

CHAPTER 10

In vivo experiment: Plant extracts active against *Penicillium* species

10.1 Introduction

Fruit forms an important part of the human diet because it supplies essential nutrients such as vitamins and minerals, which are necessary for prevention of harmful diseases in humans. Citrus (*Citrus sinensis* L. Osbeck, belonging to the family Rutaceae) is well known and is grown in over 100 countries on six continents, with a worldwide crop of about 70 billion kg in 2004 (Anon 2005), and its production exceeds that of any other fruit. However, there are serious problems encountered in citrus production, in particular, postharvest losses which are frequently caused by plant pathogenic fungi. Up to 25% of the total production of harvested fruit is subject to fungal attack in both industrialized and developing countries, and damage is often higher, exceeding 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. However, there are some implications in the market place where transport from producer to consumer may take several weeks to deliver the fruit and storage in packing houses can exceed the maximum period. These factors all influence fruit decay.

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). *Penicillium expansum* is also harmful to humans since it produces patulin, a mycotoxin known to cause immunological, neurological, and gastrointestinal toxic effects in animals (Pitt 1997). Exposure to high levels of patulin results in vomiting, salivation, anorexia, polypnea, weight loss and leukocytosis.

Green mold 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 though 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 (albedo) (Kavanagh and Wood 1971).

Postharvest decay is the major factor limiting the extension of storage life of many fresh harvested commodities. All fresh fruits and vegetables for domestic or export markets should be free of dirt, dust, pathogens and chemicals before they are packed. Chemical fungicides are used widely to prevent the spreading of plant diseases in fruit and also to avoid losses in the commercial fruit industry. There are some difficulties involved, for example residues in fruit which can cause serious complications in human health when the fruit is consumed (Cabras et al. 1999). 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 1988).

Penicillium digitatum is relatively resistant to the currently available antifungals, for example imazalil, thiabendazole and sodium *o*-phenylphenate (Harding 1972, Holmes and Eckert 1999, Kuramoto 1976). However, 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 new fungicides for postharvest citrus disease control (Kanetis et al. 2007). Azoxystrobin is very effective in controlling a broad range of plant diseases including green mold of citrus. However, because of their site specific modes of action, quinone outside inhibitor fungicides are at high risk of resistance development in the targeted phytopathogenic fungal populations (Uesugi 1998, Gullino et al. 2000).

There is a need to introduce a new fungicide with a different mode of action that can be used to combat plant fungal pathogens. An alternative way to combat plant fungal pathogens would be to consider natural plant defence mechanisms. Many plant species have excellent activity against fungi (Masoko et al. 2007). Phenolic compounds can play a vital role as phytoalexins in some citrus species (Arcas et al. 2000). The peel of citrus fruit is a rich source of flavonones and many polymethoxylated flavones, which are rare in other species (Horowitz 1961). Naringin and hesperidin are the principal flavones in *Citrus paradise* (grape fruit) and *Citrus sinensis* (oranges).

Because acetone leaf extracts of *Breonadia salicina* had excellent activity *in vitro* against *P. janthinellum* (Chapter 3), the potential use of these extracts and isolated compounds from leaves of *B. salicina* on *Citrus sinensis* artificially infected with *Penicillium* species was examined.

10.2 Materials and methods

10.2.1 Microplate dilution assay

Dried acetone leaf extracts (10 mg) of *B. salicina* were dissolved in acetone (1 ml) and tested for antifungal activity against *Penicillium expansum*, *P. digitatum* and *P. janthinellum* using the serial dilution assay described in section 3.3.2.

10.2.2 Isolate and culturing of fungi

To obtain a naturally occurring *Penicillium digitatum*, a sterile loop was rubbed carefully on the skin of infected oranges purchased from the market (Fruit and Vegetable) and then streaked on Potato Dextrose agar (PDA) plates. The plates were then incubated overnight at 24°C. The growth of fungi was observed after 24 hours and the agar plates were removed from the incubator and stored in the refrigerator until further used. The fungal culture was rubbed carefully with a sterile cotton swab and transferred to a sterile tube with fresh Potato Dextrose broth (50 ml).

Penicillium expansum and *P. janthinellum* from Department of Microbiology and Plant Pathology at the University of Pretoria were subcultured from their original fungal strains on Potato Dextrose (PD) agar plates and incubated overnight. Fungal cultures were subcultured (1% inoculum) in PD broth before being used in the experiment.

The identity of the fungus isolated from the infected orange was confirmed as *Penicillium 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.

10.2.3 Quantification of fungal inoculum

For quantification of the fungal cultures, the haemocytometer cell-counting method described by Aberkane et al. (2002) with some modifications was used. The method is described in section 3.3.1.

10.3. In vivo experiment

10.3.1 Fruit Decay test

A modification of the method described by Muñoz et al. (2007) was used for the fruit decay test using orange fruit (*Citrus sinensis* L. Osbeck). Experiments were carried out on freshly harvested navel orange fruits (*Citrus sinensis* L. Osbeck) purchased at a greengrocer. Approximately 10 ml of Sunlight liquid dishwashing soap were added into a container containing five litres of water and freshly harvested fruits were soaked for five minutes. This was done to remove the wax layer on the oranges, which were then rinsed repeatedly with tap water and allowed to air dry. The oranges were marked on the outer skin into eight sections labelled N (acetone negative control), H (highest concentration of 10 mg/ml of extract), L (lower concentrations 0.16, 0.08 and 1.25 mg/ml of the extract and (P) the positive control, which was amphotericin B (0.16 mg/ml based on the MIC previously determined), in one experiment. Each of the 8 sections on the orange had a duplicate treatment leading to a total of sixteen treatments and four replicates per treatment on each orange. Four oranges per replicate were prepared for each treatment. 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. Acetone extracts was used in this experiment since the solvent is not toxic to the fungi at the concentration tested (Eloff et al. 2007). The extraction procedure is described in section 2.2.4.1 (Chapter 2).

We completed three experiments to test the efficacy of the isolated compound and plant extract. In the first experiment, 1.25, 0.16 and 0.08 mg of crude extract were dissolved in 1 ml acetone and then 100 µl of the acetone extracts were applied on the wounds. These concentrations were chosen on the basis of the MICs of the extracts on the growth of *P. expansum*, *P. digitatum* and *P. janthinellum* respectively when tested for antifungal activity in section 10.2.1. The oranges were allowed to dry completely for 20 minutes to remove traces of acetone. Ten µl inocula contained 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 equated to about 10 000 fungal cells on the wound. Because four oranges were used for each fungus there were 16 replicates per treatment. After treatment the fruits were placed in a container with the following dimensions: 27cm wide, 27 cm long and 14.5 cm high. The container had a tightly closing lid sealed with a tape. The growth chamber

was maintained at 20°C and 100% relative humidity by putting moistened cotton wool at the bottom of the container. The zone of growth on the infected area was visible after 4 days and minor changes were observed on the fifth and sixth days. Measurements were then recorded after 4 and 7 days as the number of infected wounds per replicate, and mean diameter in mm \pm SD (standard deviation).

In the 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 mg of ursolic acid was dissolved in 1 ml acetone and then 100 μ l was applied on the wound as described above. In this experiment each orange was marked on the outer of the skin into four sections and each section had two duplicates to make eight replicates per orange fruit. Acetone was used as the negative control and amphotericin B (0.16 mg/ml) was the positive control.

In order to test the dose response of the extract and to determine the LC₅₀ in the third experiment, different concentrations of the extract were tested 0.0, 1.0, 2.0, 4.0 and 8.0 mg/ml. In the case of amphotericin B, it was difficult to determine the low dose effect in experiments mentioned above (1 and 2) since we tested the highest concentration of 0.16 mg/ml. The concentration of the positive control was decreased to 0.04 and 0.08 mg/ml.

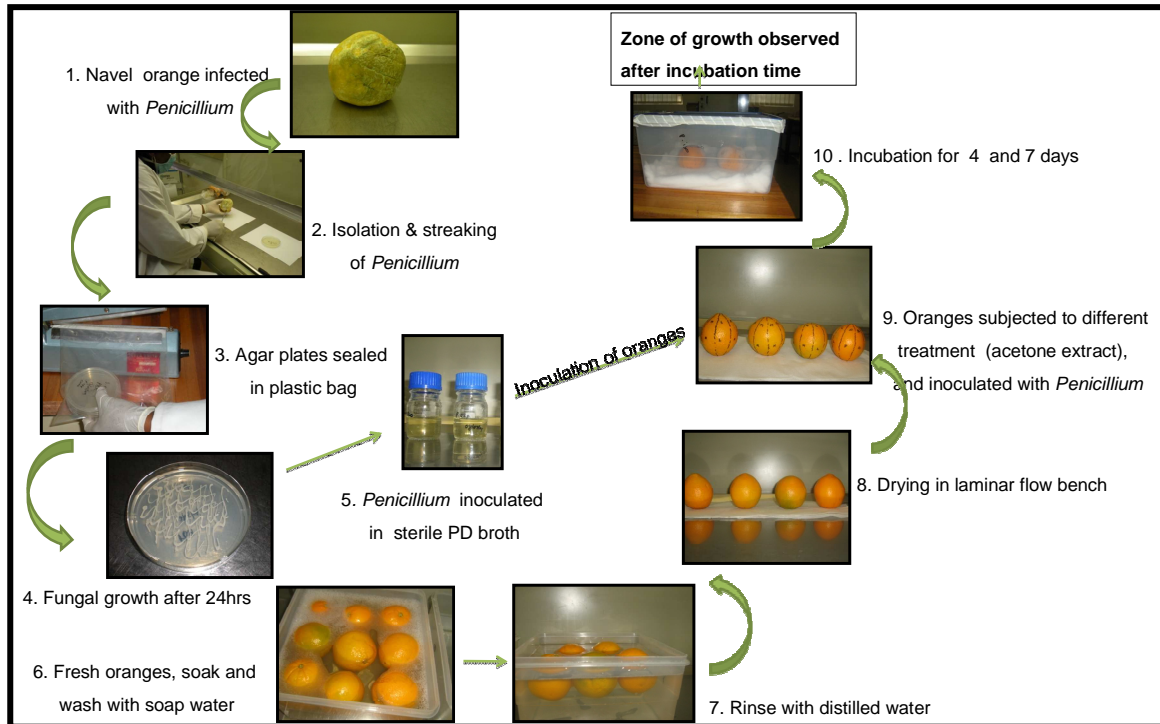


Figure 10-1 Schematic representation of orange infection experiment using plant pathogen isolated from rotten orange and *P. expansum* and *P. janthinellum*. (2) Sterile loop was used to streak *P. digitatum* on agar plate and (3) incubate overnight. (4) Observation of fungal growth after 24 hrs. (5) The fungal culture was adjusted to approximately 1.0×10^6 cells/ml (6-8) Navel oranges were soaked for 5 minutes and then washed with soap and allowed to dry. (9) Each orange was divided into eight sections with two duplicates in each section to make sixteen treatments. The oranges were punctured and then the extracts were applied into the wound at different concentrations and allowed to dry for 20 minutes. Acetone and amphotericin B were used as negative and positive controls. The fungal culture was applied to the wound and (10) the oranges were placed in a container and incubated until the zone of growth was visible.

10.4 Results and discussion

10.4.1 Microplate dilution assay

The minimum inhibitory concentration (MIC) values of acetone extracts against three plant pathogenic fungi were determined (Table 10-1). Acetone leaf extracts had good antifungal activity against *P. janthinellum* with MIC value of 0.08 mg/ml. The *Penicillium digitatum* and *P. expansum* were more resistant, both with MIC values of 1.25 mg/ml. Ursolic acid also had antifungal activity against *P. expansum*, *P. janthinellum* and *P. digitatum* with MIC value of 0.13 and 0.25 mg/ml. The *Penicillium digitatum* was 25 times more resistant to amphotericin B than the two pure cultures, possibly indicating why it was not inhibited by the treatment the oranges received prior to marketing.

Table 10-1 Minimum inhibitory concentration (MIC) 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	MIC (mg/ml)		
	Extract	Ursolic acid	AmpB
<i>Penicillium expansum</i>	1.25	0.13	3.2
<i>Penicillium janthinellum</i>	0.08	0.13	3.2
<i>Penicillium digitatum</i>	0.16	0.25	80

10.4.2 First experiment to evaluate the procedure

The experiments were designed to evaluate the potential use of the extract or isolated compound(s) against a plant fungal pathogen. In the first experiment, we determined the potential toxicity of the solvent and the crude extract and amphotericin B. After four days incubation time, the acetone treatment gave the highest growth with a diameter of 5.7 to 6.0 mm in all cases and probably did not inhibit the growth of fungi against three *Penicillium* species. This suggests that the solvent was not harmful to the fungi or had evaporated to such an extent that the residues left had no effect (Figure 10-2). 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 between the values between 4 and 7 days.

From the low standard deviation within the different treatments it appears that the method gives reproducible results. The method used was further validated by the good correlation between the MIC obtained with the serial dilution method and the growth rate on the oranges. The results provided confidence to continue with the next steps.

Table 10-2 Growth of different *Penicillium* isolates in mm treated with acetone (0), acetone extracts and amphotericin B after 7 days incubation

	<i>Penicillium expansum</i>			<i>Penicillium digitatum</i>			<i>Penicillium janthinellum</i>		
	0.0	10	1.25	0.0	10	0.16	0.0	10	0.08
Diameter (mm)	6.2	1.32	5.17	5.89	0.0	4.73	6.77	2.01	6.7
AmpB	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

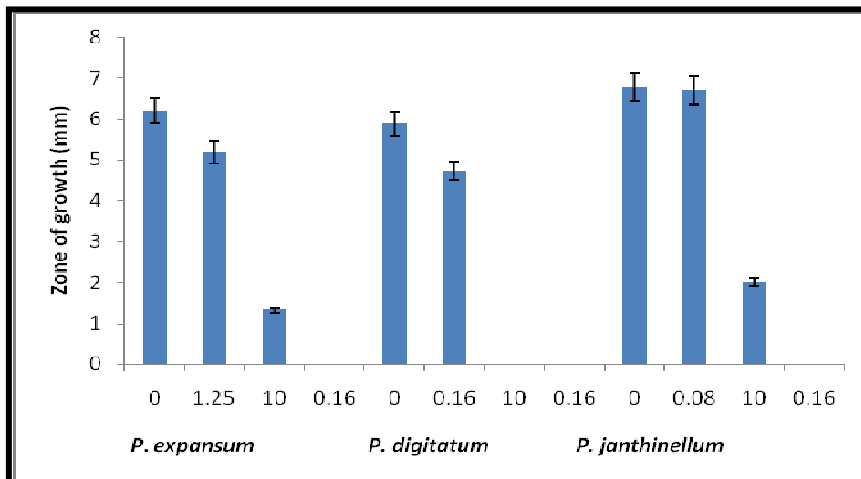
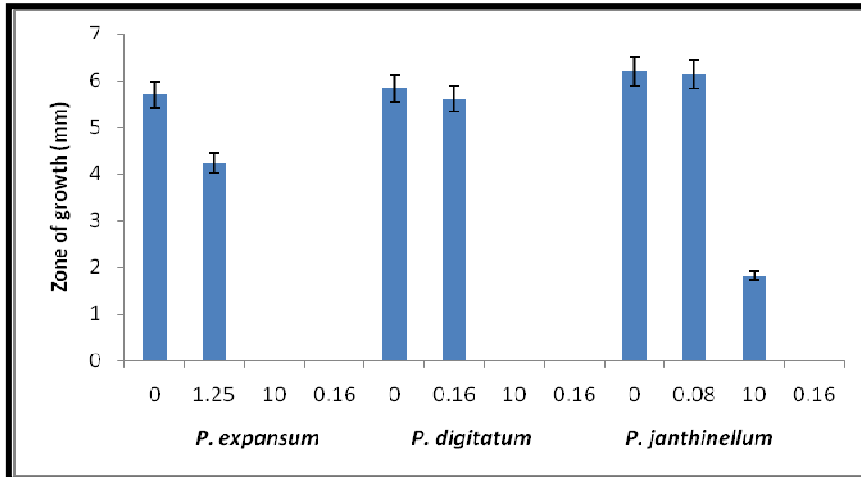


Figure 10-2 Orange fruits were inoculated with about 10 000 cells of *P. expansum*, *P. digitatum* and *P. janthinellum* on each wound and subjected to different concentrations of the crude extract. Diameters of growth (mm) were measured after 4 (top) and 7 (bottom) days incubation time. Results are shown as the average diameter of the infected wounds. Error bars show standard deviation. **Lanes from left to right:** Acetone extracts at different concentrations (0.0, 1.25 and 10 mg/ml), Amphotericin B (0.16 mg/ml).

10.4.3 Determining the dose related effect of the treatments

To determine the activity of ursolic acid the second experiment was repeated for four days using acetone, 1 mg/ml ursolic acid and 0.16 mg/ml amphotericin B on the *P. digitatum*. The

values for the acetone control (0 mg/ml) were in the same order as in the previous experiments and the value for 1 mg/ml ursolic acid was 2.89 mm (figure 10-3).

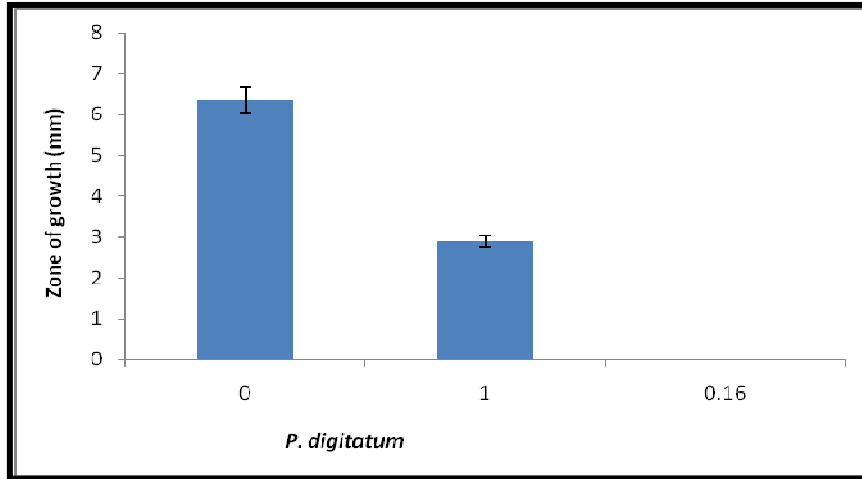


Figure 10-3 Orange fruits were inoculated with about 10 000 cells of *P. digitatum* on each wound and subjected to 1 mg/ml concentration of ursolic acid. Diameters of growth (mm) were measured after 4 days incubation time. Results are shown as the average diameter of the infected wounds. Error bars shows standard deviation. **Lanes from left to right:** Acetone (0.0), ursolic acid (1 mg/ml) and Amphotericin B (0.16 mg/ml).

Figure 10-4 shows the average diameter of the zone of growth tested on oranges against three plant pathogenic fungi, *P. expansum*, *P. digitatum* and *P. janthinellum*. In the first experiment, the lowest dose of amphotericin B was too high to determine the LC_{50} . In the third experiment different concentrations were investigated: 1.0, 2.0, 4.0 and 8.0 mg/ml, amphotericin B (0.04 and 0.08 mg/ml). The highest zone of growth (2.7 mm) were observed in the negative control (acetone) while at 1 mg/ml the average diameter was reduced (2.2 mm) when acetone extracts were used as treatment against *P. expansum*. There was a good dose related inhibition by the crude acetone extract. Noticeably, when amphotericin B was used, there was no zone of growth observed on the fruit even at the lowest concentration of 0.04 mg/ml (Fig 10-4, Table 10-3).

Acetone (the negative control) had the highest zone of growth when oranges were treated against *P. digitatum* with average diameter of 2.9 mm and was reduced to 2.4 mm at 1 mg/ml.

There were slight differences in the average diameter at acetone extract concentration of 4 and 8 mg/ml (with average diameters of 1.1 and 0.9 mm, respectively). In contrast to *P. expansum* where there was no growth with amphotericin B at a concentration of 0.04 mg/ml, the *P. digitatum* was much more resistant with an average growth diameter of 2.9 mm (0.08 mg/ml) and 2.1 (0.04 mg/ml).

The zone of growth in the acetone negative control tested against *P. janthinellum* was 5.2 mm and this was reduced to 3.2 mm at an extract concentration of 1 mg/ml. In general, the zone of growth on the fruit was reduced when the concentration of the acetone extracts increased, for example, 1 mg/ml (average diameter 3.2 mm) and 2 mg/ml (2.7 mm), 4 mg/ml (1.7 mm) and 8 mg/ml (0mm). In contrast to *P. expansum* some growth of *P. janthinellum* took place at 0.04 mg/ml amphotericin B.

10.4.4 Determining the effect of dose and time of exposure

When the growth was determined after 4 and 7 days on the same oranges, there were not many changes in the dose related response. There were minor differences between measurements taken after 4 and 7 days with *P. expansum*. After 7 days there was also some growth at a dose of 8 mg/ml (Figure 10-4). With the *P. digitatum* there was a doubling of the area of fungal growth between day 4 and 7 without much change in the dose related response. After ten days however the whole orange was covered when infected with *P. digitatum* (Figure 10-6). It is possible that in the case of *P. digitatum* that the growth form changed from a yeast-like single cell to a mycelial growth form. With *P. janthinellum* more or less the same effect was found as with *P. expansum*, with not much growth between days 4 and 7.

P. expansum was sensitive to amphotericin B at the lowest concentration tested after both periods. *P. janthinellum* growth was inhibited by amphotericin B up to 4 days, but after 7 days the inhibition was overcome. In the case of the isolate it was resistant to the highest concentrations and between days 4 and 7 it grew even better than the negative control. This can probably be explained by changing from a yeast to a mycelial growth form.



Table 10-3 Growth of different *Penicillium* species in mm treated with different concentrations of the crude extract and two concentrations of amphotericin B after 7 days incubation

Pathogen						AmpB	
	0.0	1	2	4	8	0.04	0.08
<i>Penicillium expansum</i>	3.2	2.7	2.2	1.7	1.2	0.0	0.0
<i>Penicillium digitatum</i>	6.2	4.7	3.6	2.7	2.5	10.6	11.5
<i>Penicillium janthinellum</i>	6.7	4.7	3.3	2.2	2.8	6.7	0.0

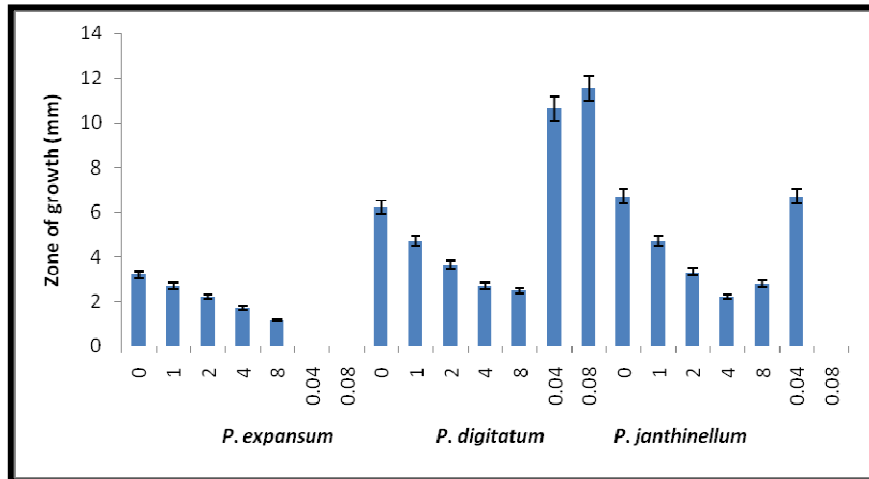
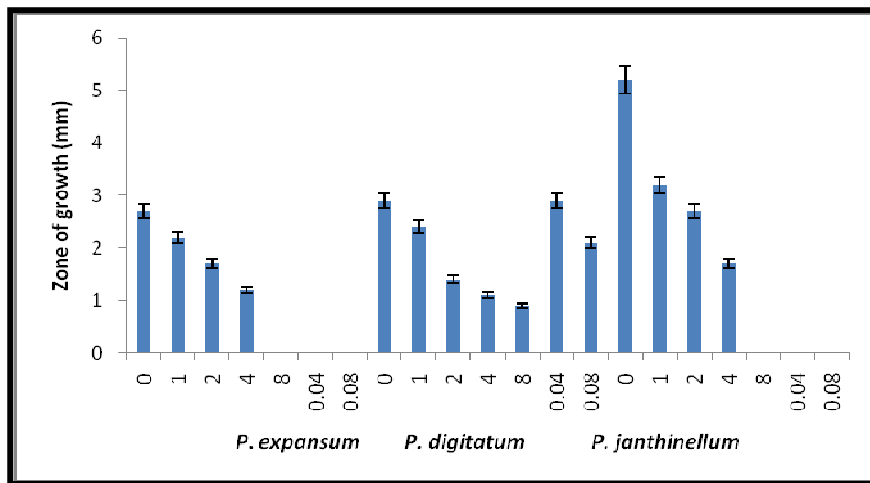


Figure 10-4 Orange fruits were inoculated with about 10 000 cells of *P. expansum*, *P. digitatum* and *P. janthinellum* on each wound and subjected to different concentrations of the crude extract. Diameters of growth (mm) were measured after 4 (top) and 7 (bottom) days incubation time. Results are shown as the average diameter of the infected wounds. Error bars shows standard deviation. **Lanes from left to right:** Acetone extracts at different concentrations (0.0), 1.25 and 10 mg/ml, Amphotericin B (0.16 mg/ml).

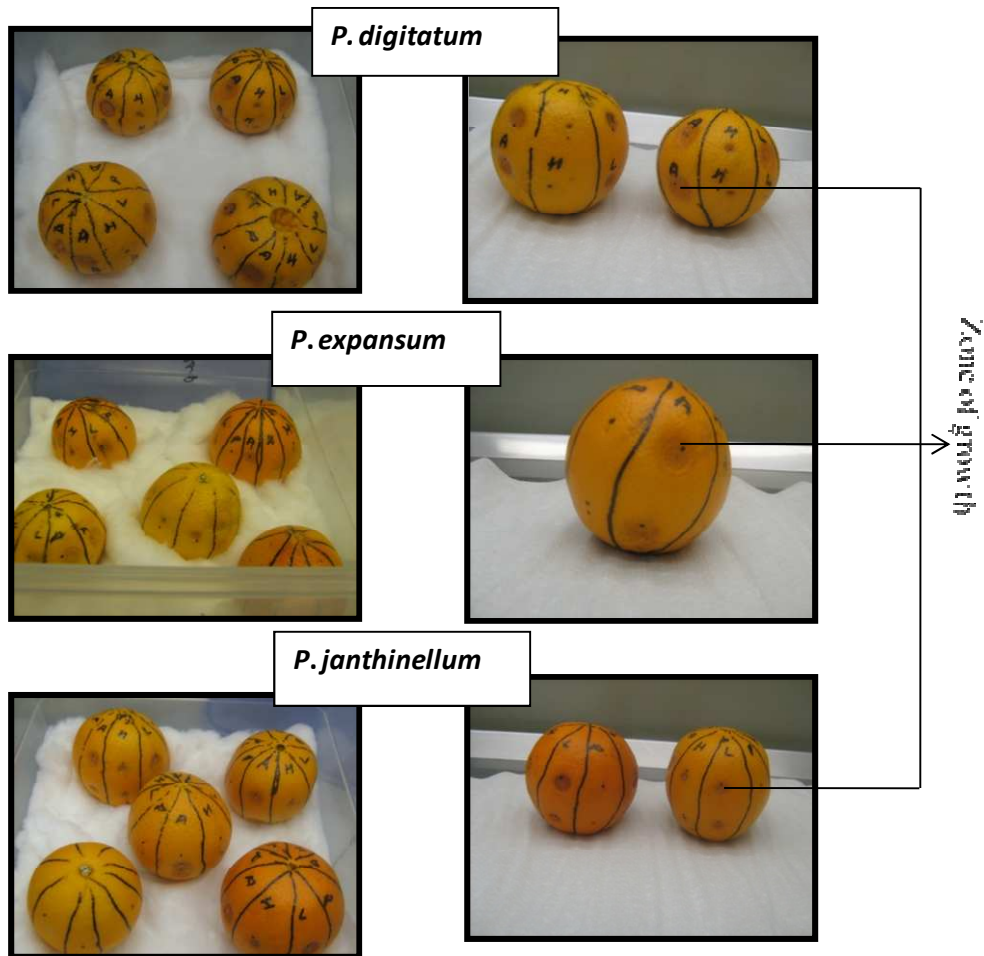


Figure 10-5 Orange fruits inoculated with 1.0×10^6 cells/ml of *P. digitatum*, *P. expansum* and *P. janthinellum*, subjected to different treatment (A) acetone, (L) lower concentration (H) higher concentration and (P) Amphotericin B and then incubated for 4 days. Zone of growth (mm) of infected wounds were observed on the skin of fruit.

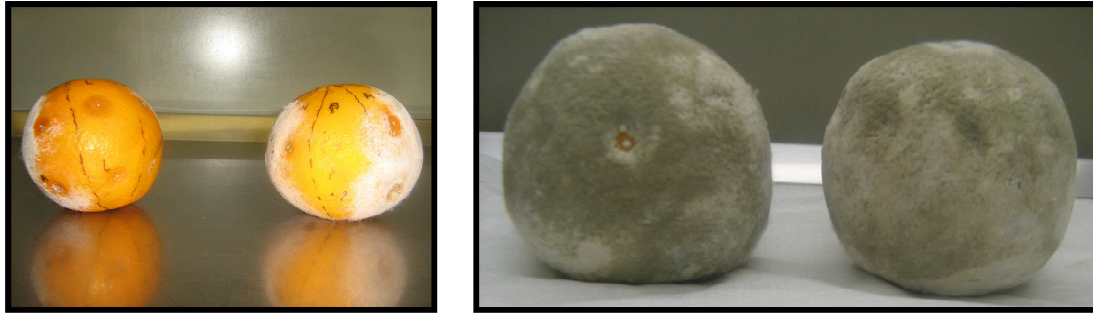


Figure 10-6 Oranges inoculated with 100 000 cells of *P. digitatum* and subjected to different treatments. **Left:** Oranges half infected with *Penicillium* after 8 days and **right:** oranges were covered with fungi after 10 days probably reflecting a change from a yeast to a mycelial growth form.

10.4.5 Determining the LC₅₀ of the crude extract

In the third experiment we wanted to determine the dose related response of the crude extract in order to compare the activity relative to ursolic acid as well as to compare the ratio of toxicity of mammalian cells to activity against the fungal pathogens. To calculate the IC₅₀ it would be more correct to calculate the growth area rather the growth diameter growth rate. The data presented in bar charts in Figure 10-4 were presented in a scatter diagram to enable calculation of LC₅₀ values in Figure 10-7. 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 dose related response in all cases (Figures 10-7 and 10-8). The smooth lines again attest to the validity of the model we have developed. In Figure 10-7, the graph shows that at a zero concentration, the area of growth of *P. expansum* was 32.2 mm² and therefore the LC₅₀ (dose that kills 50% of the cells) was 1.8 mg/ml, because this would have led to a 50% growth of 16.1 mm². In the case of the *P. digitatum*, the IC₅₀ was 1.6 mg/ml and with *P. janthinellum* it was 1 mg/ml. The above analysis revealed that when the area of growth was very high, then the IC₅₀ (inhibition concentration of fungi) was reduced.

There was a slight difference in IC₅₀ when comparing data between 4 and 7 days of incubation (Figure 10-8). With *P expansum* the IC₅₀ 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 IC₅₀ of 1 mg/ml with a longer period of incubation.

It is remarkable 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 a yeast to a mycelial growth form by the two other isolates.

The effect of 0.04 and 0.08 mg/ml amphotericin B on the three isolates was also determined in the same experiment as shown in Table 10-4. The resistance of the *P. digitatum* in comparison to the other *Penicillium* species to amphotericin B may explain why it was isolated from commercially available oranges. It has been reported that *P. digitatum* isolate from citrus fruit were relatively sensitive to the three fungicides, sodium *o*-phenylphenate (*o*-phenylphenol), imazalil, and thiabendazole with mean EC₅₀ 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 EC₅₀ ranging between 0.87 and 0.92 µg/ml (Holmes and Eckert 1999).

Table 10-4 Growth of different *Penicillium* isolates in mm² treated with two concentrations of amphotericin B after 7 days incubation

Pathogen	0.04 mg/ml	0.08 mg/ml
<i>Penicillium expansum</i>	0	0
<i>Penicillium digitatum</i>	89	105
<i>Penicillium janthinellum</i>	35	0

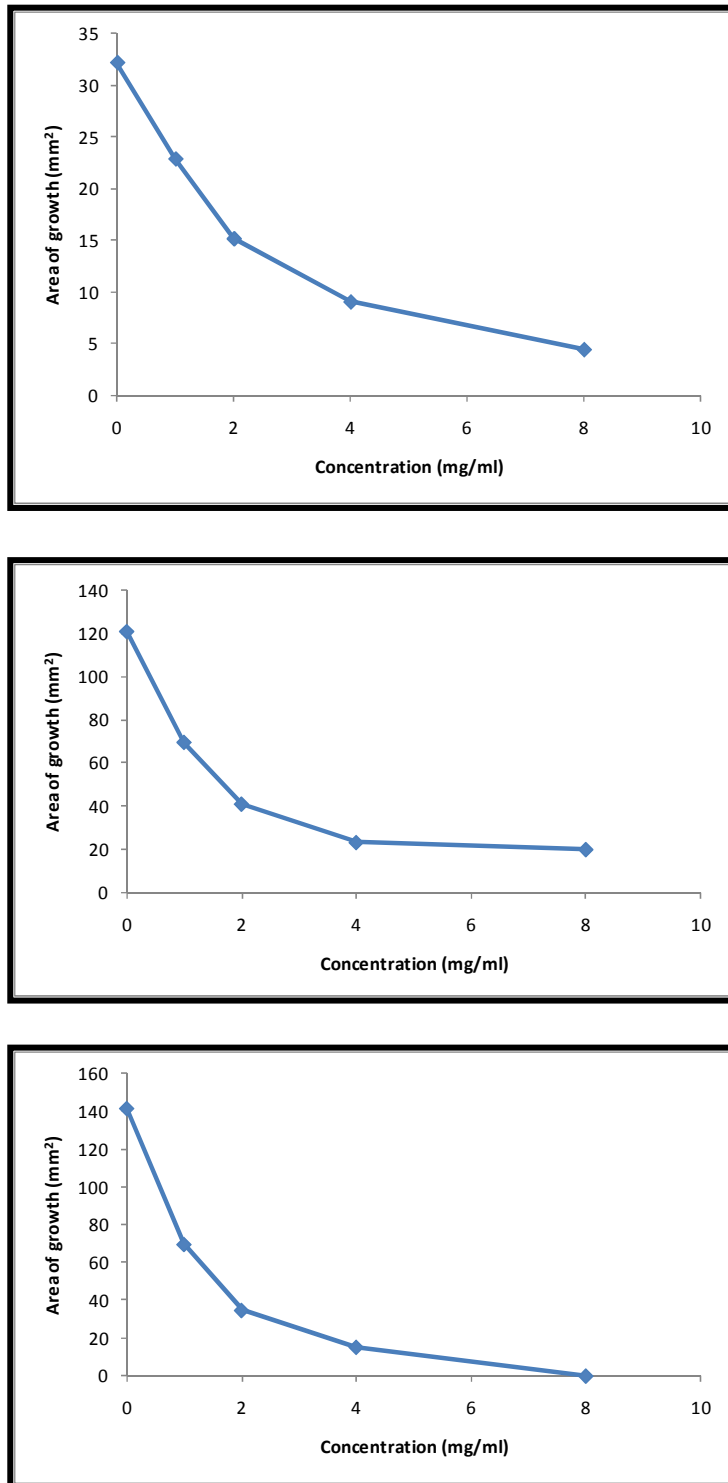


Figure 10-7: Oranges inoculated with 100 000 cells of *P. expansum* (top) *P. digitatum* (middle) and *P. janthinellum* subjected to different concentrations of the crude acetone leaf extract of *Breonadia salicina*. The area of growth in mm² was calculated and results are shown as the average infected area after 4 days of incubation time.

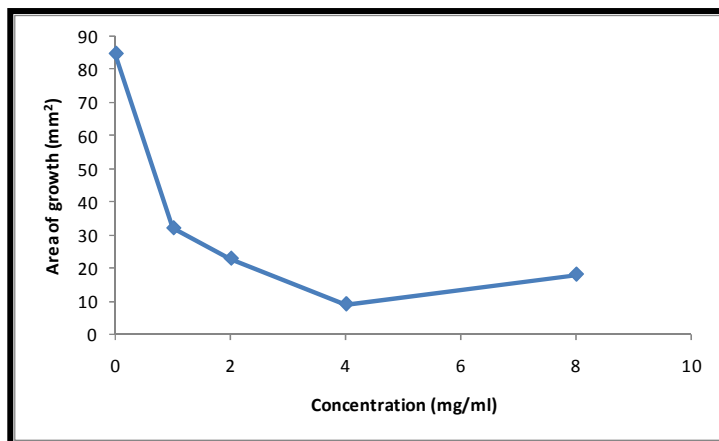
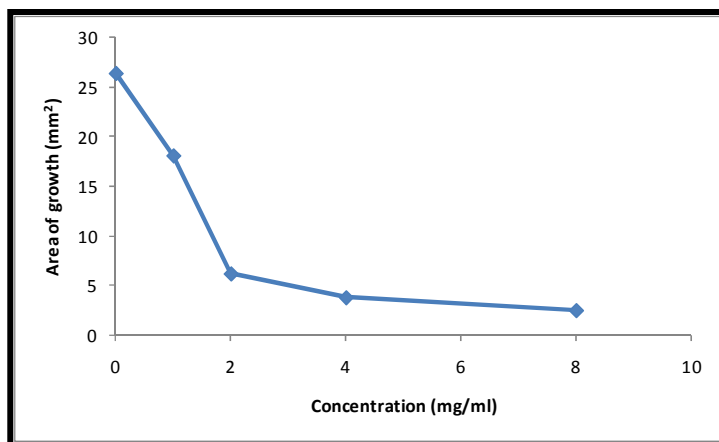
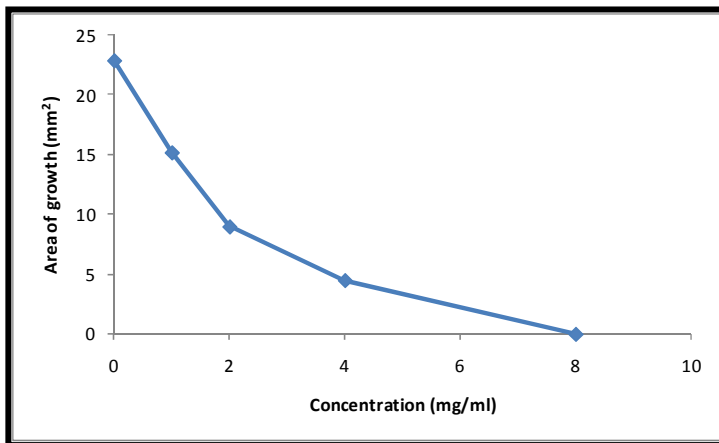


Figure 10-8: Oranges inoculated with 100 000 cells of *P. expansum* (top) *P. digitatum* (middle) and *P. janthinellum* (bottom) subjected to different concentrations of the crude acetone leaf extract of *Breonadia salicina*. Area of growth in mm² was calculated and results are shown as the average infected area of the after 7 days of incubation time.

Table 10-5 LC₅₀ and area of growth after 4 and 7 days incubation time against three *Penicillium* species.

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

It is interesting that the LC₅₀ of ursolic acid was in the same order as that of the crude extract. This indicates that the other antifungal compounds present in the crude extract probably play a role in the total antifungal activity.

10.5 Therapeutic Index

10.5.1 Therapeutic index of the crude extract and isolated compounds

The therapeutic index for the acetone extracts (treatment) was calculated using the cytotoxicity concentrations of the crude extracts (Chapter 9).

The therapeutic index for each fungus was 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.

Because LD₅₀ or LC₅₀ are inversely related to activity the therapeutic index can be calculated by dividing the LC₅₀ for animal cells (Vero) by the LC₅₀ for the pathogen determined in section 10.5.5.

Table 10-6 The Therapeutic Index (TI) of acetone extracts against three plant pathogenic fungi calculated by dividing LC₅₀ with MIC using values after 4 days.

Fungi	LC₅₀ in mg/ml for fungus	LC₅₀ Vero cells mg/ml	Therapeutic Index
<i>Penicillium expansum</i>	1.8	0.082	0.05
<i>Penicillium digitatum</i>	1.6	0.082	0.05
<i>Penicillium janthinellum</i>	1.0	0.082	0.08

The therapeutic index of 0.05 to 0.08 means that the acetone leaf extract of *B. salicina* may be much too toxic to become a useful product to control *Penicillium* infections in oranges. Many fungicides are very 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 equates to mammalian toxicity without any 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.

10.6 Conclusion

The method that was used to evaluate the protection by crude leaf extracts of *B. salicina* gave reproducible results. A crude leaf extract at a concentration of less than 1 mg/ml gave the same level of protection as 1 mg/ml ursolic acid (2.89 mm diameter, Figure 10-3), the main antifungal compound in the extract. The crude extract therefore gave practically the same protection as the isolated compound. This indicates that a low cost product could be developed from leaves of *B. salicina*. This LC₅₀ was however two to three orders of magnitude higher than that of the commercially used antifungals. In practical terms all antifungal compounds have some toxicity, unfortunately our extract was more toxic to Vero monkey cells than to the fungi. It may be possible to remove the toxic component from the extract without major changes.

These results point to the feasibility of using plant products as biopesticides. This degree of success was attained with a plant species that had relatively low activity compared to the antifungal activity of other plant extracts investigated in the Phytomedicine Programme. It is likely that wide screening against *Penicillium* species may be a viable option to develop useful antifungal preparations for post harvest protection of plant products.

CHAPTER 11

Summary and conclusion

The aim of this study was to identify plant species with good antifungal activity and to isolate and characterize compounds or extracts with strong antifungal activity, which could be used to develop a product with good activity against plant fungal pathogens.

To attain this aim the following objectives were identified:

1. To select and identify plant species active against plant fungal pathogens for further phytochemical investigation based on the proven activity of extracts against animal fungal pathogens.
2. To determine the antifungal activity of leaf extracts of the selected plant species against *Aspergillus fumigatus* due to its potential use in protecting production animals against this pathogen.
3. To screen leaf extracts of plant species for qualitative antioxidant activity as an additional parameter for selecting the most promising species for in-depth investigation.
4. To isolate antifungal compounds from the selected plant species and to determine the structure of these compounds.
5. To determine the biological activity of the crude extract and the isolated compounds in antimicrobial and cytotoxicity assays.
6. To evaluate the potential use of the extract or isolated compound(s) against a plant fungal pathogen.

Objective 1. To select plant species to be tested for activity against plant fungal pathogens

The species selected for evaluation were: *Bucida buceras*, *Breonadia salicina*, *Harpephyllum caffrum*, *Olinia ventosa*, *Vangueria infausta* and *Xylothea kraussiana*. Acetone, hexane and methanol extracts of *B. buceras* had the best antifungal activity against *Penicillium expansum*, *P. janthinellum*, *Trichoderma harzianum* and *Fusarium oxysporum* with MICs ranging between 0.02 and 0.08 mg/ml. All the extracts of *B. salicina* had the best activity against *P. janthinellum* with MIC values of 0.08 mg/ml. Acetone extracted a large quantity of plant

material (108.3 mg) and MeOH was the second best extractant, extracting 100.4 mg. Some of the plant extracts had strong antifungal activity, but individually separated compounds were not active based on the bioautography assay. All extracts of *O. ventosa* had the highest activity against *T. harzianum* with MIC value of 0.04 mg/ml. Although *Olinia ventosa* extracts gave very promising results, the plant was not readily available for collection of test material. *Breonadia salicina* was selected for further phytochemical investigation based on the good antifungal activity and bioautography results.

Objective 2. To determine the antifungal activity of plant extracts against *Aspergillus fumigatus*

Based on the antifungal activity against plant pathogenic fungi, further investigation was carried out to test the activity of the plant extracts against an animal fungal pathogen with serious economical implications in poultry production. Leaf extracts of the six plant species had antifungal activity against *A. fumigatus*. All extracts of *B. salicina* had a strong antifungal activity against *A. fumigatus* with MIC values as low as 0.08 mg/ml. In general, extracts were not as active against animal fungal pathogens although all of the plant extracts were active against both plant and animal pathogenic fungi. In the bioautography assay, the same antifungal compounds were apparently present in extracts of *B. salicina* against plant and animal pathogenic fungi. Since the extracts had good antifungal activity against *A. fumigatus*, a clinical study could in future be carried out to test the efficacy of the extracts using chickens as done by Suleiman (2009) with other extracts.

Objective 3. To screen leaf extracts of plant species for qualitative antioxidant activity

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. Antioxidant activity was observed in the methanol extract of *V. infausta* and *X. kraussina* with the yellow band. However, the activity was not strong since the yellow band was not very clear. The acetone, hexane and DCM extracts were less effective than the

methanol extract. No plant extract had strong antioxidant activity and quantitative analysis was not investigated. It appears that the effect of extracts of *B. salicina* used traditionally may therefore be due to direct inhibition of microbial growth.

Objective 4. To isolate antifungal compounds from the selected plant species

Preliminary fractionation of *B. salicina* was carried out using serial extraction with solvents of increasing polarities. Acetone and chloroform were the most promising fractions of the serial extraction procedure. Solvent-solvent fractionation was performed on the chloroform fraction and led to the separation of three fractions (n-butanol, chloroform and aqueous). Amongst the three fractions, the chloroform fraction of the solvent-solvent fractionation was the most active and showed several antifungal compounds in the bioautography assay.

Column chromatography using silica gel was used for gradient elution of extracts, leading to isolation of four compounds. Nuclear Magnetic Resonance (NMR) spectroscopy and MS were used for identification of isolated compounds. Only one compound was identified as the triterpenoid ursolic acid while MS revealed that the other three compounds appeared to be mixtures of fatty acids and the structures were not elucidated.

Objective 5. To determine the biological activity of the crude extract and the isolated compounds in antimicrobial and cytotoxicity assays

Biological activities of all isolated compounds were determined. Although many compounds have been isolated, frequently the biological activity has not been determined. All the isolated compounds had antifungal activity against the tested organisms, with MIC values ranging between 10-125 $\mu\text{g/ml}$. In the bioautography assay, antifungal compound was visible in the bands represented by ursolic acid, C2 and C3, but not in compound C4. Based on the bioautography assay, the four isolated compounds had both antibacterial (against *E. coli*, *P. aeruginosa* and *S. aureus*) and antifungal activity.

It is important to determine cytotoxicity to know whether the compounds are potentially harmful to humans and animals. The cytotoxicity of the crude acetone extract and isolated

compounds were investigated against Vero monkey kidney cells using the MTT assay. The crude acetone extract had an LC₅₀ of 82 µg/ml. Ursolic acid and C4 were toxic towards Vero cells at an LC₅₀ of 25 and 36 µg/ml respectively. Compound 2 and C3 were not toxic toward Vero cells with LC₅₀ greater than 200 µg/ml. Ursolic acid was very toxic despite showing good antibacterial and antifungal activity against the tested microorganisms. The crude acetone extract, ursolic acid and compound 4 were too toxic to investigate further to develop drugs that can be used to combat fungal infectious diseases in plants, animals and humans. If the structures of compounds 2 and 3 can be determined there may be some possibility for further use.

The crude extract of *B. salicina* was active against the animal fungal pathogen *Aspergillus fumigatus*, with an MIC of 80 µg/ml, but was also too toxic to consider for further use. In all cases, changes of the basic chemical structure may increase the activity and decrease the toxicity of the compound to animal cells, but this was beyond the scope of this study.

Objective 6. To evaluate the potential use of the extract or isolated compound(s) against a plant fungal pathogen

In *in vivo* studies, the potential use of the extract or isolated compound(s) against three plant fungal pathogens (*Penicillium expansum*, *P. janthinellum* and *P. digitatum* isolated from infected oranges) were investigated after treating the oranges with the crude acetone extract and ursolic acid. The method used in the experiment gave good, reproducible results. The crude leaf extracts and ursolic acid inhibited the growth of fungi at 8 and 1 mg/ml. The LC₅₀ for the three fungi were *P. janthinellum* 1 mg/ml, *P. digitatum* 1.6 mg/ml and *P. expansum* was 1.8 mg/ml. These values are three orders or magnitude lower than the LC₅₀ values of commercial fungicides such as sodium *o*-phenylphenate (*o*-phenylphenol), imazalil, and thiabendazole. *Penicillium digitatum* was more resistant to amphotericin B in comparison to other *Penicillium* species. These results nevertheless suggest that a low cost product could be produced from leaves of *B. salicina* to protect fruit from plant pathogenic fungi. The therapeutic index for each fungus ranged between 0.05 and 0.08. This means that the acetone leaf extract of *B. salicina* may be much too toxic to be a useful product to control *Penicillium* infections in oranges.

The results did however illustrate the feasibility of developing a biopesticide from a plant product. The low cost of developing such a product may make it economically feasible if there were no safety concerns. Using a plant based fungicide may also find acceptance in the organic growth market.