South African Journal of Botany 93 (2014) 48–53

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Acetone leaf extracts of *Breonadia salicina* (Rubiaceae) and ursolic acid protect oranges against infection by *Penicillium* species

S.M. Mahlo [1], J.N. Eloff *

Phytomedicine Programme, Department of Paraclinical Sciences, University of Pretoria, Private Bag X04, Onderstepoort 0110, South Africa

ARTICLE INFO

Article history:
Received 6 February 2013
Received in revised form 20 December 2013
Accepted 7 March 2014
Available online 18 April 2014

Edited by M Gryzenhout

Keywords:
Antifungal activity
Breonadia salicina
Minimum inhibitory concentration (MIC)
Penicillium digitatum
P. expansum
P. janthinellum
Citrus sinensis
Therapeutic index

ABSTRACT

The activity of acetone leaf extracts of *Breonadia salicina* and the main antifungal compound isolated from the extract, ursolic acid, was determined against three important plant fungal pathogens (*Penicillium expansum*, *P. janthinellum* and *P. digitatum*) to evaluate their potential use in combating post-harvest infections of oranges. In an in vitro assay, acetone extracts had good antifungal activity against *P. janthinellum* with an MIC (minimum inhibitory concentration) of 0.08 mg/ml. *P. digitatum* and *P. expansum* were more resistant both with MICs of 1.25 mg/ml. We evaluated the potential use of an acetone extract and ursolic acid against these fungal pathogens in artificially infected oranges. A crude leaf extract at a concentration of 1 mg/ml gave the same level of protection as 1 mg/ml ursolic acid indicating synergistic activities within the crude extract. The acetone extract had an MIC of 0.16 mg/ml compared to the MIC of 0.08 mg/ml of amphotericin B against *P. digitatum*. Cytotoxicity of the crude extract and ursolic acid was determined using a tetrazolium-based colorimetric assay (3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT)) against Vero monkey kidney cells. The acetone extract had sufficient antifungal activity in vitro against these organisms to consider its use in the citrus industry after it has been tested under production and natural infection conditions and if it does not affect the fruit quality. The extracts were however more toxic to the kidney cells than to the fungi. The results show the potential use of plant extracts to combat plant fungal infections if extracts with lower cellular toxicity can be found or if the toxicity of the extract can be decreased without changing the antifungal activity.

1. Introduction

Citrus (*Citrus sinensis* L. Osbeck, Rutaceae) is well known and grown in over 100 countries on six continents, with a worldwide crop of about 70 billion kg in 2004 (Anon, 2005). Its production exceeds that of any other fruit. However, there are serious problems impeding citrus production. Postharvest losses, frequently caused by plant pathogenic fungi, are a major concern. Up to 25% of the total production of harvested fruit is subject to fungal attack in both industrialized and developing countries and the damage may exceed 50% (Spadaro and Gullino, 2004). Infection occurs through injury during the picking or handling of fruit and results in decay during storage or marketing.

It is essential to control postharvest diseases in order to maintain the quality and improve the shelf life of citrus fruit. Storage and transportation from producer to consumer may take several weeks to deliver the fruit. The susceptibility of freshly harvested products to postharvest diseases increases during prolonged storage as a result of physiological changes that enable pathogens to develop in the fruit (Eckert and Ogawa, 1998). All fresh fruits and vegetables for domestic or export markets should be free of dirt, dust, pathogens and chemicals before they are packed. Recently the European Union has prohibited the importation of citrus from South Africa due to the presence of black spot on a few oranges in a large consignment.

Penicillium expansum is one of the most common fruit pathogens, causing a condition known as "blue rot" on nectarines and peaches (Karabulut and Baykal, 2002; Karabulut et al., 2002; Vero et al., 2002). It is also harmful to humans since it produces patulin, a mycotoxin known to cause immunological, neurological, and gastrointestinal toxic effects in animals. Exposure to high levels of patulin results in vomiting, salivation, anorexia, polypnea, weight loss and leukocytosis (Pitt, 1997).

Green mould caused by *Penicillium digitatum* (Pers.Fr.) Sacc. is generally the most serious postharvest disease of citrus and this results in significant economic losses to the fruit industry. The fungus infects fruit through injuries where moisture and nutrients are available to stimulate spore germination. Infection can occur through very minor injuries that involve damage to individual oil glands of the fruit exo-and mesocarp (flavedo), and through more extensive puncture injuries encompassing oil glands that extend deeper into the mesocarp (Kavanagh and Wood, 1971).

Chemical fungicides are used widely to prevent or treat fungal infections in fruit and to avoid production loss in the commercial fruit

* Corresponding author.
E-mail address: kobus.eloff@up.ac.za (J.N. Eloff).
[1] Permanent address: Department of Botany, University of Limpopo, Turfloop, South Africa.

http://dx.doi.org/10.1016/j.sajb.2014.03.003
0254-6299/

industry. However, there are some difficulties involved, for example fruit contaminated with toxic pesticides can cause serious complications in human health (Kaushik et al., 2009).

There is a need to introduce new fungicides with a different mode of action that can be used to combat plant fungal pathogens such as *P. digitatum*, *P. expansum* and *Aspergillus parasiticus*. *P. digitatum* has developed resistance to currently available antifungals such as imazalil, thiabendazole and sodium *o*-phenylphenate (Harding, 1972; Holmes and Eckert, 1999; Kuramoto, 1976). The efficacy of these fungicides is compromised due to the emergence and dominance of resistant fungal populations (Holmes and Eckert, 1999). Azoxystrobin, fludioxonil and pyrimethanil are recently registered fungicides for postharvest citrus disease control (Kanetis et al., 2007). Azoxystrobin is very effective in controlling a broad range of plant diseases including green mould of citrus. However, because of their site specific modes of action, these quinone inhibitor fungicides are also prone to resistance development (Uesugi, 1998; Gullino et al., 2000).

An alternative way to combat plant fungal pathogens would be to consider using natural plant products. Plant extracts contain many compounds with antifungal activity (Masoko and Eloff, 2005, Mdee et al., 2009). If some of these compounds have different mechanisms of activity, it could limit the development of resistance. *Breonadia salicina* was the most promising species to investigate for antifungal activity from several plant species (Mahlo et al., 2010). Ursolic acid was the main antifungal compound isolated from acetone leaf extracts of *B. salicina* (Mahlo, 2009).

In this contribution, we investigate the activity of acetone extracts and ursolic acid from leaves of *B. salicina* and the potential use on *C. sinensis* artificially infected with *P. digitatum*, *P. expansum* and *P. digitatum* because these pathogens are relatively resistant to the currently available fungicides and cause postharvest problems in the fruit industry. The low cost of developing a plant-based product may have economic implications if there are no safety concerns. Furthermore, using a plant-based fungicide in citrus pathogen control may also find acceptance in the organic fruit production market.

2. Materials and methods

2.1. Plant collection

Plant leaves were collected from a labelled tree growing in the Lowveld National Botanical Garden in Nelspruit, Mpumalanga during the summer. The tree labels indicated year of planting as well as the collection number from which the origin of the plants could be determined from the herbarium database. To ensure efficient drying, leaves were collected in open mesh orange bags and kept in the shade to minimise photo-oxidative changes.

2.2. Plant storage

Collected fresh leaves were examined and the old, insect and fungus-infected leaves were removed. Leaves were dried at room temperature (c. 25 °C) in suspended open mesh bags for a month. The dried plant material was ground to a fine powder to pass through a sieve of 1 mm using laboratory grinding mill (Telemecanique/MACSALAB model 200 LAB) and stored in airtight bottles in the dark until extraction.

2.3. Extraction procedure

Acetone was used as extractant because it extracts antimicrobial compounds with a wide variety of polarities (Eloff, 1998a) and it is not toxic to fungi (Eloff et al., 2007). Finely ground leaf material (4 g) of *B. salicina* was extracted with 40 ml of acetone (technical grade-Merck) in polyester tubes, by shaking vigorously for 3–5 min on a Labotec model 20.2 shaking machine. After centrifuging at 3500 rpm for 5 min, the supernatants were decanted into labelled, weighed glass vials. The process was repeated 3 times on the marc and the extracts were combined. The solvent was removed under a stream of cold air at room temperature.

2.4. Isolation and culturing of fungi

Two *Penicillium* cultures *P. expansum*, and *P. janthinellum* of unknown plant origin were obtained from the Department of Microbiology and Plant Pathology at the University of Pretoria to compare with *P. digitatum* that we isolated from infected oranges bought apparently healthy from a greengrocer. The identity of the fungus isolated from the infected orange was confirmed as *P. digitatum* by Ms Candice Johnston from the Department of Microbiology and Plant Pathology at the University of Pretoria. This is a well known vigorous citrus pathogen. Fungal strains were maintained on potato dextrose agar (PDA) and incubated for 24 h before being used. The growth of fungi was observed after 24 h and the agar plates were removed from the incubator and stored in a refrigerator at 4 °C until further used. Fungal cultures were subcultured (1% inoculum) in PD broth before being used in the experiment.

2.5. Quantification of fungal inoculum

A haemocytometer cell-counting method 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 PD agar slants for 7 days at 35 °C. The slant was stroked carefully with a sterile cotton swab to remove conidia 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 count the number conidia using a haemocytometer (Neubauer chamber; Merck S.A.) and a microscope. The final inoculum size was adjusted to approximately 1.0×10^6 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 the colonies were counted after the observation of visible growth and used to calculate the corresponding cells/ml.

3. Determining antifungal activity

3.1. Microdilution assay

The microplate method of Eloff (1998b), modified for antifungal activity testing by Masoko et al. (2005), was used to determine the MICs of the plant extracts. The activity of the plant extract was determined in triplicate in each assay, and the assays were repeated in their entirety to confirm results. The extract was dissolved in acetone to a concentration of 10 mg/ml. The plant extract (100 μl) was 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 positive control and 100% acetone as the negative control. 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 minimise 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 reduction in intensity of colour after incubation with INT.

4. In vivo experiment

4.1. Fruit decay test

A modification of the method described by Muñoz et al. (2007) was used for the fruit decay test using orange fruit (*C. sinensis* L. Osbeck).

Experiments were carried out on freshly harvested navel orange fruits (*C. sinensis* L. Osbeck) purchased at a greengrocer. Approximately 10 ml of Sunlight liquid dishwashing soap was added into a container containing 5 l of water and freshly harvested fruits were soaked for 5 min to remove the wax layer on the oranges. The oranges were then rinsed repeatedly with tap water and allowed to air dry. The oranges were then wounded with a sterile needle by making punctures approximately 5 mm in depth and 0.6 mm in diameter at appropriate places. Several treatments including the negative and positive controls were placed in labelled sections on the same orange to minimise biological variability between oranges (Fig. 1). Acetone extracts were used in this experiment since the solvent is not toxic to the fungi at the concentration tested (Eloff et al., 2007). Ten microlitre inoculums containing c. 1.0×10^6 cells/ml of *P. expansum*, *P. digitatum* and *P. janthinellum* of the culture was applied to each puncture wound to simulate infections after oranges were treated. This equates to about 10,000 fungal cells on the wound. Four oranges per replicate (16 replicates) were prepared for each treatment.

A hundred microlitre of plant extract was placed on the wound. After treatment, the fruit were placed in a 27 cm wide, 27 cm long and 14.5 cm high plastic container. After placing moistened cotton wool at the bottom of the container the tightly closing lid was sealed with masking tape to ensure the maintenance of 100% relative humidity (Fig. 1). The growth chamber was maintained at 20 °C. The zone of growth on the infected area was visible after 4 days and minor changes were observed on the fifth, sixth and seven days. Measurements were then recorded after 4 and 7 days as the number of infected wounds per replicate, and mean diameter in mm ± SD (standard deviation).

4.2. Determining the dose related effect of the treatments

In a second experiment, we wanted to test the activity of the isolated ursolic acid and to confirm that acetone did not have a negative indirect effect by e.g. interacting with the surface of the orange. One milligram of ursolic acid was dissolved in 1 ml acetone and then 100 μl was applied on the wound as described above. Four oranges per replicate (8 replicates) were prepared for each treatment. Acetone was used as the negative control and amphotericin B (0.16 mg/ml) was the positive control.

4.3. Determining the IC_{50} of the crude extract

In order to test the dose response of the extract and to determine the IC_{50} in a third experiment, different concentrations (0.0, 1.0, 2.0, 4.0 and 8.0 mg/ml) of the extract were tested. In the case of amphotericin B, the concentration of the positive control was decreased to 0.04 and 0.08 mg/ml.

Fig. 1. Oranges with growth after treatment with acetone (A), a high (H) and a low (L) concentration of the extract and incubation.

4.4. Cytotoxicity assay

The cytotoxicity of the crude extract and compound was determined against Vero monkey kidney cells using a tetrazolium-based colorimetric assay (3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT), Sigma) described by Mosmann (1983). Stock solutions of the plant extracts (200 mg/ml) and ursolic acid (20 mg/ml) were prepared by dissolving it in DMSO. Serial 10-old dilutions of each extract and isolated compounds were prepared in growth medium and added to the cells. The concentration of the pure compound at which 50% of the Vero cells were killed (IC_{50}) after 120 h was determined. The absorbance was measured on a Versamax microplate reader at 570 nm. Berberine chloride (Sigma) was used as a positive control. The intensity of colour was directly proportional to the number of surviving cells. Tests were carried out in quadruplicate and each experiment was repeated three times.

5. Results and discussion

5.1. In vitro antifungal activity

Acetone leaf extracts had good antifungal activity against *P. janthinellum* with an MIC of 0.08 mg/ml. *P. digitatum* and *P. expansum* were more resistant, both with MICs of 1.25 mg/ml (Table 1). Ursolic acid was also active against *P. expansum*, *P. janthinellum* and *P. digitatum* with MICs of 0.13, 0.13 and 0.25 mg/ml respectively. *P. digitatum* isolated from the infected orange was 25 times more resistant to amphotericin B than the two other species cultures, possibly explaining why it was not inhibited by the treatment the oranges received prior to marketing. It is interesting that in contrast with the activity of amphotericin B, the crude plant extract was much more active against *P. digitatum* than against the other fungi. The crude extract was only two times less active against *P. digitatum* than amphotericin B indicating a potential use in the industry. In a field experiment one should however compare the activity of the plant extract with that of the commercially used fungicides.

5.2. Testing the procedure

This experiment was designed to evaluate the activity of the extract or isolated compound(s) against a plant fungal pathogen. In the first experiment, we determined the in vitro efficacy of the solvent and the crude extract and amphotericin B. After four days of incubation, the acetone treatment led to the highest growth (or lowest inhibition) with a growth diameter of 5.7 to 6.0 mm in all cases. It appeared not to inhibit the growth of the three *Penicillium* species. This confirms the low level of toxicity of acetone to fungi (Eloff et al., 2007). Acetone appears not to be harmful to the fungi or had evaporated to such an extent that the residues left had no effect (Table 2). The observations were repeated after seven days but with the exception of some growth of *P. expansum* dosed with 10 mg/ml acetone extract, there were no striking differences in the values between 4 and 7 days.

From the low standard deviation within the different treatments it appears that the method gives reproducible results. To determine the activity of ursolic acid, the experiment was repeated for four days using

Table 1
Minimum inhibitory concentration (MIC) in mg/ml after 48 h of crude extract, ursolic acid and amphotericin B against three *Penicillium* species. The results show the average of three replicates with a standard deviation of 0.

Plant pathogens	Extract	Ursolic acid	Amp B
Penicillium expansum	1.25	0.13	0.003
Penicillium janthinellum	0.08	0.13	0.003
Penicillium digitatum	0.16	0.25	0.08

acetone, 1 mg/ml ursolic acid and 0.16 mg/ml amphotericin B on *P. digitatum*. The values for the negative acetone control (i.e. 0 mg/ml) were in the same order (5.7 to 6.0 mm) as in the previous experiments and the value for 1 mg/ml ursolic acid was 2.89 mm.

The method used was further validated by the good correlation between the MIC obtained with the serial dilution in vitro method and the growth rate on the oranges. At a concentration of 10 mg/ml the growth of *P. digitatum* was completely inhibited. The results provided sufficient confidence in the model to continue with the next steps.

5.3. Determining the IC_{50} of the crude extract

It is not possible to determine the MIC in the experiment infecting the oranges. To compare the efficacy of the ursolic acid with the crude acetone extracts and the positive control the concentration that would lead to 50% inhibition of the maximum growth (IC_{50}) can however be determined. This would also make it possible to compare the activity with cellular toxicity. The aim was therefore to determine the dose related response of the crude extract in order to compare the activity relative to ursolic acid and to the toxicity to mammalian cells. To calculate the IC_{50} accurately we calculated and used the growth area rather the growth diameter as a measure of the growth rate. To overcome the change of a yeast growth form to a mycelial growth form, experiments were only carried out for 4 days.

There was an excellent logarithmic dose related response in all cases (4 and 7 days) with R^2 values varying from 0.956 to 0.9995 (Fig. 2). These values attest to the validity of the model we have developed. With no plant extract added the area of growth of *P. expansum* was 32.2 mm^2 and therefore the IC_{50} (dose that kills 50% of the cells) leading to a growth of 16.1 mm^2 was 1.8 mg/ml. In the case of the *P. digitatum*, the IC_{50} was 1.6 mg/ml and with *P. janthinellum* it was 1 mg/ml. It appears that when the area of growth rate was high, then the IC_{50} (inhibition concentration of fungi) was reduced.

There was a slight difference in IC_{50} when comparing data between 4 and 7 days of incubation. With *P expansum* the IC_{50} increased from 1.8 mg/ml to 1.9 mg/ml with the longer incubation. With the *P. digitatum*, the value changed from 1.6 to 1.7 mg/ml and for *P. janthinellum* there was no difference in IC_{50} of 1 mg/ml with a longer period of incubation.

It was surprising that in this experiment the growth area for *P. janthinellum* was substantially lower than that for the two other isolates whereas the diameter of growth was very similar in the first experiment. This may be related to changing from yeast to mycelial growth form by the two other isolates.

The higher resistance of the *P. digitatum* in comparison to the other *Penicillium* species to amphotericin B may explain why it was isolated from infected commercially available oranges. It has been reported that *P. digitatum* isolated from citrus fruit were relatively sensitive to the three fungicides, sodium *o*-phenylphenate (*o*-phenylphenol), imazalil, and thiabendazole with mean EC_{50} values of 0.026, 0.1, and 6.3 μg/ml. However, in the case of packing house fruit *P. digitatum* was more resistant to imazalil with an EC_{50} ranging between 0.87 and 0.92 μg/ml (Holmes and Eckert, 1999).

It is interesting that the IC_{50} of ursolic acid was in the same order as that of the crude extract. In one case the MIC for the crude extract was actually lower than that of ursolic acid. This indicates that other compounds present in the crude extract probably play a role in the antifungal activity. These other compounds may not necessarily have individual antifungal activity because ursolic acid was by far the most important antifungal compound in bioautography (results not shown). One or more of these other compounds could have an effect on the uptake or metabolism of ursolic acid or other minor antifungal compounds.

Table 2
Growth area diameter of different *Penicillium* isolates in mm treated with three concentrations of 0, 1.25 and 10 mg/ml in acetone extracts and amphotericin B (0.16 mg/ml) after 7 days of incubation. The results show the average of three replicates with a standard deviation of 0. There was no growth in the amphotericin B treatments.

	Penicillium expansum			*Penicillium digitatum*			*Penicillium janthinellum*		
Concentration	0.0	1.25	10	0.0	0.16	10	0.0	0.08	10
Diameter (mm)	6.2	5.17	1.32	5.89	4.73	0.0	6.77	6.7	2.01

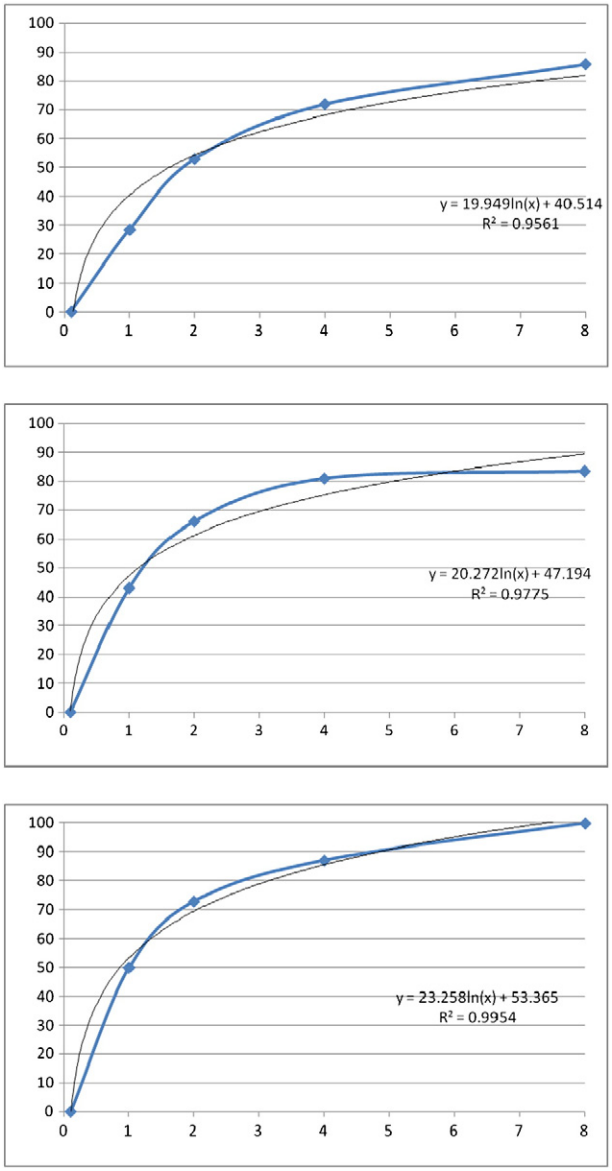

Fig. 2. Percentage inhibition of growth after four days of *P. expansum* (top) *P. digitatum* (middle) and *P. janthinellum* (bottom) (y axis) on oranges after application of different concentrations of the acetone leaf extract in mg/ml (x-axis). The growth rates of the controls were 32.5, 120 and 140 mm^2 respectively after four days of incubation.

6. Therapeutic index

6.1. Therapeutic index of the crude extract and isolated compounds

The therapeutic index (or selectivity index) for the acetone extract treatments was calculated by comparing the concentration required to kill the fungi with the cytotoxic concentration of the crude extracts.

The therapeutic index for each fungus was therefore calculated by dividing the concentration that would kill the pathogen by the concentration that would kill animal cells. The higher the value the safer the extract would be. The therapeutic index of 0.05 to 0.08 means that the

Table 3
Growth area diameter of different *Penicillium* species in mm treated with different concentrations of the crude extract and two concentrations of amphotericin B after 7 days of incubation. The results show the average of three replicates with a standard deviation of 0.

Pathogen	Concentration crude extract					Amp B	
	0.0	1	2	4	8	0.04	0.08
Penicillium expansum	3.2	2.7	2.2	1.7	1.2	0.0	0.0
Penicillium digitatum	6.2	4.7	3.6	2.7	2.5	10.6	11.5
Penicillium janthinellum	6.7	4.7	3.3	2.2	2.8	6.7	0.0

acetone leaf extract of *B. salicina* may be much too toxic to become a useful product to control *Penicillium* infections in oranges (Table 5). Many fungicides are toxic to mammal cells and it may be interesting to compare the safety of the fungicides commonly used to protect plant products with the extract we used.

One cannot accept that cellular toxicity necessarily equates to mammalian toxicity without carrying out animal experiments. If toxic components are not taken up from the digestive system or if toxins are quickly metabolized to inactive compounds by liver enzymes, the inherent toxicity may be much lower than the value reflected by the cellular assay. Some antibiotics for example are highly toxic when introduced parentally, but are safe when taken orally.

7. Conclusion

The main conclusion in this paper is that plant extracts may be useful to protect agricultural products against post-harvest losses caused by fungi. Even within the same fungal genus there appears to be selectivity in antifungal activity and in one case the activity of a crude extract that can be produced at very low cost was close to that of a widely used antifungal agent. Several plant species are not susceptible to fungal infection since they contain inhibitory compounds (Ghosh et al., 2000). Previously, extracts of some plants have been reported to offer protection from *Aspergillus flavus* infection during storage and enhance seed emergence and seed germination (Nwachukwu and Umechuruba, 2001; Donli and Dauda, 2003).

It is satisfying that the activity of the extracts and ursolic acid in the in vivo fruit model used closely matched that of the MIC determined by serial microplate dilution. The activity of ursolic acid was in the same order as that of the crude acetone leaf extracts of *B. salicina*. This indicates that there must be synergistic activities present in the extract. This may lead to a lower development of resistance by the pathogens.

Some antifungal compounds are toxic to mammalian cells, unfortunately our extract and ursolic acid were more toxic to Vero monkey kidney cells than to the fungi. This complicates the use on edible plant products. Because ursolic acid on its own was also toxic to the cells it does not seem likely that one would be able to lower the cellular toxicity with decreasing antifungal activity. This degree of success was attained with a plant species that had a relatively low activity compared to the antifungal activity of other plant extracts investigated in the Phytomedicine Programme (Mdee et al., 2009). It is likely that wide screening of plant extracts against *Penicillium* species and other plant fungal pathogens may be a viable option to develop useful antifungal preparations for post-harvest protection of plant products.

To evaluate the potential use of a plant extract under agricultural growth conditions field experiments would also have to be carried out. The efficacy of the extract compared to the antifungal agent used in agriculture and under natural infection conditions would be the real test of the potential value of a plant extract. It would also be valuable to determine the mechanism of activity of plant extracts to see if it could not control fungi that have developed resistance by another mechanism. The work described in this paper presents one step further than determining the MIC in in vitro assays, but still requires confirmation studies under agricultural production conditions.

Table 4
LC_{50} and area of growth after 4 and 7 days of incubation time against three *Penicillium* species.

Plant pathogens	Time (days)	LC_{50} (mg/ml)	Area of growth (mm^2)
P. expansum	4	1.8	11.45
P. digitatum	4	1.6	13.2
P. janthinellum	4	1.0	42.4
P. expansum	7	1.9	16.1
P. digitatum	7	1.7	60.4
P. janthinellum	7	1.0	70.5

Table 5
The therapeutic index of acetone extracts against three plant pathogenic fungi was calculated by dividing IC_{50} with MIC using values after 4 days.

Fungi	LC_{50} in mg/ml for fungus	LC_{50} Vero cells mg/ml	Therapeutic index
Penicillium expansum	1.8	0.082	0.05
Penicillium digitatum	1.6	0.082	0.05
Penicillium janthinellum	1.0	0.082	0.08

Acknowledgements

The National Research Foundation (NRF) (ICD2006072600003) and the University of Pretoria provided financial support.

Ms Candice Johnston from the Department of Microbiology and Plant Pathology at the University of Pretoria identified *P. digitatum* and provided cultures of the other *Penicillium* species.

References

Anonymous, 2005. Naringin, a bitter flavouring available hing-purity grades. Food Product Development 16, 29.
Donli, P.O., Dauda, H., 2003. Evaluation of aqueous moringa seed extract as seed treatment biofungicide for groundnuts. Pest Management Science 59, 1060–1062.
Eckert, J.W., Ogawa, J.M., 1998. The chemical control of postharvest diseases: deciduous fruits, berries, vegetables and root/tuber crops. Annual Review of Phytopathology 26, 433–469.
Eloff, J.N., 1998a. Which extractant should be used for the screening and isolation of antimicrobialcomponents from plants? Journal of Ethnopharmacology 60, 1–8.
Eloff, J.N., 1998b. The presence of antibacterial compounds in *Anthocleista grandiflora* (Loganiaceae). South African Journal of Botany 64, 209–212.
Eloff, J.N., Picard, J., Masoko, P., 2007. Resistance of animal fungal pathogens to solvents used in bioassays. South African Journal of Botany 73, 667–669.
Ghosh, S.K., Sanyal, B., Ghosh, S., 2000. Screening of some angiospermic plants to antimicrobial activity. Journal of Mycopathological Research 38, 19–22.
Gullino, M.L., Leroux, P., Smith, C.M., 2000. Review: uses and challenges of novel compounds for plant disease control. Crop Protection 19, 1–11.
Harding Jr., P.R., 1972. Differential sensitivity to thiabendazole by strains *of Penicillium digitatum*. Plant Disease 56, 256–260.
Holmes, G.J., Eckert, J.W., 1999. Sensitivity of *Penicillium digitatum* and *P. italicum* to postharvest citrus fungicides in Carlifornia. Phytopathology 89, 716–721.
Kanetis, L., Forster, H., Adaskaveg, J.E., 2007. Comparative efficacy of the new postharvest fungicides azoxystrobin, fludioxonil, and pyrimethanil for managing citrus green mold. Plant Disease 92, 1502–1511.
Karabulut, O.A., Baykal, N., 2002. Evaluation of the use of microwave power for the control of postharvest diseases of peaches. Postharvest Biology and Technology 26, 237–240.
Karabulut, O.A., Cohen, l, Wiess, B., Daus, A., Lurie, S., Droby, S., 2002. Control of brown rot and blue mold of peach and nectarine by short hot water brushing and yeast antagonists. Postharvest Biology and Technology 24, 103–111.
Kaushik, G., Satya, S., Naik, S.N., 2009. Food processing a tool to pesticide residue dissipation—a review. Food Research International 42, 26–40.
Kavanagh, J.A., Wood, R.K.S., 1971. Green mould of oranges caused by *Penicillium digitatum* Sacc.: effect of additives on spore germination and infection. The Annals of Applied Biology 67, 35–44.
Kuramoto, T., 1976. Resistance to benomyl and thiophanate-methyl in strains of *Penicillium digitatum* and *P. italicum* in Japan. Plant Disease 60, 168–172.
Mahlo, S.M., 2009. Characterization and biological activity of antifungal compounds present in *Breonadia salicina* (Rubiaceae) leaves. (PhD thesis) Phytomedicine ProgrammeUniversity of Pretoria.
Mahlo, S.M., McGaw, L.J., Eloff, J.N., 2010. Some tree leaf extracts have good activity against plant fungal pathogens. Crop Protection 29, 1529–1533.
Masoko, P., Eloff, J.N., 2005. The diversity of antifungal compounds of six South African Terminalia species (Combretaceae) determined by bioautography. African Journal of Biotechnology 4, 1425–1431.

Masoko, P., Picard, J., Eloff, J.N., 2005. Antifungal activities of six South African *Terminalia* species (Combretaceae). Journal of Ethnopharmacology 99, 301–308.
Mdee, L.K., Masoko, P., Eloff, J.N., 2009. The activity of extracts of seven common invasive plant species on fungal phytopathogens. South African Journal of Botany 75, 375–379.
Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65, 55–63.
Muñoz, A., López-Garcia, B., Marcos, J.F., 2007. Compartive study of antimicrobial peptides to control citrus postharvest decay caused by *Penicillium digitatum*. Journal of Agricultural and Food Chemistry 55, 8170–8176.
Nwachukwu, E.O., Umechuruba, C.I., 2001. Antifungal activities of some leaf extracts on seed-borne fungi of African yam bean seeds, seed germination and seedling emergence. Journal of Applied Sciences and Environmental Management 5, 29–32.
Pitt, J.I., 1997. Toxigenic *Penicillium* species. In: Doyle, M.P., Beuchat, L.B., Montville, T.J. (Eds.), Food Microbiology: Fundamentals and Frontiers. ASM Press, Washington, DC, p. 411.
Spadaro, D., Gullino, M.L., 2004. State of the art and future prospects of the biological control of postharvest fruit diseases. International Journal of Food Microbiology 91, 185–194.
Uesugi, Y., 1998. Fungicide classes: chemistry, uses and mode of action. In: Hutson, D., Miyamoto, J. (Eds.), Fungicidal Activity—Chemical and Biological Approaches to Plant Protection. John Wiley & Sons, New York, pp. 23–56.
Vero, S., Mondino, P., Burgueno, J., Soubes, M., Wisniewski, M., 2002. Characterizarion of biocontrol activity of two yeast strains from Uruguay against blue mold of apple. Postharvest Biology and Technology 26, 9198.