones or bands of inhibition. Plates developed with the solvent systems CEF and EMW showed inhibition bands only at the solvent front, implying that the active compounds were relatively non-polar. From *C. laevigatum* leaf and flower extracts, a compound with R_f value 0.13 strongly inhibited the growth of nine phytopathogenic fungi

tested. This active compound(s) might possess unspecific cytotoxicity. *Lantana camara* leaf extract contain active compounds against *A. niger*, *A. parasiticus*, *P. ultimum F. oxysporum, P. janthinellum* and *R. solani*.

5. Conclusion

The fungitoxic effects of the extracts indicate the potential of some plant species as a natural source of fungicidal material. Antifungal activity was confirmed in all the plant species tested, although the results showed that different plant extracts varied in their effectiveness in inhibiting the mycelial growth of different pathogens tested. Extracts from different parts of the same plant had varying degrees of inhibition.

If *in vitro* results can be confirmed *in vivo L. camara* acetone leaf extract would be a viable option for controlling *Fusarium oxysporum* because leaves are available in all seasons and it is a world wide invasive species. *C. macrocephalum* is one of the most serious invasive species in grass veldt in southern Africa and the high antifungal activity of acetone extracts on *C. gloeosporioides* and *F. oxysporium* may point to potential use of these invasive weeds. If hot water extracts of some of these species also have good activity is may be useful in crop protection in poor rural areas where people do not have access to organic solvents. Work is continuing on other invasive and weedy species on isolating antifungal compounds and on field trials with promising extracts or compounds.

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