

Evaluation of alien invasive weedy plants for activity against plant pathogenic fungi

Moraba Macdonald Meela
(s26180783)

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Supervisor: Prof J.N Eloff

Co-promoter: Dr L.K Mdee

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Declaration

This research was carried out in the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, under the supervision of Prof J.N Eloff and Dr L.K Mdee.

This thesis represents work done by Moraba Macdonald Meela and its results have not been submitted anywhere else before, except where the work of others is referenced.

.....
Moraba Macdonald Meela

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Conferences and proceedings

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Meela, M.M., Mdee, L.K. and Eloff, J.N. 2008. Prospects for use of alien invasive weed extracts against fungal phytopathogens. *Oral presentation at World conference on Medicinal and Aromatic Plants (WOCMAP IV), Cape Town, South Africa.*

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List of abbreviations

ATCC	American Type Culture Collection
BEA	Benzene/Ethanol/Ammonium hydroxide (90:10:1)
BuOH	n-Butanol
CEF	Chloroform/Ethyl acetate/Formic acid (5:4:1)
CHCl ₃	Chloroform
DCM	Dichloromethane
DMSO	Dimethyl sulphoxide
EMW	Ethyl acetate/methanol/water (40:5.4:4)
EtOAc	Ethyl acetate
F	Fraction
Fig	Figure
Hex	Hexane
HPLC	High Performance liquid Chromatography
Hrs	Hours
INT	p-iodonitrotetrazolium violet
LC ₅₀	Lethal concentration for 50 % of the cells
LC-MS	Liquid Chromatography Mass Spectroscopy
MeOH	Methanol
MIC	Minimum inhibitory concentration
MTT	3-(4,5-dimethyliazolyl)-2,5-diphenyl-tetrazolium bromide dye
MH	Müller-Hinton
NMR	Nuclear magnetic resonance
OC	Open column
PDA	Potato dextrose broth
PDB	Potato dextrose broth
R _f	Retardation factor
TLC	Thin layer chromatography
UV	Ultraviolet radiation

Abstract

Plant fungal pathogens are a major threat to food security worldwide. The most important method of protecting plants against fungal attack is the use of fungicides, but the development of resistance towards synthetic fungicides is of great concern. Moreover, the health risks associated with the use of chemical fungicides increase the need to search for safe, efficacious and environmentally friendly fungicides. Plants produce antifungal agents by secondary metabolism to protect themselves from fungal attack, and therefore many plant species have substantial antifungal activity. The use of plant extracts could enable the development of inexpensive and environmentally acceptable fungicides based on locally available natural products. This study was undertaken to investigate weedy and invasive plant species for antifungal activity against plant pathogens in order to develop a useful product using a widely available resource.

Acetone leaf extracts of seven invasive species (*Chromolaena odorata*, *Ipomoea alba*, *Tecoma stans*, *Passiflora suberosa*, *Passiflora subpeltata*, *Aristolochia sp*, *Solanum seafortianum*) were screened against eight plant fungal pathogens viz *Rhizoctonia solani*, *Fusarium oxysporium*, *Penicillium janthinellum*, *Penicillium expansum*, *Aspergillus parasiticus*, *Aspergillus niger*, *Pythium ultimum* and *Phytophthora nicotiana*, using microdilution assay and bioautography. The acetone extract of *Tecoma stans* had reasonable antifungal activity with an average minimal inhibitory concentration (MIC) value against all the fungi of 550 µg/ml and clear zones on bioautograms indicating inhibition of fungal growth of a compounds with an R_f of 0.082 in BEA against several of the fungal pathogens. Due to the clear compound on bioautography and availability of *Tecoma stans*, this species was selected for further work.

Bioassay-guided fractionation of the leaves of the *Tecoma stans* dichloromethane (DCM) extract obtained from solvent-solvent fractionation resulted in one major compound, oleanolic acid. The isolated compound had antifungal activity with an average MIC value of 130 µg/ml against the 10 plant pathogenic fungi and clear bands with an R_f value of 0.082 on bioautograms, indicating fungal growth inhibition. It was surprising that the MIC value of the crude DCM extract was as high as that of the only compound with antifungal activity based on bioautography. These results clearly indicated the possibility of synergisms especially since the average total activity of the extract was nearly 6.5 times higher than that of

oleanolic acid with total activity values of 60154 ml for the extract and 9262 ml for oleanolic acid.

Cellular cytotoxicity of DCM extract and oleanolic acid was investigated using tetrazolium-based colorimetric assay (MTT) on Vero monkey kidney cells. The toxicity of the extract and oleanolic acid was determined by LC_{50} values. The DCM extract and oleanolic acid were toxic with LC_{50} of 0.413 mg/ml and 0.129 mg/ml respectively, lower than that of berberine the toxic compound used as control. However therapeutic index which can be defined here as the LC_{50} in (μ g/ml)/MIC in (μ g/ml), indicated that though the extract and oleanolic acid were toxic, they could be used under controlled conditions against infections of certain of the fungal pathogens. The crude extract had a high therapeutic index value of 21 against microorganisms *T. harzianum*, *R. solani*, *F. oxysporium* and *P. expansum*; and oleanolic acid had high therapeutic index values of 16 and 64 of against *T. harzianum* and *R. solani* respectively. This high therapeutic index value of crude extract and oleanolic acid means that, crude extract and oleanolic acid may be used for treatment of infections by these tested fungi with very little toxicity under controlled conditions.

Oleanolic acid had very low antibacterial activity (MIC >250 μ g/ml). against two Gram-positive (*Staphylococcus aureus*, ATCC 29213 and *Enterococcus faecalis*, ATCC 29212) and two Gram-negative bacteria (*Escherichia coli*, ATCC 27853 and *Pseudomonas aeruginosa*, ATCC 25922). Animal pathogenic fungi were more resistant than the plant fungal pathogens.

Based on the good activity of the DCM crude extract, the surprising selectivity in activity against different fungi coupled with reasonably good therapeutic indexes and the wide availability of *T. stans* leaves opens up the possibility that a commercial product to protect plants against certain pathogens may be developed from *T. stans* leaves.

Chapter 1

Background and literature review

1. Introduction

Different plant pathogens threaten food security worldwide as more than 800 million people have inadequate food supplies and at least 10% of food production is lost to plant diseases (Strange and Scott, 2005). Plant fungal pathogens in particular, pose a major threat since about 5000 fungal species destroy economically valuable crops (Prescott *et al.*, 1996). Plant pathogenic fungi attack most crops in the field and also post harvest thereby decreasing production and shelf life of many agricultural crops.

The most important method of protecting plant against fungal attack is the use of synthetic fungicides, but the development of resistance towards synthetic fungicides is of great concern. Moreover, the health risk and hazardous effects on environment associated with the use of chemical fungicides increase the need to search for safe, efficacious and environmentally friendly fungicides (Anonymous, 1987; Hostettman *et al.*, 2000).

Plants produce antifungal agents by secondary metabolism to protect themselves from fungal attack, and therefore many plant species possess substantial antifungal activity. (Angeh, 2006; Eloff *et al.*, 2006), developed a procedure that yielded excellent activity against plant fungal pathogens from *Melianthus comosus* in the laboratory and in a preliminary field trial. Thus, the use of plant extracts with inhibitory activity against fungal plant pathogens could lead to the development of environmentally acceptable fungicides based on the availability of natural products (Athukoralage *et al.*, 2000).

1.1. Fungal plant pathogens and their effect

Some notorious plant pathogenic fungi genera include *Pythium*, *Phytophthora*, *Fusarium* and *Rhizoctonia*, which cause root and corn rot and seedling damping-off in many fruits, vegetables and ornamental plants. Different areas of the USA suffered severe agricultural losses due to their corn (maize) crop completely destroyed by the fungus *Cochliobolus heterostrophus* (Strange and Scott, 2005). When many agricultural crops are infected by

certain fungal pathogens they produce mycotoxins that are harmful to both humans and livestock (Richard *et al.*, 1996).



Fig 1.1: Apple with blue mold caused by *Penicillium expansum* (Janisiewicz, 1999).

1.1.1. Mycotoxins

Mycotoxins are a group of secondary metabolites produced by fungi that are natural contaminants of agricultural products. Ingestion of food and feed made toxic by these metabolites cause diseases called mycotoxicoses (Marasas and Nelson, 1987). Patulin, a mycotoxin produced by *Penicillium* spp during food spoilage causes severe acute and chronic toxicity, including carcinogenic, mutagenic and teratogenic effects (Bretta *et al.*, 2000; Hassan, 2000; McCallum *et al.*, 2002).

Aflatoxins, of which aflatoxin B1 (AFB1) is the most potent and commonly occurring compound, are produced by the fungus *Aspergillus flavus*. AFB1 and mixtures of aflotoxins were classified by the International Agency for Research on Cancer (IARC) as Group 1 carcinogens, i.e. substances that can cause cancer in humans (www.sciencein africa.co.za/2001/august/peanut.htm).

Fumonisin, mainly B1 and B2, contaminate maize-based foods and feed throughout the world, and have been linked to several diseases such as equine leukoencephalomalacia (ELEM) in horses, porcine pulmonary oedema (PPE) in swine, liver toxicity in many other animals and oesophageal and liver cancer in humans (Chu and Li, 1994; Gelderblom *et al.*,

1991; Harrison *et al.*, 1990; Keller *et al.*, 1990; Lino *et al.*, 2004; Marasas *et al.*, 1998; Ross *et al.*, 1990; Thiel *et al.*, 1992).

1.2. The use of synthetic fungicides

Synthetic fungicides are the primary means of controlling plant pathogens. About 23 million kg of fungicides are applied to fruit and vegetables annually, and it is generally accepted that production and marketing of these perishable products would not be possible without their use (Ragsdale and Sisler, 1994). However, safety risks, high costs, side-effects and development of resistance towards the use of these fungicides are raising serious concerns (Tripathi and Dubey, 2004).

Furthermore, chemical fungicides commonly used in agriculture are a source of concern to the Environmental Protection Agency due to their health risk concerns and negative effect on environment (Anonymous, 1987). Their carcinogenicity and off-odour effects have limited their use (Ling, 1991; Unnikrishnan and Nath, 2002). It is in light of these drawbacks that other means of fungal control have to be investigated.

1.3. Other treatment of fungal control

The fundamental approach to the management of a disease is to ensure that it is not present through exclusion (quarantine) or proper sanitation (Ebbels, 2003). The use of physical treatments such as heat therapy, low temperature storage and hot water treatment can significantly reduce pressure on harvested commodities (Eckert, 1991; Lurie, 2001). Harvesting and handling techniques that minimize injury to the commodity and storage conditions that are optimum for maintaining host resistance will also help in suppressing post harvest disease development (Sommer, 1985). Biocontrol agents have also been considered in management of post harvest diseases of fruit and vegetables as a viable alternative to the use of present day synthetic fungicides (Wilson and Wisniewski, 1989; Wilson *et al.*, 1999; Pang *et al.*, 2002).

However, when these methods are applied as treatment alone, they prove to be less effective and inconsistent in their commercial applicability (Droby *et al.*, 2001), limiting their acceptance as an alternative to synthetic fungicides. Lack of efficacy in these methods and

negative perception in the public towards synthetic chemicals lead to growing interest towards natural alternatives, particularly of plant origin.

1.4. Use of plants

Plants are good candidates in the search for fungicidal compounds since they have to exist under difficult conditions and attack by all manner of parasites (Hostettman *et al.*, 2000). Their use as natural additives emerged from an increasing tendency to replace synthetic antimicrobial agents with natural ones (Sharma and Tripathi, 2006).

Some plants extracted in different organic solvents have an inhibitory action against different storage fungi (Singh *et al.*, 1993; Mohamed *et al.*, 1994; Hiremath *et al.*, 1996; Kapoor, 1997; Radha *et al.*, 1999; Rana *et al.*, 1999). Extracts from *Melianthus comosus* and a compound isolated phytochemically were tested in our laboratory and had excellent activity against plant pathogenic fungi (Eloff *et al.*, 2006). Research has also shown fewer cases of undesirable side-effects when dealing with extracts (Zishiri, 2004).

Phyto-compounds are also expected to be more advantageous than synthetic chemicals for their sheer magnitude of complexity, diversity and novelty of chemicals (Sharma, 1998) since they may be biodegradable in nature, non-pollutant and may possess no residual or phytotoxic properties (Badei *et al.*, 1996; Bishop and Thornton, 1997; Tewari, 1990). Therefore, plant extracts or plant secondary metabolites which are not toxic and specific in their action could be considered as an alternative to synthetic fungicides based on the availability of material, hence our exploration of more available weedy plants.

1.5. Weeds and alien invasive species

Weeds are usually aggressive growers, making them compete for water, light, space and nutrients; hence they are frequently present in large quantities. They are adaptable, being able to easily invade a wide range of ecological niches and most are exotic in origin (Bromilow, 2000; Enright, 2000).

1.5.1. Why choose to work on weeds

1. Weeds and alien invasive species may be such successful competitors due to resistance towards different pathogens. These plant species may therefore contain active principles to resist fungal attack.
2. Extracts of a plant such as *M. comosus* has excellent antifungal activity, but the cost of cultivating the plant have prompted the investigation of readily available material such as weeds.
3. If weeds or alien invasive plant species are used as raw material for plant-derived fungicides then there are large quantities of material readily available for use. Additionally collection may protect indigenous plants and at the same time may create economic uses and jobs based on these unwanted species.

1.6 Aim and Objectives

1.6.1 Aim

To investigate weedy plant species for antifungal activity against plant pathogens in order to develop a useful product.

1.6.2. Objectives

1. To evaluate seven selected weedy plants species for antifungal activity and select one or two plant species for further study.
2. To isolate the active antifungal compound(s) from the selected plant(s) species.
3. To determine the *in vitro* biological activity of the isolated compound(s).
4. To discuss the potential value of the selected plant species in protecting plants against fungal attack

Chapter 2

***In vitro* screening of acetone extracts of seven invasive weeds against plant fungal pathogens.**

2.1 Introduction

Acetone was used as an extractant based on its efficacy with reference to quantity and diversity of compounds extracted, ease of removal, safety and toxicity to assay organisms in the case of bioassay work (Eloff, 1998b). Screening of plants for active compounds has become increasingly vital (Rabe and van Staden, 1997). Eloff (2004) stated that finding new lead compounds for developing pharmaceuticals and developing the phytomedicines for use as herbal medicines as some of the important reasons for plant screening. However, problems such as unsustainable harvesting methods of medicinal plants could endanger these plants (Goldrings, 1990) and result in extinction before their potential as sources of pharmaceutical drugs is discovered. Therefore the rationale behind screening of readily available invasive weeds which can survive adverse conditions, for potential as alternative antifungal source is strong.

2.1.1 Alien invasive and weedy species

The information in this section was obtained from the website of Gateway to knowledge on alien plant management in South Africa.

(<http://fred.csir.co.za/plants/global/continen/africa/safrica/index.html>).

There are about 750 tree species and approximately 800 shrubby and herbaceous species which were introduced into South Africa. At this stage 161 of these introduced species of which 38 are herbaceous, 13 succulent and 110 woody are regarded as invasive although many more will be declared weeds in time. Amongst these plants 44 species have been legally declared noxious weeds (i.e their removal is enforced by law) while 31 have been declared as invaders (i.e their spread have to be monitored).

Of the invasive plants introduced into South Africa, 64 species are from South and Central America and 14 from North America, 26 from Australia, 19 from Europe and 25 from Asia. Plants introduced from Australia are more invasive since 45% of plants introduced from this

continent have become important weeds, compared to 12% of those from South America and 3% of those from Europe and Asia. It is vital to highlight that not all alien plants are invasive since only 110 of the 744 (15%) of alien species are regarded invasive; the remainder pose little threat and can be beneficial

Table 2.1: Weedy plants species selected for investigation.

1, Lowveld National Botanical Garden, Nelspruit; 2, JN Eloff's garden, 219 Theuns Road, Murrayfield, Pretoria.

Species	Family	Common name	Part used	Collection site	References
<i>Aristolochia gigantea</i>	Aristolochiaceae	Dutchman's pipe	Leaves	1	http://www.ntbg.org/plantsresource
<i>Chromolaena odorata</i>	Asteraceae	Triffid weed	Leaves	1	(Bromilow, 2001)
<i>Ipomoea alba</i>	Convolvulaceae	Moonflower	Leaves	1	(Bromilow, 2001)
<i>Passiflora suberosa</i>	Passifloraceae	Indigo berry	Leaves	1	(Bromilow, 2001)
<i>Passiflora subpeltata</i>	Passifloraceae	Granadina	Leaves	1	(Bromilow, 2001)
<i>Solanum seafortianum</i>	Solanaceae	Potato creeper	Leaves	1	(Bromilow, 2001)
<i>Tecoma stans</i>	Bignoniaceae	Yellow bells	Leaves	2	http://cloudbridge.org/trees/tecoma stans.html

2.2 Materials and Methods

2.2.1 Plant collection

The Phytomedicine Programme has been collecting leaves of trees from the Lowveld National Botanical Garden for more than a decade. With the excellent climate in the Lowveld in Mpumalanga invasive species represent a major problem. The plants selected to work on were those that were available during a collection trip in the natural areas and borders of the Lowveld National Botanical Garden. Leaves of the plants were collected, in the summer of 2008, as detailed in (Table 2.1) below. Voucher specimens in the garden herbarium verified the identity of the species.

2.2.2 Plant storage

Leaves were separated from stems and dried at room temperature. Most scientists have tended to use dried material because there are fewer problems associated with large-scale extraction of dried plants 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.

2.2.3 Extraction procedure

Plants samples from each species were individually extracted by extracting 4g of finely ground plant material with 40 ml acetone (technical grade-Merck) in polyester centrifuge tubes. Tubes were vigorously shaken for 30 minutes in a Labotec model 20.2 shaking machine at high speed. After centrifuging at 3500 rpm for 10 minutes the supernatant was decanted into pre-weighed labelled containers. The process was repeated 3 times to exhaustively extract the plant material and the extracts were combined. The solvent was removed under a stream of air in a fume cupboard at room temperature, to quantify the extraction.

2.2.4 Phytochemical analysis

Chemical constituents of the extracts were analyzed by thin layer chromatography (TLC) using aluminium-backed TLC plates (Merck, silica gel 60 F₂₅₄). The TLC plates were developed under saturated conditions with one of the three eluent systems developed in our laboratory that separate components of plant extracts well i.e.

- Ethyl acetate/methanol/water (40:5.4:5): [EMW] (polar/neutral);
- Chloroform/ethyl acetate/formic acid (5:4:1): [CEF] (intermediate polarity/acidic);
- Benzene/ethanol/ammonia hydroxide (90:10:1): [BEA] (non-polar/basic) (Kotze and Eloff, 2000).

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

2.2.5 Fungal test organisms

Eight microorganisms namely, *Rhizoctonia solani*, *Fusarium oxysporium*, *Penicillium janthinellum*, *Penicillium expansum*, *Aspergillus parasiticus*, *Aspergillus niger*, *Pythium ultimum* and *Phytophthora nicotiana* were obtained from the Department of Plant Pathology, University of Pretoria. All fungal strains were maintained on potato dextrose agar (Oxoid, Basingstoke, UK).

2.2.6 Culture preservation techniques

All organisms were maintained on potato dextrose agar (Merck). The agar was prepared by dissolving 25 g of agar into one litre of distilled water. The mixture was boiled while stirring with a magnetic stirrer for 15 minutes to completely dissolve the powdered agar. The agar was autoclaved for 15 minutes, allowed to cool slightly and poured into sterile Petri dishes. The agar was allowed to cool completely and solidify before being used for maintaining fungal cultures. The fungal organisms were sub cultured from the mother cultures by cutting the agar on which the fungus was growing into small squares with a sterile surgical blade and transferred to newly prepared agar in petri dishes. The new cultures were incubated at room temperature for 3-7 days in the dark after which they were sealed with parafilm and stored at 8°C. These cultures were then ready to be used for preparing the fungal inoculum for use in bioassays.

2.2.7 Preparation of fungal inoculum

Potato dextrose broth (Merck) was used as the liquid medium for fungal culture. The broth was prepared by dissolving 30g of broth powder into one litre of distilled water. The mixture was boiled while being stirred by a magnetic stirrer for 15 minutes and then autoclaved for 15 minutes. The conidia of fungi were harvested from the agar plate using a sterile cotton swab and suspended uniformly in the broth. The inoculum was quantified immediately before being used for bioassay.

2.2.8 Quantification of fungal inoculum

The conidia of each fungal species from one agar plate were harvested with cotton swabs and suspended uniformly in 25 ml of potato dextrose broth each. The inoculum of each fungal species was quantified by counting the number of conidia using a haemocytometer and their number was adjusted to c. $1 \times 10^6 \text{ ml}^{-1}$ of the suspension.

The haemocytometer (Merck) was cleaned with 70% ethanol. The glass cover slip was cleaned with 70% ethanol and placed over the grooves and semi-silvered counting area. A micropipette was used to transfer approximately 20 μl of conidial suspension to the edge of the haemocytometer and allowed to spread evenly by capillary action. A compound microscope (Zeiss) was used at 100 x magnification to focus on the grid lines in the chamber. The slide was adjusted to view one corner (16 square grid area), which is a 1 mm^2 area. The conidia in the two corners diagonally opposite to each other were counted and averaged to obtain the number of conidia (n). For standard subculture, count between 100 and 300 conidia (cells) per mm^2 are ideal. The accuracy of the results increases when more cells are counted. The concentration of the sample was calculated as follows:

$$c = n/v$$

c = cell concentration (conidia/ml)

n = number of cells counted

v = volume counted

For a haemocytometer, the depth of the chamber is 0.1 mm and the area counted is 1 mm^2 . Therefore, the volume is $0.1 \text{ mm} \times 1.0 \text{ mm}^2 = 0.1 \text{ mm}^3$ or 10^{-4} ml . Hence, $c = n/v = n/10^{-4} \text{ ml} = n \times 10^4/\text{ml}$. After quantification and appropriate dilution, the inoculum was used for antifungal assay.

2.2.9 Antifungal assays

2.2.9.1 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).

Residues of the different extracts were dissolved in acetone to a concentration of 10 mg/ml. The plant extracts (100 μl) were serially diluted 50 % with water in 96 well microtitre plate

(Eloff, 1998c). Fungal cultures were transferred into fresh Potato dextrose broth, and 100 μ l of this was added to each well. Amphotericin B was used as the reference antibiotic and positive control, and appropriate solvent blanks were included. As indicator of growth, 40 μ l of 0.2 mg/ml of p-iodonitrotetrazolium violet (Sigma®) (INT) dissolved in water was added to each of the microplate wells. The covered microplates were incubated for two to three days at 35 °C and 100% relative humidity. The experiment was performed in triplicates and repeated 3 times. The MIC was recorded as the lowest concentration of the extract that inhibited antifungal growth after 24 hours of incubation.

2.2.9.2 Bioautography method

A bioautographic method developed in our laboratory (Masoko and Eloff, 2005) was used to determine active compounds. TLC plates (10 x 10 cm) were loaded with 100 μ g of each of the extracts. The prepared plates were developed in the three different mobile system used: CEF, BEA and EMW. The 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 3-5 days. Potato dextrose broth was prepared in 250 ml bottles. Cultures were transferred into broth from agar with a sterile swab. The 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 the other fungi in a Biosafety Class II cabinet (Labotec, SA) cupboard. The plates were sprayed until they just wet, incubated overnight and then sprayed with a 2 mg/ml solution of p-iodonitrotetrazolium violet (Sigma®) (INT) and further incubated overnight or longer at 35 °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 compounds that inhibited the growth of tested fungi. To minimize fungal spreading and infection in our laboratory, the bioautograms were sealed in the clear plastic envelopes before scanning in for permanent record. R_f values of active compounds were calculated using the following formula:

$R_f = \text{distance moved by analyte} / \text{distance moved by solvent front}$

2.3 Results and discussion

2.3.1 Extraction yields.

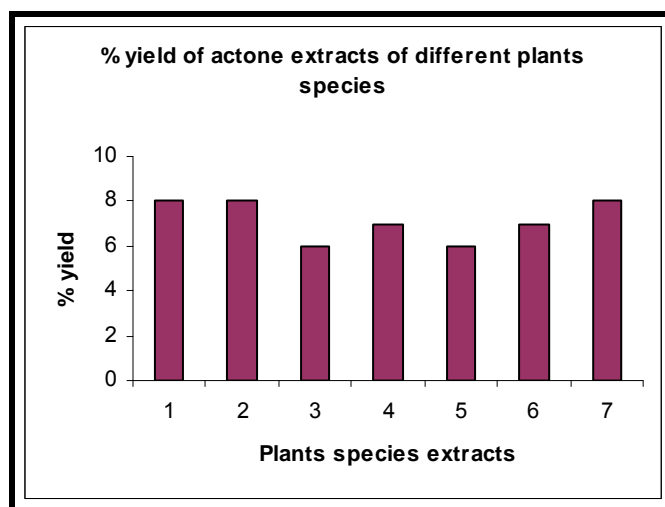


Fig 2.1: Quantity extracted as percentage (%) of the dry mass extracted. 1-*Chromolaena odorata*, 2-*Ipomoea alba*, 3-*Passiflora suberosa*, 4- *Passiflora subpeltata*, 5-*Tecoma stans*, 6-*Aristolochia gigantea*, 7-*Solanum seaforthianum*.

To analyze the extracts the concentration has to be known therefore it is important to dry the extract (Eloff, 2004). The acetone extract was very easy to dry, therefore facilitating the analysis of the extract.

Acetone extracted most of the material from *Chromolaena odorata*, *Ipomoea alba*, and *Solanum seaforthianum* yielding 8%, followed by *Passiflora subpeltata* and *Aristolochia gigantea* yielding 7%. *Tecoma stans* and *Passiflora suberosa* had reasonably good extraction yield of 6%.

These values were very similar and were more or less in line with the quantity extracted from 27 Combretaceae species that varied from 2.6-18.4% with an average of 10.6% (Eloff, 1999). Methanol could have been used as a polar solvent however it is considered to be toxic and water could also been considered as an alternative, however it takes a long time to dry in a stream of air at room temperature (Kotze and Eloff, 2002) and may contain sugars that lead to contamination by microorganisms.

2.3.2 Phytochemical analysis

The spraying reagent vanillin-sulphuric acid was used to detect the compounds which were not observed on chromatograms under UV light. BEA separated most of the components very well in almost all the plants species crude extracts. CEF and EMW separated some of the components well however they moved most of the components up leaving them packed to the solvent front and making it difficult to notice good separation of compounds. Most of the compounds in the extracts were therefore relatively non-polar (**Fig 2.2**).

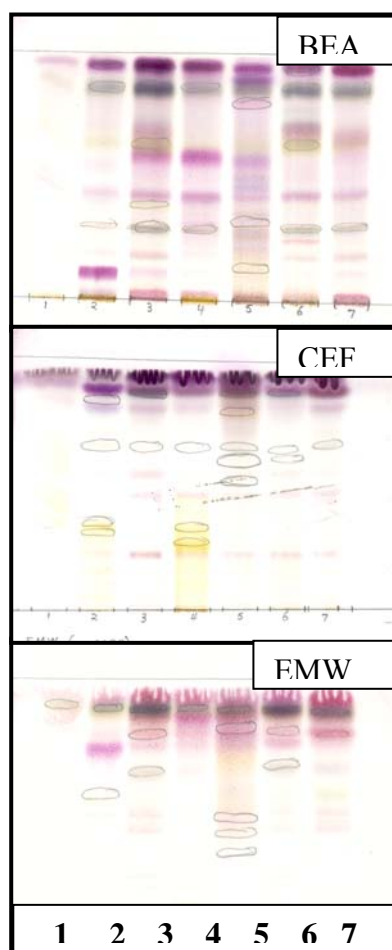


Fig 2.2: Chromatograms using three eluent systems BEA, CEF and EMW sprayed with vanillin-sulphuric acid. Lanes from left to right are (1) *Ipomoea alba*, (2) *Tecoma stans*, (3) *Passiflora suberosa*, (4) *Passiflora subpeltata*, (5) *Chromolaena odorata*, (6) *Aristolochia gigantea* and (7) *Solanum seaforthianum*.

2.3.3 Quantification of fungal inoculum

The number of cells of *Aspergillus niger* were counted on haemocytometer and averaged. The average was 144 cells; hence the concentration of the sample was 1.44×10^6 cells/ml. This concentration exceeds the required concentration; hence the inoculum was diluted with more broth to obtain an average of 1.0×10^6 cells/ml. The same procedure was followed for other fungal species to obtain a concentration of 1.0×10^6 cells/ml.

2.3.4 Bioautography

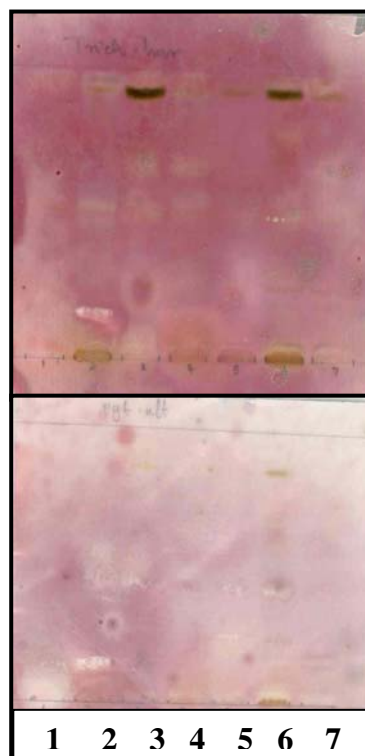


Fig 2.3: Bioautography results of the plates developed in BEA and spayed with two fungi *Trichoderma harzianum* (Top) and *Pythium ultimum* (Bottom). The clear zones on the plates against pink colour resulting from INT show inhibition of fungal growth by the compounds in the extracts. Lanes from left to right are extracts of plants species (1) *Ipomoea alba*, (2) *Tecoma stans*, (3) *Passiflora suberosa*, (4) *Passiflora subpeltata*, (5) *Chromolaena odorata*, (6) *Aristolochia gigantea* and (7) *Solanum seafortianum*.

The *T. stans* extract was active with a clear zone on TLC bioautograms of all the tested organisms indicating growth inhibition. The other extracts had no growth inhibition in most

tested organisms, however some growth inhibition was observed in the two tested organism in **Fig 2.3**. The clear zone of these extracts except *T. stans* diminish very quick and this could be due to volatility of active compounds.

The R_f value which is the distance from baseline (point of application) to the centre of a zone of a visible compound divided by the distance from the baseline to the solvent front was determined. The degree of inhibition by *T. stans* extract was higher with an active band of R_f value of 0.082 in BEA mobile system. The volatility of some compounds and poor separation of active constituents by CEF and EMW mobile phases made it difficult to detect of R_f values of active bands in these mobile phases.

Table 2.1: Average MIC values in $\mu\text{g/ml}$ of acetone extracts of different plant species.

1-*Passiflora subeltata*, 2-*Chromolaena odorata*, 3-*Passiflora suberosa*, 4-*Ipomoea alba*, 5-*Aristolochia gigantea*, 6-*Solanum seforthianum*, 7-*Tecoma stans*.

Microorganisms	Average MIC values ($\mu\text{g/ml}$) recorded after 24hrs incubation.								Amphotericin B ($\mu\text{g/ml}$)
	1	2	3	4	5	6	7	Average	
<i>A. parasiticus</i>	160	160	80	640	160	160	1880	460	0.31
<i>R. solani</i>	60	60	40	40	30	40	60	50	20
<i>A. niger</i>	80	160	160	640	640	640	640	420	0.31
<i>F. oxysporium</i>	40	80	160	240	80	80	240	130	7.5
<i>P. janthinellum</i>	320	80	80	640	160	160	160	230	40
<i>P. nicotiana</i>	40	80	80	2500	160	320	640	550	10
<i>P. ultimum</i>	20	20	20	240	20	120	120	80	25
<i>P. expansum</i>	80	60	80	210	80	80	640	180	40
Total average	100	90	90	640	170	200	550		17.8

2.3.5 Microdilution assay

The lower the MIC value is, the more active the extract is. The extracts of *C. odorata* and *P. suberosa* had promising antifungal activity with a low average MIC value of 90 $\mu\text{g/ml}$ for both extracts compared to the other species extracts tested (**Table 2.2**). *P. subpeltata* extract

was relatively active with an average MIC value of 100 µg/ml. *C. odorata* and *P. suberosa* extracts had good antifungal activity with a lower average MIC value of 90 µg/ml but growth inhibition of the fungi by the extracts was not observed on the bioautography results (**Fig 2.3**).

Lack of activity of extracts of these plants species in bioautography could be due to evaporation of active compounds during removal of the TLC eluents or disruption of synergism between active constituents caused by TLC (Masoko and Eloff, 2005). *T. stans* extracts had a total average MIC value of 550 µg/ml which is not considered very active, however fungal growth inhibition on bioautography was observed. The low activity of *T. stans* extracts in MIC may be due to lack of synergistic effects of other compounds in the extract possibly due to lack of antifungal activity. In most cases good activity of the extracts may have been caused by synergistic effects of other compounds in the extracts because single compounds isolated from the same extract did not give good activity when the extracts were subjected to bioautography. There was no inhibition of growth in wells of acetone used as our solvent blank which means, acetone did not have effect on the tested organisms, proving it good solvent system for bioassays confirming published data (Eloff, *et al.*, 2007). Amphotericin B was used as positive control and had average MIC value of 17.8 µg/ml making it much more active than the tested extracts. *A. parasiticus* and *P. nicotiana* were most resistant against the plant extracts with MIC values of 460 µg/ml and 550 µg/ml while *R. solani* and *P. ultimum* were the most sensitive with MIC values of 50 µg/ml and 80 µg/ml respectively.

To compare the total activity of the extracts, the quantity extracted from the plant should be taken into account (Eloff, 2000; 2004). Total activity calculated by dividing the mass in mg of the extracts by MIC in mg/ml determines to what volume the extract can be diluted and still kills the organisms (**Table 2.3**).

Table 2.3: Total activity of the total average MIC values of the tested extracts of different plants species.

Plants species extracts	Mass in mg	Average MIC in mg/ml	Total activity in ml
1	80	0.09	889
2	80	0.64	125
3	60	0.09	667
4	70	0.1	700
5	60	0.55	109
6	70	0.17	412
7	80	0.2	400

1-*C. odorata*, 2-*I. alba*, 3-*P. suberosa*, 4-*P. subpeltata*, 5-*T. stans*, 6-*A. gigantea*, 7-*S. seaforthianum*.

C. odorata and *P. suberosa* had the highest total activity of 889 ml and 667 ml respectively, which means the extract can be diluted with 889 ml and 667 ml of solvent and still kill the organisms. *T. stans* which indicated good activity on bioautograms had total activity of 109 ml which is not bad considering it was obtained 60 mg quantity extracted. This total activity value of 109 ml is reasonable for the plant to be investigated further.

2.4 Conclusions

Bioautography worked well with *T. stans* extracts indicating growth inhibition by one compound and other plant species had little or no inhibition based on bioautography possibly due to volatile compounds or disruption of synergistic active components. *T. stans* was selected for further work of isolation because its active compounds on TLC bioautograms which is a key technique in bio-assay fractionation. It would therefore enable isolation of active compounds observed on bioautograms. Moreover *T. stans* plant material was easily accessible to us, so since isolation of compounds require extraction of large quantities of material; I had to focus on active and accessible plant species.

Chapter 3

Isolation of bioactive compounds from *Tecoma stans*

3.1 Introduction

Bioactive compounds can be isolated from plants by bioassay-guided fractionation. This procedure employs various screening methods that locate the desired activities in the crude extracts and in fractions eluted from the different separation steps (Hostettmann *et al.*, 2000). TLC is vital for the isolation of natural products since is regarded reproducible and accurate, and is appropriate for bioautography techniques (Wen *et al.*, 2004; Iscan *et al.*, 2002). About 250 000 living plants species consists of greater diversity of bioactive compounds than any chemical library created by man (Raskin *et al.*, 2002), therefore isolation of bioactive compounds from *T. stans* has rational basis.

Tecoma stans also known as yellow elder is a member of the family Bignoniaceae. This tropical tree is found predominantly in Central and South America, although, its native environment extends from the southern states of the U.S. to north of Argentina. However, *T. stans* has also become established in other tropical and subtropical areas such as Africa, Asia, Pacific islands and Australia. Certain tropical countries have reported it being invasive and difficult to control. *T. stans* is a drought tolerant shrub and is relatively resistant to pests. The tree has green leaves of opposite configuration with blade approximately 5 cm to 8 cm in length and narrow, serrated and sharply pointed. The flowers are 2.5 cm to 5 cm long, bell shaped and yellow in colour (**Fig 3.1**) ([http://cloudbridge.org/trees/tecoma stans.html](http://cloudbridge.org/trees/tecoma_stans.html)).



Fig 3.1: Photo of *Tecoma stans* tree showing its green leaves and bright yellow blooms (Photograph JN Eloff).

The bright yellow flowers have made the species a popular ornamental tree throughout the tropics. In addition to its popular ornamental purposes the leaves, bark and roots have been used for various medicinal purposes in herbal medicine. (<http://cloudbridge.org/trees/tecoma-stans.html>). The control of diabetes is the most mentioned traditional use of the plant, and is said to be used traditionally in Mexico to control diabetes (Luca *et al.*, 2003). The plant leaf extract reduces blood sugar of fasting rabbits (Hammouda *et al.*, 1964). The plant contains monoterpene alkaloids and two of them (tecomine and tecostanine) are responsible for the hypoglycaemic activity of the plant (Hammouda and Amer, 1966)., The antimicrobial activity of a *T. stans* extract was determined on fungi and a wide range of Gram-positive and Gram-negative bacteria. The methanol extract of *T. stans* leaves was active only against *Candida albicans* (Binutu and Lajubutu, 1994). There is therefore still large scope for a study on the activity of different extracts of *T. stans* leaves against fungal plant pathogens.

3.2 Materials and Methods

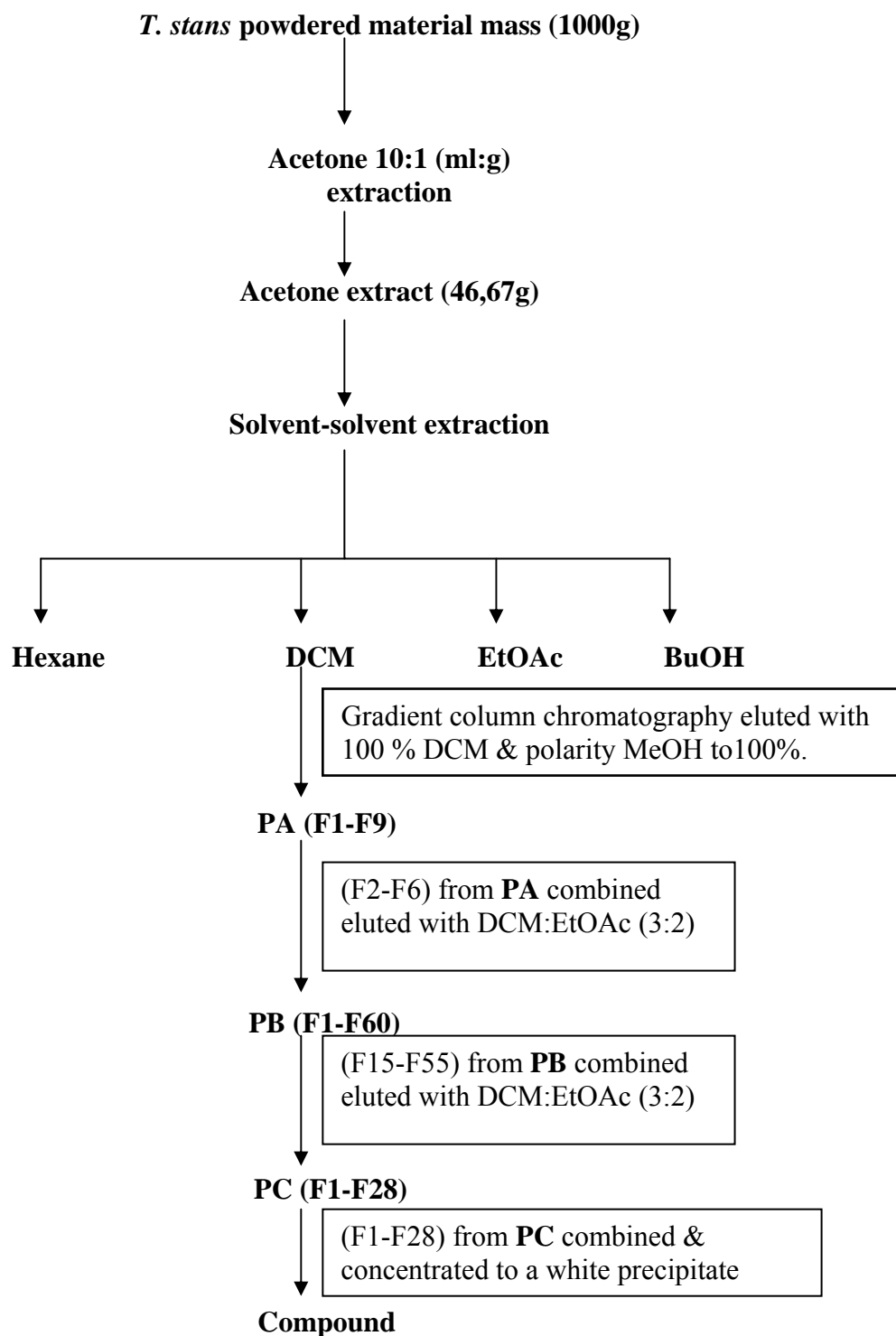


Fig 3.2: Schematic representation of isolation procedure of the active compound from *T. stans*. **PA**, **PB** and **PC** designate fraction issued from columns.

3.2.1 Extraction procedure.

One Kg of *T. stans* powdered leaves was exhaustively extracted with acetone 3 times and dried under a stream of air in a fume cupboard at room temperature. The extract was stored in pre-weighed labelled containers to quantify extraction.

3.2.2 Solvent-solvent fractionation of crude extract.

Solvent-solvent extraction is one of the popular techniques used in the preparation of samples for qualitative and quantitative analysis. It is a process of separating one constituent from a mixture by dissolving it into a solvent, in which it is soluble while the other constituents of the mixture are not, or are at least less soluble (Holden, 1999). The acetone crude extract of *T. stans* was separately suspended in methanol and 20% distilled water in separatory funnel (2000 ml) and successively partitioned with hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc) and n-butanol (BuOH) respectively in this order. These solvents have varying polarities to extract different plant's constituents of the plant with different polarities. These afford four partition fractions of each plant extract which were subjected to pilot work in TLC fingerprints and bioautograms, and microdilution activity assay.

3.2.3 General isolation procedure (column chromatography).

Open columns were be packed with silica gel 60 to fractionate the fractions obtained from the solvent-solvent fractionation to pure or semi pure compounds. Solvents system developed from the TLC were used as the mobile phase for column chromatography. Silica gel 60 was used in a 100:1 gram ratio to the analysis of sample. Silica gel 60 was measured, poured into a beaker with a relevant packing eluent and stirred to remove air bubbles. Bubbles were removed further by placing the beaker in an ultrasonic bath. The columns were firmly clamped vertically and the suspension carefully poured into the column, while tapping the wall of the column gently to ensure air bubbles rise to the top and for good packing of silica gel. The mobile phase was left to run until the level of mobile phase is just above the stationary phase. A mixture of extract with small quantity of silica gel was placed between filter papers, one on top of the gel and the other on the extract and gel. A small quantity of clean cotton wool was placed on top of the filter paper to make sure there is no disturbance on the surface when solvent was poured into the column. Fractions were collected under

gravitational force at c. 3 ml/min in test tubes. The tubes were placed in a fume cupboard and concentrated under a stream of air for bioassay and further TLC. The isolated compounds were purified by recrystallization in different solvents of varying polarity.

3.2.4 Stepwise isolation process of bioactive compound(s) from *T. stans*.

3.2.4.1 Isolation of active compound(s) from DCM extract.

Silica gel 60 (Merck) was mixed with DCM to form a slurry and packed to a height of 42 cm in a glass column of 48 cm x 10 cm. DCM extract was dissolved in a small volume of DCM, mixed with silica gel 60 (Merck), allowed to dry under a stream of cold air and loaded on top of the packed column. The column was eluted with 100% DCM and subsequently, the polarity of the eluting solvent was increased with methanol (MeOH). A volume of 1000 ml of 100% DCM was initially used followed by solvent mixture of 10% MeOH/DCM, 20% MeOH/DCM, 30% MeOH/DCM, 40% MeOH/DCM, 50% MeOH/DCM, 60% MeOH/DCM, 80% MeOH/DCM, and finally the column was eluted with 100% MeOH. The fractions were labelled F1 to F9. Fractions of 1000 ml were collected, concentrated using a rotary evaporator at 40°C, and transferred to preweighed glass vials to dry completely under a stream of air. The dried fractions were resuspended to 10 mg/ml in chloroform (CHCl₃) and 100 µg (10 µl) was loaded onto each of six 10 cm x 20 cm TLC plates. Two plates were each developed in BEA, CEF and EMW. Chromatograms eluted with each of the three solvent systems were sprayed with vanillin-sulphuric acid and served as reference chromatograms, and the remaining three plates were sprayed with *A. parasiticus* suspensions for bioautographic assay.

3.2.4.1a Column separation of Fractions F2-F6

Silica gel 60 was mixed with DCM: EtOAc (3:2) and packed in 42 cm x 5 cm column. The fractions F2 to F6 from the first column were combined, dissolved in small volume of CHCl₃ and mixed with silica gel 60, dried and loaded on top of the packed column. The column was eluted with DCM:EtOAc (3:2). Fractions of 1ml were collected.

3.2.4.1b Column separation of Fractions F15-F35 and F35-F40

The procedure above in **Section 3.2.4.1a** was used in 38 cm x 3.5 cm and 49 cm x 2.8 cm columns, with fractions F15 to F35 and F40 to F50 from the second column loaded on top of the packed columns. The columns were eluted with DCM:EtOAc (3:2). Fractions of 1ml were collected.

3.2.4.2 Phytochemical analysis and bioautography

Similar fractions obtained from the columns in **Section 3.2.4.1b** were combined, loaded on TLC plates and developed in an appropriate mobile phase and sprayed with vanillin spray reagent heated in an oven at 110 °C until colour development to visualize the single band depicting the antifungal compound. To confirm isolation of the antifungal compound, bioautography was carried out on the isolated compound and the DCM crude extract.

3.2.5 Nuclear Magnetic Resonance Spectroscopy

Nuclear Magnetic Resonance Spectroscopy (¹H NMR and ¹³C NMR) was carried out to confirm the structure of the compound using a Bruker 300 MHz spectrometer. The isolated compound from *T. stans* was dried, weighed (15-20 mg) and dissolved in c. 2 ml of chloroform into NMR tubes using a clean Pasteur pipette and analyzed in the Department of Chemistry, University of Botswana.

3.3 Results and discussion

3.3.1 Quantity of *T. stans* extracted using solvent-solvent extraction

A quantity of 46.67g of serially extracted acetone extracts of *T. stans* leaves powder was subjected to solvent-solvent extraction and afforded four fraction of increasing polarity *viz* Hexane, DCM, EtOAc and BuOH. Hexane was the best extractant in terms of quantity extracted with the quantity of 46.23% (21.58g) followed by DCM with 16.75% (7.82g), EtOAc with 1.79% (0.84g) and BuOH with 0.64% (0.3g) respectively.

3.3.2 TLC fingerprints and bioautograms

TLC fingerprint of *T. stans* fractions developed in BEA are shown **Fig 3.3**. TLC plates were developed in BEA, CEF and EMW mobile phases as outlined in **Section 2.2.4**. BEA was the best mobile phase yielding a good separation of several non-polar compounds. Hexane and DCM fractions were separated into several compounds by BEA, but understandably not in the polar CEF and EMW systems. No compounds were separated from the EtOAc and Butanol fractions by the non-polar BEA solvent system.

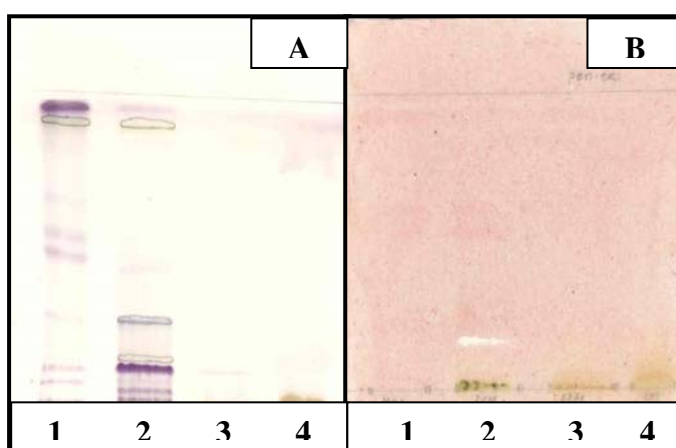


Fig 3.3: TLC of fractions of *T. stans* acetone extract after solvent-solvent extraction.

[Fractions from **left to right**: Hexane (1), DCM (2), EtOAc (3) and Butanol (4)]. TLC plates were developed in BEA and sprayed with vanillin spray reagent (A) and sprayed with *A. Parasiticus* (B).

TLC bioautograms of *T. stans* were developed against *A. parasiticus*. TLC plates were developed in three mobile systems (BEA, CEF and EMW). BEA was the best solvent system again, with the DCM fraction indicating activity as shown in **Fig 3.3 (B)**. The clear zone indicates inhibition of *A. parasiticus* growth since in this area INT was not reduced to the red product by actively growing organisms. Bioautograms developed in CEF and EMW did not indicate activity by showing clear zones because they did not separate components in the DCM fraction well. The bad separation could have resulted in masking the clear area with the colour of the non separated compounds. These results are consistent with the screening results since *T. stans* acetone extract showed activity on BEA TLC bioautograms and no activity in CEF and EMW due to disruption of active constituents from these two polar mobile systems. Microdilution assay results supported the TLC bioautograms with DCM

extract having good activity against *A. parasiticus* and *P. expansum* with an MIC value of 80 $\mu\text{g/ml}$ and 20 $\mu\text{g/ml}$ respectively. The activity by the DCM fraction led to its selection for isolation of active compounds via bio-assay guided fractionation.

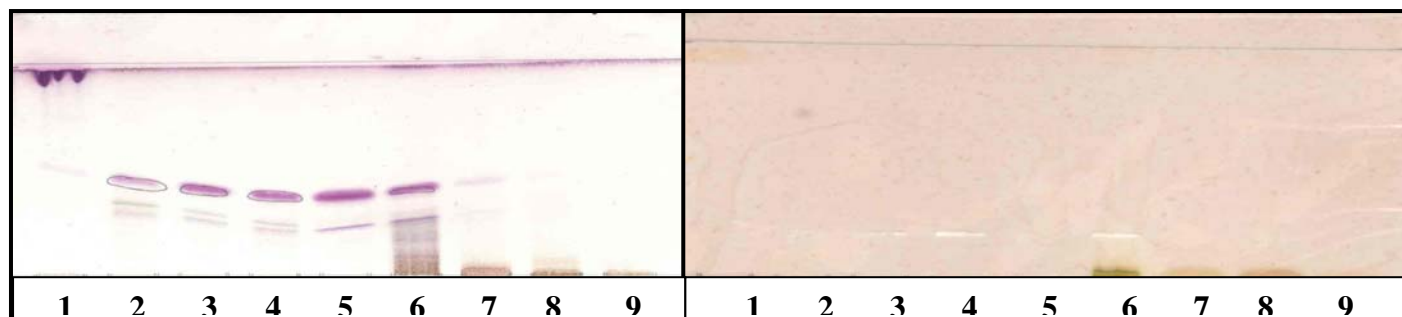


Fig 3.4: TLC of fractions of the dichloromethane extract after open column chromatography. [Fractions from left to right were eluted with: 100% DCM (1), 10% MeOH/DCM (2), 20% MeOH/DCM (3), 40% MeOH/DCM (4), 50% MeOH/DCM (5), 60% MeOH/DCM (6), 70% MeOH/DCM (7), 80% MeOH/DCM (8) and 100% MeOH (9)]. TLC plates were developed in BEA and sprayed with vanillin sulphuric acid spray reagent (A) and sprayed with *A. parasiticus* (B).

Fractions F1-F6 in **Fig 3.4** that had activity on bioautograms were combined and yielded impure precipitate which was subjected to second open column chromatography (PA). Fractions (F1-F60) obtained from (PB) were loaded onto 10 cm x 20 cm TLC plates and developed in DCM:EtOAc (3:2) as shown in **Fig 3.5**. The developed fractions were sprayed with vanillin spray reagent and heated in an oven at 110°C until optimal colour development.

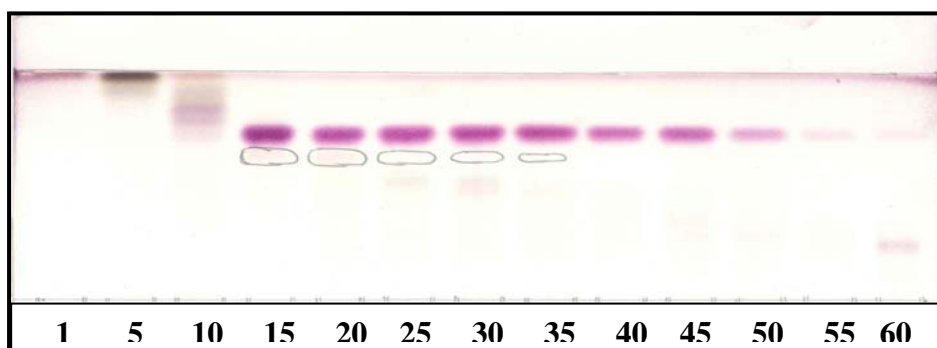


Fig 3.5: Fractions (F1-F60) of PB developed in DCM:EtOAc (3:2), sprayed with vanillin spray reagent.

Fractions of **(PB)** F15-F55 were combined and yielded impure precipitate which was subjected to third open column **PC**. Fractions (F1-F28) eluted from **PC** were loaded 10 cm x 10 cm on TLC plates and developed in DCM:EtOAc (3:2) as shown in **Fig 3.6**. The developed fractions were sprayed with vanillin spray reagent and heated in an oven at 110°C until optimal colour development.

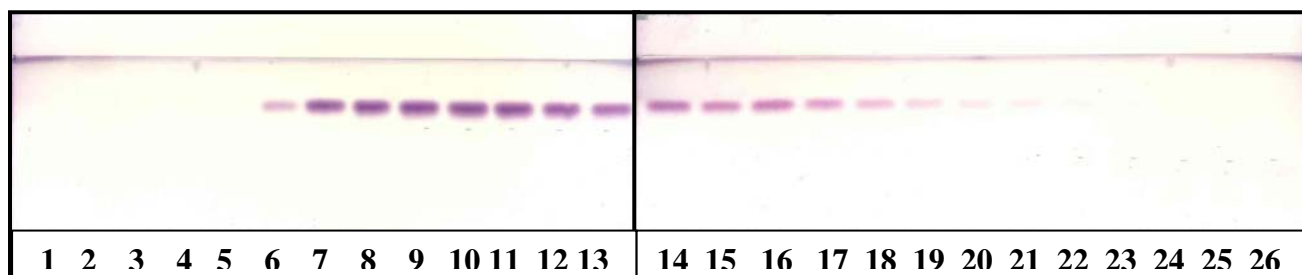


Fig 3.6: Fractions of **PC** developed in DCM:EtOAc (3:2), sprayed with vanillin spray reagent.

Fractions (F6-F21) eluted from **PC** were combined and yielded a white precipitate. To confirm the activity of the isolated compound, bioautography was undertaken by profiling the combined fractions on TLC plate simultaneously with the DCM extract which the column fractions were obtained and sprayed with *P. janthelium* suspension (**Fig 3.7**).

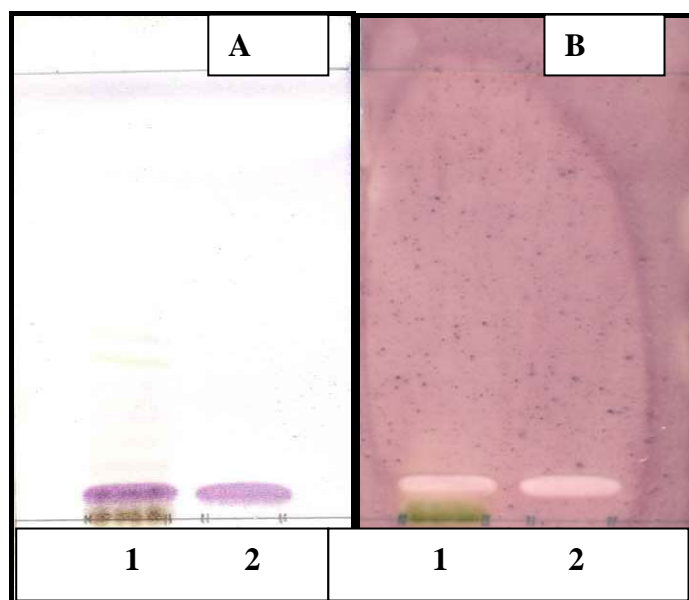


Fig 3.7: TLC plates loaded with DCM extract and compound isolated from the extract, sprayed with vanillin sulphuric acid spray reagent (**A**) and fungal suspension (**B**). From left to right (1) represent crude extract and (2) represent isolated compound.

R_f value helps in dereplicating isolation of antifungal compounds from other plants species. The R_f value of the extract and isolated compound was 0.081 in BEA (**Fig 3.7**). This confirms that compound isolated was probably the antifungal compound present in the crude extract.

3.3.3 Identification of compound.

The compound was isolated as a white precipitate (**Fig 3.7 (A)**). NMR spectroscopy identified the compound as oleanolic acid, see (**Fig 3.8**) and (**Appendix A**). Oleanolic acid is a triterpenoid compound present in the leaves and bark of many plant species. This isomer of ursolic acid has been recognized to have hepatoprotective, anti-inflammatory, and anti-hyperlipidemic properties; and have recently been noted for antitumor promotion effects. It has been marketed as an oral drug in China for human liver disorders ([www.alibaba.com/product-gs/203129384/Oleanolic acid.html](http://www.alibaba.com/product-gs/203129384/Oleanolic%20acid.html)). The compound is well known but literature reveals little about its antifungal activity and whether it was isolated from *Tecoma stans* before.

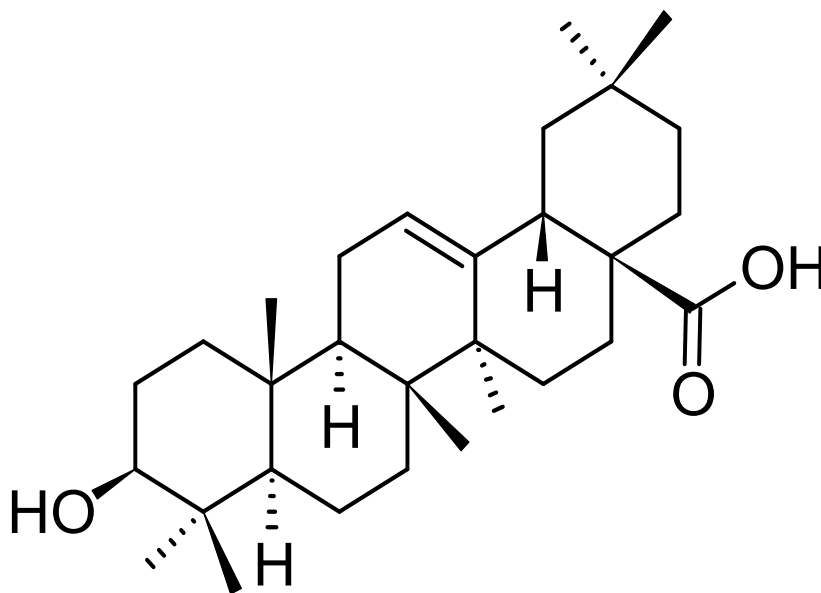


Fig 3.8: Structure of oleanolic acid isolated from *T. stans* DCM extract.

3.3.4 Conclusions

Solvent-solvent extraction and 100% DCM column elution of the DCM extract helped to remove inactive components. The DCM fraction from solvent-solvent fractionation was the most active containing a single active compound based on bioautography. The isolation of active compound worked well and resulted in one active compound oleanolic acid being characterized.

In the next chapter the biological activity against several fungi and bacteria as well as the cytotoxicity will be determined.

Chapter 4

Biological activity of DCM extract and the isolated oleanolic acid

4.1 Introduction

Many plant extracts and their active single compounds are biologically active. The Phytomedicine Programme at the University of Pretoria [www.up.ac.za/phyto] has worked on biological activity of many plant extracts and their compounds. It has happened frequently that known compounds were isolated but the biological activity of the isolated compound was not known. The biological activity on other organisms was therefore also determined. The general idea that plant extracts are safe to use is wrong in many cases. Therefore, studying the toxicity of the plant extracts and isolated compounds is very important.

4.2 Materials and Methods

4.2.1 Bioautography

TLC plates were prepared and loaded with extract and oleanolic acid concentrated to a final concentration of 10 mg/ml and 1.0 mg/ml respectively. Ten microlitres was applied and the plates were developed in mobile phase, dried and sprayed with fungal organisms as described in **Section 2.2.9.2**.

4.2.2 Microdilution assay

The microdilution method of Eloff (1998c) modified by Masoko *et al.*, (2005) was used to determine the MIC values of *T. stans* extracts and oleanolic acid against 10 plant pathogenic fungi viz *Rhizoctonia solani*, *Fusarium oxysporium*, *Penicillium janthinellum*, *Penicillium expansum*, *Aspergillus Parasiticus*, *Aspergillus niger* *Collectotrichum glaeosporiodes*, *Trichoderma harzianum*, *Pythium ultimum* and *Phytophthora nicotiana*. This method is detailed in **Section 2.2.9.1**.

4.2.3 Cytotoxicity assay

The samples were tested for cytotoxicity against the Vero monkey kidney cell line (Department of Veterinary Tropical Disease, University of Pretoria). The cells were maintained in minimal essential medium (MEM, Highveld Biological, Johannesburg, South Africa) supplemented with 0.1 % gentamicin (Virbac) and 5 % foetal calf serum (Adcock-Ingram). To prepare the cells for the assay, cell suspensions were prepared from confluent monolayer cultures and plated at a density of 0.5×10^3 cells into each well of a 96-well microtitre plate. After overnight incubation at 37 °C in a 5% CO₂ incubator, the subconfluent cells in the microtitre plate were used in the cytotoxicity assay. Stock solutions of the plant extract were prepared in growth medium (1-1000 µg/ml). The viable cell growth after 120 h incubation with plant extracts/samples was determined using the tetrazolium based colorimetric assay (MTT assay) described by Mosmann (1983). The absorbance was measured on a Titertek Multiscan MCC/340 microplate reader at 540 nm test wavelength and reference wavelength of 690 nm. Berberine chloride (Sigma Chemical Company) was used as a positive control. Tests were carried out in quadruplicate and each experiment was repeated three times

4.3 Results and discussion

4.3.1 TLC bioautograms

In isolating bioactive compounds from plant extracts it is always possible that the major biologically active compounds may be inactivated during the isolation process and that a minor compound may be isolated in the end. To examine this possibility bioautography was carried out on the crude extract and the isolated oleanolic acid.

The bioautography results (**Fig 4.1**) indicate inhibition of fungal growth by the extract and oleanolic acid by compounds with the same R_f value, by showing a clear zone which resulted from INT that was not reduced to the red product by actively growing organisms. The two identical bands observed in the chromatography of the extract and oleanolic acid shows that the active compound present in the extract was isolated because the active bands had the same R_f value of 0.08 in BEA confirming the isolation of the main active compound present in the crude extract.

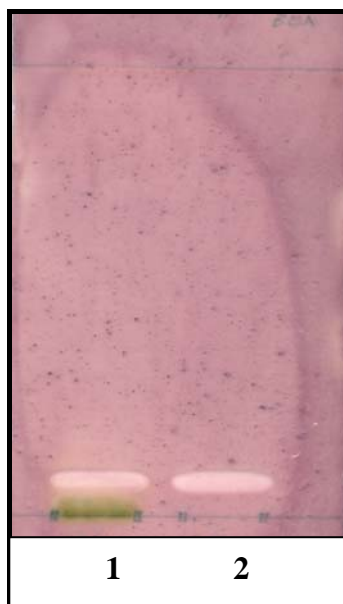


Fig 4.1: TLC bioautograms of *T. stans* crude extract (1) and oleanolic acid (2) sprayed with *P. expansum* cell suspension.

4.3.2 Microdilution assay

To evaluate the degree to which the activity has increased by isolating oleanolic acid and the sensitivity towards different fungal pathogens, microdilution assays were carried out against several pathogens.

Table 4.1: MIC values in $\mu\text{g/ml}$ of *T. stans* DCM fraction and oleanolic acid tested against 10 plant pathological fungi.

Microorganisms	Average MIC values ($\mu\text{g/ml}$)			
	<i>T. stans</i> crude	Oleanolic acid	Average	Amphotericin B ($\mu\text{g/ml}$)
<i>C. glaeosporioides</i>	310	250	280	0.31
<i>A. parasiticus</i>	160	250	210	0.78
<i>A. niger</i>	120	250	190	0.31
<i>P. nicotiana</i>	120	190	150	7.5
<i>P. janthinellum</i>	470	250	360	40
<i>T. harzianum</i>	20	8	10	7.5
<i>R. solani</i>	20	2	10	25
<i>F. oxysporium</i>	20	50	40	20
<i>P. expansum</i>	20	20	20	40
<i>P. ultimum</i>	60	16	40	40
Average	130	130		18.1

It was a major surprise that the *T. stans* extract and oleanolic acid had the same average MIC value of 130 $\mu\text{g/ml}$ against 10 tested fungal pathogens, (**Table 4.1**). However different

microorganisms responded differently to the extract and the isolated compound. *P. janthinellum* and *C. glaeosporioides* were relatively resistant and *T. harzianum*, *R. Solani*, *F. oxysporium* and *P. expansum* were relatively sensitive. The extract had good activity against some fungi with MIC values of 20 µg/ml and 60 µg/ml.

Because the activity of the extract was comparable with the activity of the compound, it means that despite removing in the order of 99% of “supposedly inactives” from the crude extract based on bioautography, the activity did not increase 100 fold.

The most probable explanation for this is that there must be a large degree of synergism taking place. The other compounds acting synergistically were not active after they were separated from other compounds during bioautography. An alternative explanation is that other volatile compounds may have been present in the crude extract and that these evaporated during the long period of removing the solvent from the chromatograms before spraying with the fungi. Alternatively some antifungal compounds may have been destroyed during the isolation process.

Amphotericin B was active with an average MIC value of 18 µg/ml but in some cases against certain fungi the crude extract had a higher activity than amphotericin B. This points to the possible use of crude extracts in protecting plants against fungi.

To confirm the conclusion reached in the previous paragraph that there must be substantial activity residing in compounds outside oleanolic acid an analysis of the total activity of the crude extract and the isolated compound can be made (Eloff, 2004) (**Table 4.2**). This takes into account not only the antimicrobial activity but also the quantity. If all the activity is presented by the isolated compound, the two values would be the same. The value for the extract was 6.5 times higher than that of the isolated compound. Even if only 50% of the active compound was isolated in the end with the rest residing in impure fractions, this only represents a third of the activity present in the crude. There can be two explanations. The other active compounds were decomposed or inactivated during the isolation procedure. Alternatively and more likely because there was only one antifungal compound present in the crude based on bioautography more than one compound played a role in determining the antifungal activity through synergism.

Table 4.2: Total activity of the *T. stans* DCM extracts and oleanolic acid against the ten fungal pathogens.

Samples	Mass in mg	Average MIC in mg/ml	Total activity in ml
DCM extract	7820	0.13	60154
Oleanolic acid	1204	0.13	9262

4.3.3 Cell viability and cytotoxicity

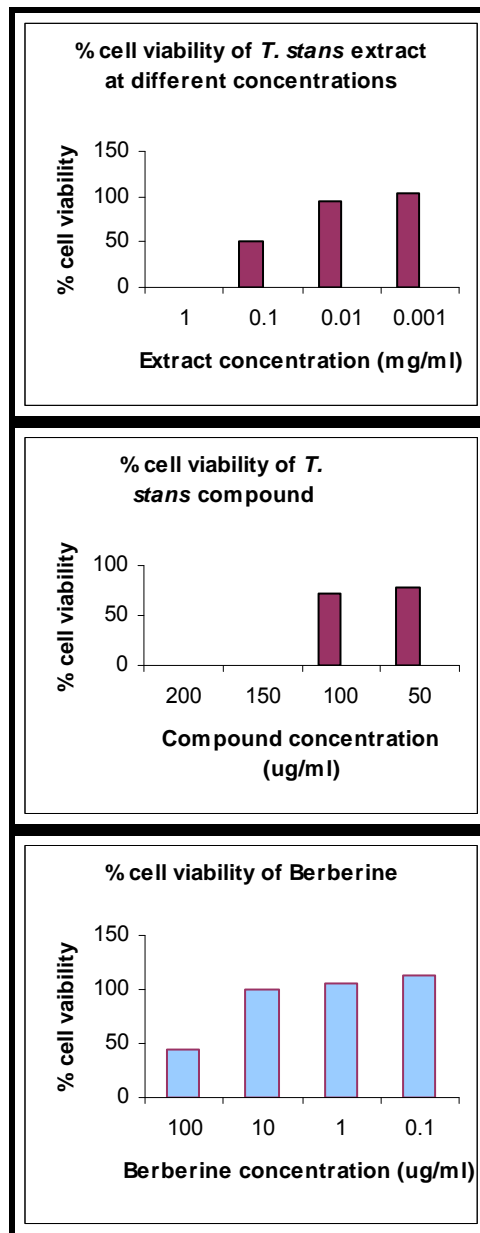


Fig 4.2: Cytotoxicity cell viability graphs of extracts, compound (oleanolic acid) and positive control. Graphs from top to bottom are: *T. stans* extract (top), *T. stans* compound (centre) and berberine as control (bottom).

Cellular cytotoxicity of the *T. stans* DCM extract and compound were determined with the toxic alkaloid berberine as positive control. The percentage cell viability increased with a decrease in concentrations of DCM extract, compound and berberine (**Fig 4.2**). The highest concentration of both DCM extract and compound killed all the Vero cells at concentrations higher than 150µg/ml. Berberine was toxic at concentrations higher than 10 µg/ml.

Cellular toxicity can also be expressed by the LC₅₀ values calculated from the regression curve. *T. stans* DCM extract had an LC₅₀ 0.413 mg/ml, the compound 0.129 mg/ml and berberine 15.48 mg/ml which means that the crude extract and oleanolic acid were more toxic than berberine. The compound was more toxic to Vero cells than the extract.

4.4 Therapeutic index

Practically all antifungal compounds are toxic because of much closer metabolic pathways between fungi and mammals than between bacteria and mammals. There are therefore fewer specific targets that can be addressed. The important question is how the toxicity to the target organism relates to the cellular toxicity. The therapeutic index can be defined here as the LC₅₀ in (µg/ml)/MIC in (µg/ml). The higher this value is, the safer the product is to use under controlled conditions (Table 4.3).

Table 4.3: Therapeutic index of crude and oleanolic acid against ten fungi

Microorganisms	Crude extract LC ₅₀ (µg/ml)	Crude extract MIC (µg/ml)	Therapeutic index (Crude extract)	Oleanolic acid LC ₅₀ (µg/ml)	Oleanolic acid MIC (µg/ml)	Therapeutic index (oleanolic acid)
<i>C. glaeosporiodes</i>	413	310	1	129	250	0.5
<i>A. parasiticus</i>	413	160	2.5	129	250	0.5
<i>A. niger</i>	413	120	3	129	250	0.5
<i>P. nicotiana</i>	413	120	3	129	190	0.7
<i>P. janthinellum</i>	413	470	0.9	129	250	0.5
<i>T. harzianum</i>	413	20	21	129	8	16
<i>R. solani</i>	413	20	21	129	2	64
<i>F. oxysporium</i>	413	20	21	129	50	2.6
<i>P. expansum</i>	413	20	21	129	20	6.5
<i>P. ultimum</i>	413	60	7	129	16	8

The crude extract had a high therapeutic index value of 21 against microorganisms *T. harzianum*, *R. solani*, *F. oxysporium* and *P. expansum* which means crude extracts can be used against infection of this fungi and pose little toxicity if used under controlled conditions. It is interesting to note that *P. expansum* which is a major problem in fruit spoilage can be controlled by the crude extract at a dosage level with low toxicity. *Fusarium spp.* which can be a big problem in maize contamination and damping off of seedlings can also be controlled with little toxicity.

The high therapeutic index values of 16 and 64 of oleanolic acid against *T. harzianum* and *R. solani* respectively, stood out as the most promising for the use of oleanolic acid against infections caused by these two fungi at the correct dose.

4.5 Conclusions

It appears that there may be greater potential in using plant extracts than the isolated oleanolic acid. There were major differences in the sensitivity of the different pathogens to the crude extract. This may be valuable because it indicates that the activity is on a basic metabolic target present in all the fungi.

Because many antifungal agents are also highly toxic it was important to determine the cellular toxicity and the therapeutic index. Under controlled conditions the crude extract and oleanolic acid can be used against infections caused by some pathogens with high therapeutic index value therefore posing relatively low toxicity threats. In the case of other pathogens neither the crude extract nor oleanolic acid would be of any potential use due to its toxicity. One should however keep in mind that cellular toxicity does not necessarily correspond to toxicity via other routes. If the toxic compound is ingested and it is destroyed at the low gut pH, or not taken up from the gut or rapidly detoxified, the toxicity may be much lower. It is also possibly that a more toxic compound could be after uptake of the extract stressing the importance of *in vivo* animal experiments on toxicity. This is however beyond the scope of this dissertation.

Chapter 5

In vitro antibacterial and antifungal activity of oleanolic acid against animal bacterial and fungal organisms

5.1 Introduction

As an extension to biological activity work, the antimicrobial activity of the compound was also determined against animal pathogens. The increasing resistance of bacterial isolates for antibiotics has spurred search for alternative antimicrobial components (Berkowitz, 1995). Many naturally occurring compounds found in plants have antimicrobial potential and could be a source of antimicrobial agents (Deans and Ritchie, 1987). If readily available invasive weeds have potential as antimicrobial agent, then the problem of antimicrobial agents' resistance and scarcity may be alleviated. This chapter is aimed at determining the usefulness of the isolated compound with regard to antimicrobial activity against other target organisms.

5.2 Materials and Methods

5.2.1 TLC bioautograms

Aliquots (10 µl) of 1 mg/ml solution in acetone (equivalent to 10 µg) of oleanolic acid were loaded on 3 x 10 cm TLC plates. TLC plates were prepared as described in **Section 2.2.9.2**. Three fungal species (*Candida albicans*, *Cryptococcus neoformans* and *Sporothrix schenckii*), two Gram-positive bacterial species (*Staphylococcus aureus*, ATCC 29213 and *Enterococcus faecalis*, ATCC 29212) and two Gram-negative bacterial species (*Escherichia coli*, ATCC 27853 and *Pseudomonas aeruginosa*, ATCC 25922) were used. Bacteria were cultured in Müller-Hinton (MH) broth at 37 °C and maintained on MH agar at 4 °C. Developed plates were sprayed with actively growing suspension of the above mentioned organisms and incubated overnight at 37 °C.

5.2.2 Microdilution assay

5.2.2.1 Antifungal assay

Aliquots (100 µl) of 1 mg/ml solution of oleanolic acid in acetone were serially diluted with distilled water in 96-well microtitre plates and the serial dilution assay was conducted as

described in **Section 2.2.9.1**. Sabouraud Dextrose (SD) broth was used to dilute the culture organisms.

5.2.2.2 Antibacterial assay

The assay was conducted as described by Eloff (1998c). The method is basically similar to the one described in **Section 2.2.9.1**, the difference is that the colour indicator INT (0.2 mg/ml) was added after overnight incubation of compounds with bacteria. Overnight cultures of the bacteria were diluted 1:100 with fresh Müller-Hinton (MH) broth before use in the assay.

5.3 Results and discussion

5.3.1 TLC Bioautograms

Oleanolic acid had antimicrobial activity on bioautograms against the fungal and bacterial organisms. The clear zones on the plate indicate fungal or bacterial inhibition by oleanolic acid (**Fig 5.1**). The activity was non selective because there were clear zones against the three tested fungi and four tested bacteria.

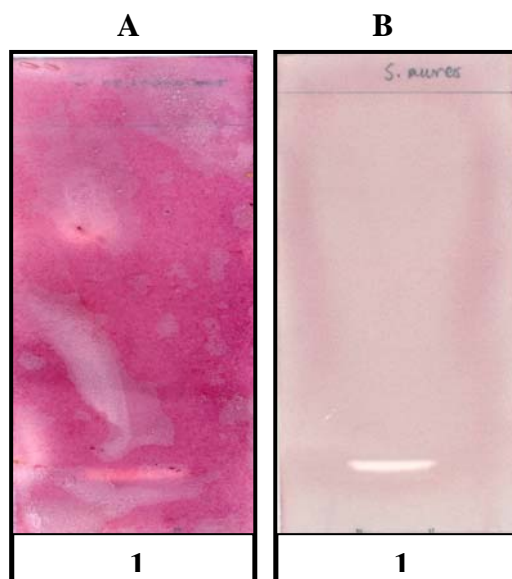


Fig 5.1: TLC bioautograms of oleanolic acid sprayed with fungus *C. neoformans* (A) and (B) sprayed with bacteria *S. aureus*.

5.3.2 Microdilution (Antifungal assays)

The MIC values for the three human fungal pathogens after 24 and 48 hrs are presented in Table 5.1.

Table 5.1: MIC values of oleanolic acid isolated from *T. stans* DCM fraction tested against three animals pathological fungi.

Microorganisms	Average MIC values (µg/ml)			Incubation time
	Oleanolic acid	Average	Amphotericin B (µg/ml)	
<i>C. neoformans</i>	250	250	2.5	24hrs
<i>S. schenckii</i>	90	90	7.5	
<i>C. albicans</i>	250	250	10	
Average	200		6.7	
<i>C. neoformans</i>	250	250	25	48hrs
<i>S. schenckii</i>	180	180	40	
<i>C. albicans</i>	250	250	20	
Average	230		28	

Oleanolic acid had a much lower antifungal activity against the human fungal pathogens with an average MIC value 200 - 230 µg/ml after 24 and 48 hrs than against several of the plant fungal pathogens with values of 20 µg/ml and lower (**Table 5.1**). *C. neoformans* and *C. albicans* were resistant towards the oleanolic acid with an MIC of 250 µg/ml for both in 24 hrs and 48 hrs incubation time. However *S. schenckii* was more sensitive to oleanolic acid with an MIC of 90 µg/ml. The MIC of oleanolic acid against *S. schenckii* increased after 48 hrs to 180 µg/ml, indicating slight fungistatic activity.

Amphotericin B the positive control had a much higher activity than oleanolic acid with an average MIC value of 6.7 µg/ml after 24 hrs and 28 µg/ml after 48 hrs incubation respectively. The response of the fungi towards positive control differed to a degree.

5.3.3 Microdilution (Antibacterial assays)

The antibacterial activity of the oleanolic acid are presented in Table 5.2

Table 5.2: MIC values of oleanolic acid isolated from *T. stans* DCM fraction tested against four animal bacteria.

Microorganisms	Average MIC values (µg/ml)			Incubation time
	Oleanolic acid	Average	Gentamicin (µg/ml)	
<i>E. coli</i>	>250	>250	16	30 min
<i>E. faecalis</i>	>250	>250	16	
<i>S. aureus</i>	>250	>250	16	
<i>P. aeruginosa</i>	>250	>250	12	
Average	>250		15	
<i>E. coli</i>	>250	>250	23.5	24 hrs
<i>E. faecalis</i>	>250	>250	23.5	
<i>S. aureus</i>	>250	>250	12	
<i>P. aeruginosa</i>	>250	>250	16	
Average	>250		18.75	

Oleanolic acid had very little antibacterial activity against both the Gram-positive and Gram-negative strains with an average MIC value greater than 250 µg/ml in all cases. Gentamicin, the positive control had antibacterial activity with an average MIC value of 15 µg/ml and 18.75 µg/ml after 30 min and 24 hrs incubation respectively. The incubation times of 30 min and 24 hrs were preferred because bacterial cultures grow quickly therefore reacting fast to INT the colour indicator.

5.4 Conclusions

It is interesting that although based on bioautography, oleanolic acid in all cases had some effect on all the tested bacteria and animal fungal pathogens tested here, in the presence of other compounds there was no activity at the highest dose tested.

There was very low activity against any of the animal microbial pathogens when mixtures were applied. One is tempted to speculate that in this case there may be inhibition of activity by other compounds present in the crude extract in contrast to the situation with plant fungal pathogens where there may be synergism. Could this be an example of evolutionary pressures yielding different products in plants and in animals?

Chapter 6

Conclusions

6.1 Introduction

The soaring food prices and scarcity is a major problem to most countries particularly developing countries. Compounding this crisis could be food and feed pathogens which could cause catastrophic food loss if left unchallenged. One major cause of these pathogens is the plant pathogenic fungi. The control of these fungi by synthetic fungicide is clouded by lots of drawbacks such as severe human and environmental toxicity effects and high costs. Their high costs make them less accessible particularly by rural farmers in developing countries. These setbacks warrant an alternative source of fungicide. Plants and their secondary metabolites have shown great potential as antifungal source.

Growing and harvesting promising plants would increase the costs substantially. Alien invasive plants and weeds known for their characteristics as aggressive growers could aid in developing a less expensive plant source if explored for antifungal activity. The aim of this study was to investigate weedy and invasive plant species for antifungal activity against plant pathogens in order to develop a useful product. The objectives were: to evaluate seven selected weedy plants species for antifungal activity and select one or two plant species for further study; to isolate the active antifungal compound(s) from the selected plant(s) species; to determine the *in vitro* biological activity of the isolated compound(s); to discuss the potential value of the selected plant species in protecting plants against fungal attack

6.2 Evaluation of selected species for further study

The selected species were evaluated using acetone as an extractant and tested against ten plant pathogenic fungi viz *Rhizoctonia solani*, *Fusarium oxysporium*, *Penicillium janthinellum*, *Penicillium expansum*, *Aspergillus Parasiticus*, *Aspergillus niger*, *Collectotrichum glaeospariodes*, *Trichoderma harzianum*, *Pythium ultimum* and *Phytophthora nicotiana*. *Tecoma stans* was for selected further work based on the presence of clear zones on TLC bioautograms indicating antifungal activity, a reasonable MIC of 550 µg/ml and easy accessibility of plant material. Lack of clear zones of inhibition and limited

access to plant material disqualified several species with higher antifungal activity from further analysis.

6.3 Isolation of active constituent and biological activity determination

The active *T. stans* extract was subjected to a solvent-solvent extraction a process preceding column separation. Solvent-solvent extraction aided in removing inactives and other unwanted components. The DCM extract obtained from solvent-solvent extraction was most active against *A. parasiticus* and *P. expansum* with MIC values of 80 µg/ml and 20 µg/ml respectively, and a clear zone of inhibition on bioautograms. Column chromatographic separation lead to the isolation and characterization of one active compound identified as oleanolic acid. Oleanolic acid was obtained in large quantity of 1.24 g but is a well known compound and some other students in the Phytomedicine Programme have already isolated it as the principle responsible for antifungal activities in other plants. Dereplication using advanced techniques such as LC-MS and HPLC or even simple TLC now that the R_f values are known could help in reducing isolation of known compounds.

The extract and oleanolic acid were active against ten tested fungi with an average MIC value of 130 µg/ml but oleanolic acid was inactive against bacteria with an MIC value of >250 µg/ml. It appeared to be less potent against animal fungal pathogens than against plant fungal pathogens. Cell cytotoxicity of the DCM extract and oleanolic acid was investigated using MTT assay on Vero monkey Kidney cells. DCM extract and oleanolic acid were toxic to Vero cells with an LC_{50} 0.413 mg/ml and 0.129 mg/ml respectively, when compared with berberine a toxic compound with LC_{50} of 15.48 mg/ml. Oleanolic acid was more toxic than the crude extract supporting the potential use of plant extracts for controlling plant fungal pathogens.

The therapeutic indexes also indicated that though the extract and oleanolic acid were toxic, they could be used under controlled conditions against infections of certain fungal pathogens. It is important to note that although the extract and oleanolic acid were toxic towards cell this is only a preliminary evaluation because many factors determine the toxicity to humans and the environment.

6.4 Potential use of weeds in protecting plants

The objectives of this work were largely met, but the aim of discussing the development of a useful product was only partially met because field trials and animal and environmental toxicity assays were beyond the scope of an MSc dissertation.

Nevertheless it became clear that there appear to be synergistic activities to such a degree that in many cases the crude extract had a higher activity than the single antifungal compound according to bioautography. This confirms the results of Angeh (2006) and (Eloff *et al.*, 2006) that crude extracts had a good potential to be developed as antifungal agents for protecting plants and plant products. A good approach would be to increase the activity of extracts by removing inactive compounds through low cost procedures such as selective extraction or solvent-solvent fractionation. This approach leads to a patentable product. Before a low cost product with excellent activities can be brought onto the market, registration and especially the requirements regarding environmental toxicity can be a formidable barrier. Because *Tecoma stans* occurs so widely and the DCM fraction had excellent activities against certain important fungal pathogens with a good therapeutic index it may be worthwhile to do more work on increasing the activity of extracts against fungal pathogens and to examine the environmental and mammal toxicity in greater detail.

The idea of isolating compound resulted in less active and toxic compound. This is because a lot of constituencies were removed during column separation. Other active constituencies present in traces amounts were probably removed and could have added synergistic effects if isolation did not take place. Moreover variation in plants constituencies plays a major role since plants growing at different locations may not possess same components. Therefore it would be better to find a way to modify the extracts and use the extract as antifungal source since extracts show great activity, owing to contribution of diversity of compounds acting synergistically. Future study such as field trials could verify the results obtained here.

This work proves that invasive weeds have potential and could be useful in combating plant fungal pathogens. People should stop overlooking the usefulness of this plants and regulation should be set in place to avoid complete eradication of the plants. More work on these plants should be encouraged and funding provided for development of alternative remedies.

Chapter 7

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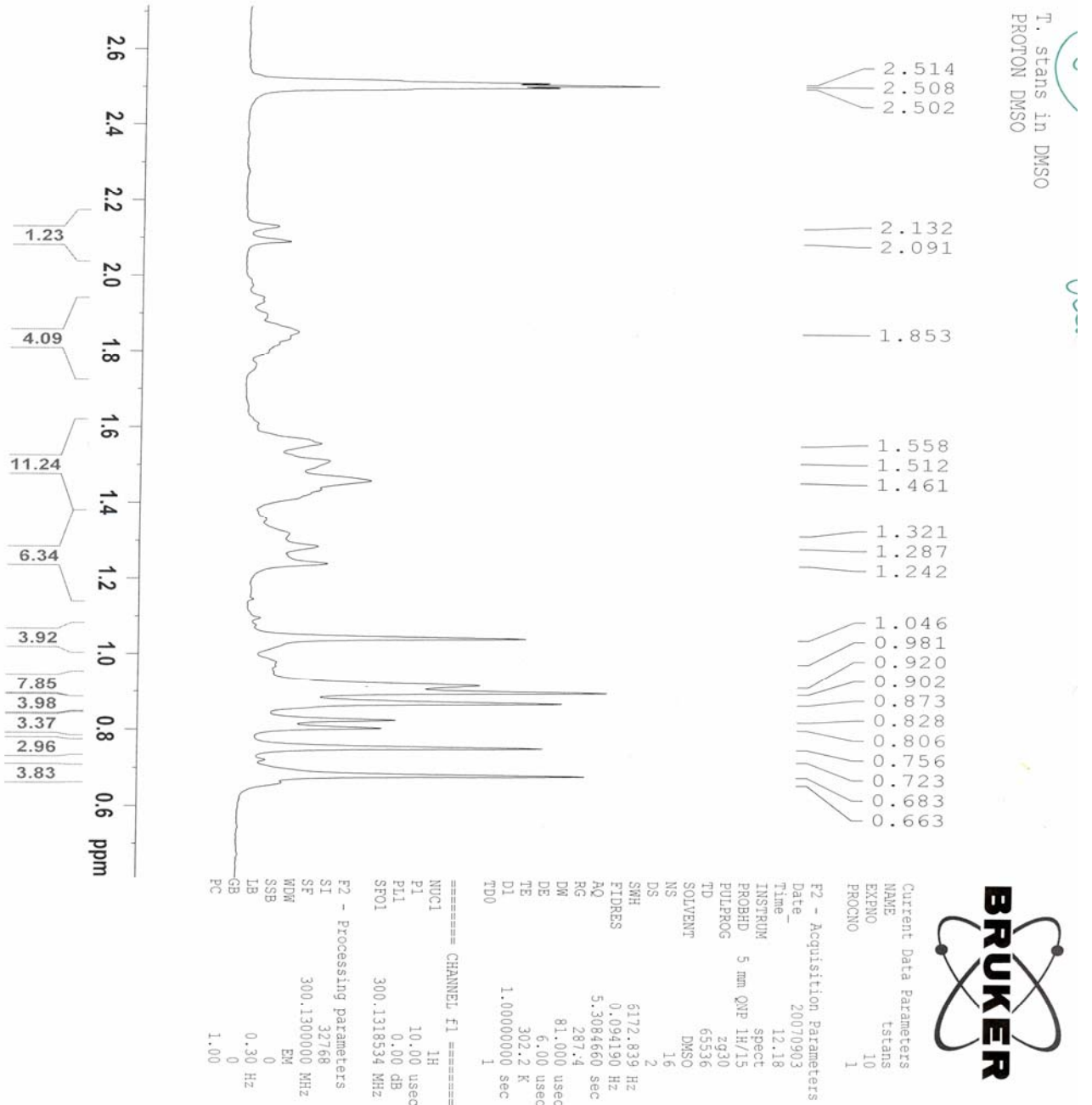
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Appendix A



NMR spectra of oleanolic acid