ANTIFUNGAL AND ANTIMYCOTOXIGENIC ACTIVITIES OF FOUR WEEDY PLANT EXTRACTS AGAINST SELECTED MYCOTOXIGENIC FUNGI

BY

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DECLARATION

I declare that the **Antifungal and Antimycotoxigenic Activities of Four Weedy Plant Extracts Against Selected Mycotoxigenic Fungi** dissertation / thesis hereby submitted to the University of Limpopo for the Degree of Doctor of Philosophy in Medical Science has not previously been submitted by me for a degree at this or any other University; that it is my work in design and in execution, and that all material contained herein has been duly acknowledged.

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ABSTRACT

Fungal contamination and the presence of mycotoxins in food is a major concern, and has received worldwide attention due to the deleterious effects both the fungi and the toxins they produce have on human and animal health, as well as international food trade. Mycotoxins are defined as low molecular weight fungal metabolites that are toxic and/or have adverse effects on the health of animals and humans that eat contaminated agricultural products. A wide variety of fungi are known to produce metabolites that are toxic to vertebrates, but this study focused on two classes of mycotoxigenic fungi of continuing importance in animal and human diseases worldwide, viz. *Aspergillus* and *Fusarium* species. *Aspergillus* and *Fusarium* species are the most common contaminants of food and feed, both in the field and in storage. These fungal species are known to produce toxic secondary metabolites called the aflatoxins and fumonisins respectively, of which aflatoxin B_1 (AFB_1) and fumonisin B_1 (FB_1) are considered to be the most toxic.

Fumonisins are mainly produced by *Fusarium verticilloides* and *F. proliferatum*. It occurs mainly in maize and maize-based products. Fumonisins are known to adversely affect human and animal health. Aflatoxins are field and storage mycotoxins, produced by *Aspergillus flavus* and *A. parasiticus.* Aflatoxin is a common contaminant of foods, particularly grains like maize, peanuts, nuts and cassava some of which are staple diets in many developing countries. Aflatoxins are potent carcinogens, mutagens and immuno-suppressing agents.

Although it is widely perceived that many synthetic fungicides used to control fungal infections are effective, concern has been expressed about their safety. There is therefore an urgent need to find affordable interventions, which can be safely used as fungicides without the cost and disadvantages of the currently used prevention strategies. The use of plant extracts may provide an alternative way to prevent fungal growth and mycotoxin production. The present study therefore, set out to investigate the antifungal and antimycotoxigenic activities of aqueous and organic extracts of four weedy plant species viz. *Tagetes minuta, Lippia javanica, Amaranthus spinosus* and *Vigna unguiculata* against isolates of four pre- and post harvest agriculturally important fungi, i.e. *Fusarium verticillioides, F. proliferatum, Aspergillus flavus* and *A. parasiticus*..

Dried powdered aerial parts of the plants were extracted sequentially with hexane, dichloromethane, methanol and water. The extracts were tested for activity using a serial microdilution and maize patty assays. All extracts except for the water extracts showed growth inhibitory activity against most isolates of *Fusarium* spp. The most active were the methanol and hexane extracts of *V. unguiculata* and *A. spinosus* with minimum inhibitory concentration (MIC) values of <0.5 mg/ml after 48hrs against *Fusarium* spp. No inhibition of the *Aspergillus* spp. tested was observed, but conidium formation was stimulated on plates treated with the plant extracts when visually compared to the growth controls.

Using the maize patty assay, an inhibitory effect on fumonisin B_1 production was observed at different concentrations of the plant extracts and was found to be dose dependent. Generally, at higher concentrations of 0.6 mg/ml all plant extracts showed apparent inhibition of FB_1 production. The methanol and hexane extracts of all the

plant species exhibited high inhibition of fumonisin B_1 production while the DCM extracts were less active.

The antioxidant activity and phenolic content of the plant extracts were also determined using the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method and ferric reducing ability of plasma (FRAP) methods. The best activities using the FRAP assay were found with extracts of *V. unguiculata* (0.06 - 0.13 mmol trolox/mg) and *T. minuta* (0.04 - 0.27 mmol trolox/mg). Using the ABTS assay, the highest antioxidant activity was obtained from the MeOH extracts (0.33 - 0.69 µmol/mg) of the four plant species. The MeOH extracts of all the plant species had the highest phenolic content (0.64 - 1.63 mg GAE/ml) and this correlated to the highest antioxidant activity. From the results obtained, it can be hypothesized that the activity of the plant extracts could be due to the presence of flavonoids identified in the plant extracts. The antifungal and antimycotoxigenic activities discussed earlier could also be attributed to these compounds.

Phytochemical analysis showed in general a great diversity of compounds eluting between 0.5 min to 16 min. However, in general and across all solvents, *L. javanica* and *T. minuta* had more compound peaks than *A. spinosus* and *V. unguiculata* (Figs. 6.1, 6.2 and 6.3). Most compounds in the range 3 – 12 min were low in molecular weight < 529 amu and also UV active.

Generally, the findings of the present study indicate that the plant extracts, particularly extracts of *V. unguiculata* and *A. spinosus* have antifungal activities and may be developed into environmentally friendly fungicides.

TABLE OF CONTENTS

CHAPTER 5: ANTIOXIDANT ACTIVITY AND PHENOLIC CONTENT IN PLANT EXTRACTS

CHAPTER 6: PHYTOCHEMICAL INVESTIGATION

CHAPTER 7

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS AND SYMBOLS

ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
AFB_1	Aflatoxins B_1
AFB_2	Aflatoxins B_2
AFG_1	Aflatoxins G_1
AFG_2	Aflatoxins G_2
APCI	Atmospheric pressure chemical ionization
$BaCl_2$	Barium chloride
BEA	Benzene/ethanol/ammonia
CEF	Chloroform/ethyl acetate/formic acid
CLA	Carnation Leaf Agar
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
EMW	Ethyl acetate/methanol/water
ESI	Electrospray ionization
EU	European Union
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB_3	Fumonisin B_3
Fig.	Figure
FRAP	Ferric Reducing Ability of Plasma
g	Gram
GAE	Gallic acid equivalent

Hex	Hexane
HIV	Human immunodeficiency virus
HPLC	High Performance Liquid Chromatography
IC_{50}	Inhibition concentration
INT	*p*-iodonitrotetrazolium
IR	Infrared
KH_2PO_4	Potassium hydrogen phosphate
JECFA	Joint Expert Committee on Food Additives
kg	Kilogram
LEM	Leukoencephalomalacia
m	Metre
MeOH	Methanol
MIC	Minimum inhibition concentration
mmol	millimoles
ml	Millilitre
MRC	Medical Research Council
MS	Mass spectrometry
Na_2HPO_4	sodium hydrogen phosphate
nm	nanometre
NMR	Nuclear magnetic resonance
NTDs	Neural tube defects
OPA	*o*-phthaldialdehyde
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PEM	Protein-Energy Malnutrition

PMTDI	Provisional maximum tolerable daily intake
PROMEC	Programme on Mycotoxin and Experimental Carcinogenesis
Rpm	Revolutions per minute
SOP	Standard operating procedure
TB	Tuberculosis
TLC	Thin Layer Chromatography
UWC	University of Western Cape
UV	ultraviolet
WHO	World Health Organisation
%	Percent
µg	Microgram
°C	Degree Celsius

PAPERS PREPARED FROM THIS THESIS

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CHAPTER 1

INTRODUCTION

1.1 Mycotoxins and food contamination

Agricultural and food commodities are the major items of international trade for many African countries (Bankole and Adebanjo, 2003). The safety of food and feed for human and animal consumption respectfully is of utmost priority so that consumers are not compromised by the intake of poor quality or unsafe food. Africa faces a crisis of rising food prices and food insecurity (Watkinson and Makgetla, 2002). Food makes up such a high share of spending by the poor that rapid inflation in food prices has a devastating impact on living standards as well as on the efficiency of the economy as a whole. In many parts of the developing world especially Africa, millions of households engage in farming to supplement what they purchase for the family (Lakew, 2006). Subsistence farming is widespread in rural Africa and is a strategy by poor rural households to reduce expenditure on food (FAO, 2005). Food production and storage therefore, play an important role in rural households.

One of the most important safety aspects of food and feed is contamination with mycotoxins. Mycotoxins are toxic secondary metabolites of fungal origin, and are known to contaminate a variety of crops worldwide, both in the field and in storage (Shephard *et al.* 1996). These toxins, when ingested, inhaled or absorbed through the skin cause lowered performance, morbidity and/or even

death in humans and animals. Mycotoxicoses is a disease or the term used for poisoning associated with exposures to mycotoxins (Bennett and Klich, 2003), that results from the ingestion of toxin-contaminated food, or indirectly from consumption of animal products such as milk from livestock exposed to contaminated feed (Marasas, 1995). The symptoms of a mycotoxicosis depend on the type of mycotoxin; the concentration and length of exposure; as well as age, health, and sex of the exposed individual (Bennett and Klich, 2003).

Hunger in many parts of Africa resulting from food insufficiency far outweighs other considerations such as food safety, and there is ample evidence that Africans are experiencing heavy dietary exposure to food-borne mycotoxins, particularly aflatoxins and fumonisins (Probst *et al.* 2007). Maize contaminated with aflatoxins has been implicated in several deadly epidemics in Kenya since 1981 (Probst *et al.* 2007) with the more recent incident reported in 2004 (Lewis *et al.* 2005). Fumonisins have been reported as a possible contributory risk factor for primary liver cancer (Ueno *et al.* 1997). Synergistic toxicological and carcinogenic effects of fumonisin B_1 and aflatoxin B_1 have been reported (Gelderblom *et al.* 2002).

1.1.1 Fumonisins

Fumonisins are secondary metabolites produced by *Fusarium* species, mainly *Fusarium proliferatum* and *F. verticillioides* (McKean *et al.* 2006). Although an increasing number of structural analogues have been isolated from fungal cultures, the most important analogues found in naturally contaminated maize

are fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) (Shephard, 1998), Fumonisins are diesters of propane-1,2,3-tricarboxylic acid (tricarballylic acid, TCA) and various 2-amino-12,16-dimethylpolyhydroxyeicosanes in which the hydroxyl groups on ^{14}C and ^{15}C, are esterified with a terminal carboxyl moiety of the TCA (Shephard, 1998). Fumonisin B_1 (Fig. 1.1) is the most toxic and occurs mainly in maize and maize-based products (Wang *et al.* 2008). Maize left on the ground due to logging, harvest operation and before storage, is predisposed to infection with *Fusarium* (Fig. 1.2) which may lead to contamination with fumonisins (Sinha and Sinha, 1992). The level of insect damage on grain kernels also influences the extent of fumonisin contamination i.e. damage to the ears of maize by stalkborers and weevils among other pests increases the probability of fungal infestation leading to mycotoxin contamination (Keetch *et al.* 2005).

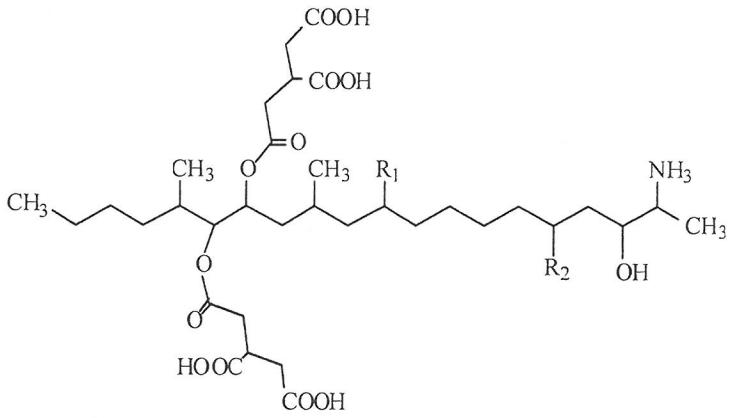


Fig. 1.1: Chemical structure of FB_1 found in naturally contaminated maize.

Fig. 1.2: Maize infected with *Fusarium verticilliodes*.

1.1.2 Aflatoxins

Aflatoxins are commonly produced by *Aspergillus flavus* and *A. parasiticus* that occur in the field and during storage (Phillips, 1999). Aflatoxins are dihydrofuran or tetrahydrofurano moieties fused to a coumarin ring (Hussein and Brasel, 2001). They have been designated as B_1 and B_2 ($AFB_1$and AFB_2), G_1 and G_2 (AFG_1 and AFG_2) (Fig. 1.3). Aflatoxins are distinguished by their flourescence properties with both AFB_1 and AFB_2 fluoresce blue and AFG_1 and AFG_2 fluoresce yellow-green under ultraviolet light (Hussein and Brasel, 2001). AFB_1 is the most potent of the aflatoxin group of mycotoxins and has been shown to be carcinogenic, mutagenic and immuno-suppressive (Mwanda *et al.* 2005). Furthermore, they are common contaminants of food, particularly maize, rice (Lewis *et al.* 2005), peanuts (Fig. 1.4), other nuts and cassava (Wareing *et al.* 2001). These food commodities form staple diets in many developing countries.

Also, Aflatoxins have also been found to contaminate herbs and spices (El-Kady *et al.* 2008).

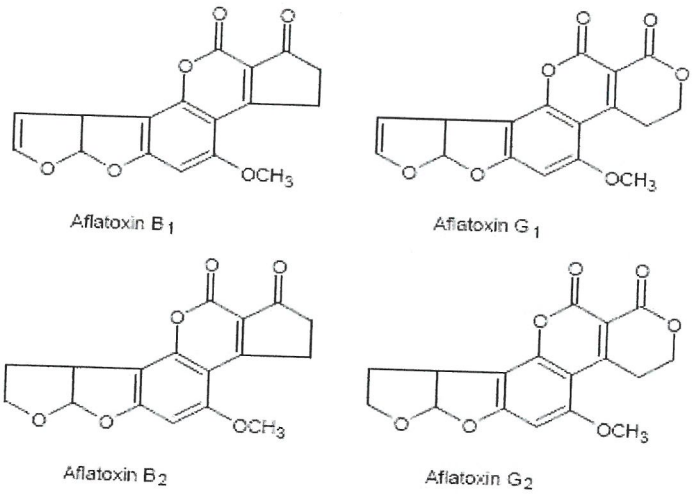


Fig. 1.3: Chemical structures of aflatoxins.

Fig. 1.4: Peanuts infected with *Aspergillus flavus.*

1.2 Implications of fumonisins and aflatoxins on animal and human health

Several studies on the major occuring fumonisin analogue, FB_1, have shown it to be the causative agent for the fatal disease syndromes, leukoencephalomalacia (LEM) in horses (Marasas, 1995, Kellerman *et al.* 1990), pulmonary oedema and

hydrothorax in swine (Ross *et al.* 1990). FB_1 has also been shown to be nephrotoxic (Riley *et al.* 1994), hepatotoxic and hepatocarcinogenic in laboratory rats (Gelderblom *et al.* 1991) and to produce toxic effects in broiler chicks (Leroux *et al.* 1992). The effect of fumonisins on human health is not certain, but it has been statistically linked to the incidence of oesophageal cancer in the Transkei region of South Africa (Marasas *et al.* 1988) and in Cixian and Linxian countries of the People's Republic of China (Sydenham *et al,* 1996), as well as neural tube defects (NTDs) in infants along the Texas-Mexico border (Missmer *et al.* 2006; Gelineau-van Waes *et al.* 2005). All these areas have high consumption of maize products in the diet.

The largest risk of aflatoxins to humans is due to chronic dietary exposure. This has been associated with human hepatocellular carcinomas (Marasas and Nelson, 1986), which may be compounded by hepatitis B virus infection (Bhat and Vasanthi, 2003). Approximately 250 000 deaths are caused by hepatocellular carcinomas in China and Sub-Saharan Africa annually (Groopman *et al.* 1992) and they are attributed to risk factors such as high daily intake (1.4 µg/kg body weight) of aflatoxins and high incidence of hepatitis B (Wild *et al.* 1992).

In developing countries poor diet is associated with malnutrition and growth faltering in infancy and childhood (WHO, 2003). Children are a susceptible population to environmental hazards such as AFB_1 and there is strong links between growth and health of the fetus and infant, and disease risk in later life (Polychronaki *et al.* 2008). Generally, if a child is suffering from a disease the

child is more susceptible to the effects of aflatoxin exposure (Shephard, 2005). Aflatoxins have been reported to be associated with Protein-Energy Malnutrition (PEM), particularly kwashiorkor (Hatem *et al.* 2005). They have been found in tissues of children suffering from kwashiorkor and Reye's syndrome and were thought to be a contributing factor to these diseases (Becroft and Webster, 1972). Reye's syndrome, which is characterized by encephalopathy and visceral deterioration, results in liver and kidney enlargement and cerebral edema (Becrotft and Webster, 1972). Peri-natal exposure to aflatoxins has been shown to stunt growth (low height for age) and may contribute to infant mortality due to PEM (Gong *et al.* 2002). Chronic exposure, which is more common, has been implicated in the high incidence of hepatocellular carcinoma in the developing world, and more recently to immunologic suppression and under-nutrition (Katerere *et al.* 2008). There is also a general consensus emerging that aflatoxin exposure is an important co-factor in HIV morbidity and infant mortality in the developing world, because of its impact on the nutritional and immune status of at risk populations (Katerere *et al.* 2008). According to Williams *et al.* (2004), aflatoxin exposure and the subsequent toxic affects on immunity and nutrition combine to negatively affect health factors that account for more than 40% of the burden of disease in developing countries. In their estimation, exposure to aflatoxins plays a part in diarrheal diseases and acute respiratory infections in infants, and predisposes the population to TB and HIV/AIDS infections. According to Katerere *et al.* (2008), this would be expected from an agent which interferes

with the functioning of the liver which is central to important processes such as basic metabolism of food and xenobiotics and immune mediation.

Recent outbreaks of acute aflatoxicosis have been reported in India, Thailand and Kenya (Lewis *et al.* 2005). In Kenya in 2004, a high Case Fatality Rate of 39% was seen (Probst et al 2007)

1.3 Regulation of fumonisins and aflatoxins

Given the above, it is no wonder that different countries have enforced different thresholds to limit the passage of mycotoxins along the food chain. Such regulations aim to provide consumers with an increased measure of protection by setting maximum levels for specific mycotoxins and environmental contaminants in foodstuffs. The regulations assist to keep contaminants at levels that are toxicologically acceptable and to exclude grossly contaminated food from entering the food chain.

Internationally, for example, the Food and Agricultural Organisation/World Health Organisation (FAO/WHO) Joint Expert Committee on Food Additives (JECFA) have assigned a provisional maximum tolerable daily intake (PMTDI) for fumonisin B_1 (FB_1) of 2 µg/kg of body weight/day (Creppy, 2002). FAO/WHO Food Standards Programme has assigned PMTDI levels for aflatoxin of 15 µg/kg for all foodstuffs. In South Africa, all foodstuffs containing more than 10 µg/kg total aflatoxin, of which aflatoxin B_1 should not be more that 5 µg/kg, are deemed contaminated, impure or decayed (Department of Health, 2004)

However it is worth noting that regulations are only relevant to situations where the food supply chain is formally commercialized. This situation does not obtain in most rural Africa where most food is home-grown and consumed within households and communities without entering commercial processing and distribution systems.

1.4 Factors that influence growth of fungi and mycotoxigenesis

The production or presence of mycotoxins in food and feed is affected by factors that can be categorized as physical, chemical, and biological (D'Mello and MacDonald, 1997). Physical factors include the environmental conditions conducive to fungal colonization and mycotoxin production such as temperature, relative humidity, and insect infestation (D'Mello and MacDonald, 1997). Many African countries have tropical climate with all year round ambient temperature and relative humidity that provide optimal conditions for the growth of toxigenic fungi (Bankole and Adebanjo, 2003). Droughts and soil moisture stress may selectively alter colonization and metabolism of mycotoxigenic fungi and thus alter mycotoxin production (Russel *et al.* 1991).

Chemical factors include the use of fungicides and/or fertilizers. Nitrogen supply to crops in the form of fertilizers and pesticides has been shown to increase incidence of *Fusarium* infected grains (Jouany, 2007). This may be as a result of altering the rate of residue decomposition, by acting on the rate of plant growth and by changing the soil structure and its microbial activity (Jouany, 2007). The use of synthetic pesticides can sometimes stimulate growth of certain fungi and

mycotoxins formation both in the soil and on the plant. Mycotoxin production is known to occur when fungi are under stress (Edward, 2004). Application of fungicides has also been reported to enhance selection of resistant fungi (Leroux *et al.* 1999).

Biological factors include:

(1) Plant stress – previous investigations prove that plants under stress are highly susceptible to infection by some fungi (Hesseltine, 1976).

(2) Insect vectors – Rajapakse and Emden, (1997) studied the association of storage fungi with the rice weevil. These authors state that the saprophytic fungi, which include some mycotoxin producers such as *Aspergillus ochraceus* and *A. flavus*, are mechanically carried in the alimentary canal of the insect along with the food.

(3) Fungal infection – Infection of one fungus makes a plant more susceptible to invasion by a second (Hesseltine, 1976). According to Fischer and Holton (1957), wheat plants infected with the smut, *Tilletia caries*, are highly susceptible to attack by root rot organisms such as *Fusarium*.

(4) Fungal strain differences - Elad *et al.* (2007) reported the presence of fungicide-resistant strains of *Botrytis cinerea*. They reported that resistance to fungicides such as benzimidazoles and dicarboximides was found in all sites in which these fungicides had been used against grey mould, but not in other sites. A new phenotype of multiple fungicide resistance was found among these strains. Furthermore, the factors contributing to contamination are based on the interactions between fungal species and substrate.

1.5 Current control methods of mycotoxigenic fungi

Control of food and feed contamination by mycotoxins will undoubtedly result in economic gains as well as health improvements worldwide. Several prevention and control methods have been proposed to minimize the loss of food and agricultural commodities as a result of fungal spoilage and also reduce exposure to mycotoxins (Huwig *et al.* 2001). These strategies include among others, the use of synthetic chemicals, fumigants, microwave or sonic drying and physical separation (Bankole and Adebanjo, 2003). Although some of these methods are effective they do not fulfill all the requirements, especially those concerning safety and safeguarding of the nutritional characteristics of treated food and feed (Galvano *et al.* 2001). Fungicides, which are commonly used, are expensive and inaccessible to rural farmers in many African countries. Most of these chemicals are residual and not biodegradable posing a huge environmental threat and most importantly, increasing instances of fungal resistance have been reported (Wilson *et al.* 1997).

Poverty and ignorance among farmers and farmworkers in sub-Saharan Africa often lead to abuse and misuse of fungicides, with fatal consequences e.g. the death of hundreds of people in Nigeria as a result of consumption of *Vigna unguiculata* (cowpea) treated with inappropriate pesticides (Bankole and Adebanjo, 2003). Although most organophosphates are banned in southern Africa, many of these substances are still used in a large scale in developing countries and continue to pose severe health hazard (Skouras *et al.* 2006). High

levels of organophosphates and carbamate pesticides have been reported in human breast milk as well as in blood samples of farm workers (Hoyer *et al.* 2000). Organophosphates and carbamates have been reported to cause serious poisoning, particularly ventricular arrhythmias, central nervous system depression or seizures, and respiratory failure (Bardin *et al.* 1994). These pesticides enter the body via dermal absorption, ingestion and inhalation (Eskenazi *et al.* 1999). Children can also be exposed by the same pathway, through consumption of contaminated food, by household use of pesticides, as a result of drift from nearby agricultural applications, through contaminated breast milk from their farmworker mothers, through playing in the fields and through pesticides tracked into their homes by their parents or other household members working in the fields (Eskenazi *et al.* 1999). Given this scenario and also what has been previously mentioned about the effect of mycotoxins on animal and human health, it is imperative that safer alternatives be investigated.

1.6 Plant-based constituents as alternatives

The role of plant extracts has been generally neglected despite their documented use by many rural farmers in Africa and Asia. For instance, there is widespread use of *Lippia javanica* and *Tagetes minuta* in Africa and *Azadiracta indica* in India as fumigants and pest repellants (Grassroots Natural Products, 2002; Kaaya, 2002). Extracts of *Allium sativum* have been reported to be potent fungicides, effectively protecting peaches against brown rot caused by *Monilinia fructicola* (Wilson *et al.* 1997). Bouda *et al.* (2001) reported that the essential oils of some

weed species such as *Lantana camara* effectively controlled insects such as *Sitophilus zeamais* and suggested that these species could be exploited for insect control in stored products. Essential oils from *Cinnamonum zeylanicum* were also reported to inhibit the growth of the bacillus, *Clostridium botulinum* and the fungal disease of damping off of cabbage caused by *Rhizoctonia solani* (Bowers and Locke, 2000).

Though results obtained from studies on some plant species that have been examined for antifungal activity against fungal plant pathogens are encouraging (Martini *et al.* 2004, Hammer *et al.* 2001, Rauha *et al.* 2000, Srinivasan *et al.* 2001), most studies tend to focus mainly on human pathogens and few lead compounds have been developed for use in food and crop protection (Cowan, 1999).

The use of plants as biopesticides in organic agriculture has also been reported (Pussemier *et al.* 2006). Organic agriculture does not use synthetic pesticides, instead relies on the use of legumes, cover-crops such as the incorporation of cowpea residue in the soil, manure, compost and relatively slow release fertilisers to meet the crops nitrogen needs (Worthington, 1998). Typically, this kind of farming allows for lower levels of nitrogen to be applied per acre, and a significant share of nitrogen is or becomes bound up in decaying organic matter, and hence tends to be released more slowly. However, organic produce may have a shorter shelf life period compared to conventional produce, and may become contaminated by fungi.

Of recent interest, has been the possible role of natural phenolic compounds obtained from plants in inhibiting growth and toxin production by fungi. Phenolic compounds have been found to be inhibitory to the production of several mycotoxins including fumonisins and aflatoxins (Beekrum *et al.* 2003). Phenolic compounds are secondary metabolites of plant origin (Dhiraj *et al.* 2005). These metabolites protect the plant against biological and environmental stresses and are generally synthesised in response to fungal and bacterial attack, high energy radiation exposure, herbivores and predators (Dharaj *et al.* 2005). The phenolic compound 2-hydroxy-4-methoxybenzaldehyde extracted from *Decalepis hamiltonii* have been reported to have high antifungal activity against 24 fungal species known to cause diseases in sorghum and maize (Mohana *et al.* 2008). According to these authors treatment of seeds with this phenolic compound significantly increased seed germination and decreased seed mycoflora.

Joseph *et al.* (2005) and Selvi *et al* (2003) reported on the antiaflatoxigenic activity of hexane and chloroform extracts of *Garcinia cowa* and *G. pendiculata.* They showed that extracts of the two plants species inhibited the growth of *A. flavus* and aflatoxin B_1 production up to 100% at a concentration of 2 mg/ml and attributed the activity of the extracts from these two plants to their antioxidative properties which, they speculated, effectively suppressed the biosynthesis of aflatoxins. Eugenol, a naturally occurring compound found in many medicinal and aromatic plants e.g. *Ocimum sanctum* and *Pimenta racemosa,* has been reported to inhibit aflatoxin production by *Aspergillus parasiticus* in a dose dependent manner (Jayashree and Subramanyam 2002). Phenolic compounds derived from

olive pomace, a by-product of olive oil production, have been reported to inhibit the growth of *Alternaria solani*, *Botrytis cinerea* and *Fusarium culmorum* (Winkelhausen *et al.* 2005). Plant phenolic compounds such as chlorophorin, benzoic acid, caffeic acid, ferulic acid and vanillic acid have been reported to significantly reduce fumonisin B_1 and aflatoxin B_1 production without affecting or inhibiting the growth of *Fusarium verticilliodes*, *F. proliferatum*, *Aspergillus flavus* and *A. parasiticus* (Samapundo *et al.* 2007, Beekrum *et al.* 2003). Given the potential effect of phenolic compounds against fungal diseases, it becomes necessary to scientifically exploit these compounds in the management of plant borne pathogenic fungi and the prevention of mycotoxin production in food and feed. The present study therefore, set out to investigate both the anti-fungal and anti-mycotoxigenic activities of four plant species, i.e. *Tagetes minuta*, *Lippia javanica*, *Vigna unguiculata* and *Amaranthus spinosus*. These plant species are commonly used for medicinal/dietary purposes, and play an important role as famine foods in rural in Southern Africa and are discussed in more detail below.

1.7 *Tagetes minuta* L.

Family: Asteraceae

Synonyms: *Tagetes glandulosa, Tagetes glandulifera* (Schrank).

Common name(s): Wild Marigold (English), kambanje (Shona), Kakiebos, (Afrikaans), insangwana, unukani (Zulu).

Botanical description and geographical distribution

T. minuta is an aromatic, branched erect, annual herb that stands 1-2m tall. The leaves are stalked, opposite and slightly glossy green and 7-15cm long. It is in leaf from April to November, in flower in October, and the seeds ripen in November. The flower heads are yellow, small and 10-15mm long. Flowers are arranged in solitary clustered panicled branches (Fig. 1.5; Singh *et al.* 2000).

Fig. 1.5*:* Flowering plant of *Tagetes minuta*.

T. minuta is native to temperate forest and mountain regions of most countries in the world including southern Africa, Australia, India, Kenya, Brazil and regions of Paraguay as a weed (Singh *et al.* 2000).

Traditional uses and biological studies

T. minuta is used as a medicinal tea. Decoction of the plant is prepared by extracting the whole herb with hot water which is used as beverage and as a remedy for common cold, digestive system complaints, stomach upsets, diarrhea and suspected liver ailments (Rios and Recio, 2005). The person of East Africa

hang tagetes plants in their huts to repel flies (Grassroot Natural Products, 2002). The whole plant of *T. minuta* is often placed in the bedding of animals as a flea repellent. Garden plants are sprayed with a warm water extraction to keep plants free from insects (aphids in particular) and fungal diseases (Grassroot Natural Products, 2002).

Essential oils of *T. minuta* have been reported to inhibit the growth of filamentous fungi (Bii *et al.* 2000; Vasudevan *et al.* 1997), and have also been shown to possess repellent and biocidal activities against different mosquito species (Vasudevan *et al.* 1997). Pyrethrins from *Tagetes* species have also been reported to be potent and safe insecticides. Synthetic pyrethrins are not as effective as natural ones and insects were found to develop resistance against them (Vasudevan *et al.* 1997). On the other hand, antibacterial activity of flavonoids has been reported against microorganisms like *Staphylococcus aureus* and *S. epidermidis*. Vasudevan *et al.* (1997) reported the potent effect of *T. minuta* and *T. erecta* on the root-infesting nematodes of tomato plants. These workers reported that tomato plants grown between *Tagetes* species had significantly greater shoot growth and fruit yield. The root extract of *Tagetes* was found to suppress nematode egg hatching and infectivity to tomato. Thiophenes from *T. minuta* roots, which are sulphurous heterocyclic compounds derived from polyacetylenes, have been reported to be toxic to nematodes, insects, fungi and bacteria (Gil *et al.* 2002).

1.8 *Lippia javanica* (Burm.f.) Spreng

Family: Verbenaceae

Synonyms: *Lippia japonica*

Common names: fever tea, lemon bush (English), lemoenbossie (Afrikaans), mutswane (Swati), umSuzwane (Zulu), musukudu/bokhukhwane (Sotho), zumbani (Shona).

Botanical description and geographical distribution

L. javanica is a 1-2m high woody shrub that stands erect and is multi-stemmed (Fig. 1.6). The stems have a square appearance when looked at in cross-section. The leaves are hairy with noticeable veins and when crushed gives off a strong lemon-like smell. It is said to be one of the most aromatic of South Africa's indigenous shrubs. Small cream flowers can be found on the shrub from summer to autumn in some areas and in others are produced all year. The fruits are inconspicuous, small and dry (van Wyk *et al.* 1997).

Fig. 1.6: Shrub of *Lippia javanica.*

L. javanica is widespread throughout large parts of South Africa, with the exception of Western Cape. They grow from the Eastern Cape, northwards, extending into tropical Africa including Botswana, Swaziland, Mozambique, Zimbabwe, Malawi, Zambia and Kenya. They grow in open veld, in the bush on forest margins as well as cultivated field (van Wyk *et al.* 1997).

Traditional uses and biological studies

L. javanica is well known medicinally to many rural Africans. The leaves and stem infusions are prepared as tea and used to treat coughs, diarrhoea, malaria, skin infections and wounds (Viljoen *et al.* 2005). The Venda people of South Africa use the leaf infusions as anthelmintics, for respiratory and febrile ailments and as prophylactic against dysentery, while the Xhosa people use *L. javanica* for the disinfection of anthrax-infected meat (Mabogo, 1990). The smoke from the herb is also known to be effective, if inhaled, against asthma, chronic coughs and pleurisy (van Wyk *et al.* 1997).

Essential oils *of L. javanica* are known for antimicrobial properties (Bassole *et al.* 2003). Essential oils of *L. javanica* have been reported to completely inhibit *Staphylococcus aureus, Escherichia coli, Salmonella gallinarum, Klepsiella pneumomiae, Candida albicans* and *Pseudomonas aeruginosa* at concentrations of 1 mg/ml (Manenzhe *et al.* 2004). Oils of *Lippia javanica* were reported to have toxic and/or repellent effects against insects when used as fumigants in grain stores (Omolo *et al.* 2005). Viljoen *et al.* (2005) showed *L. javanica* volatile oils to be active against gram-negative bacteria and yeast-borne respiratory disorders.

1.9 *Amaranthus spinosus* L.

Family: Amaranthaceae

Common name(s): spiny amaranth, thorny pigweed (English), thepe (Sotho), mowa (Shona), imbuya (Zulu).

Botanical description and geographical distribution

A. spinosus is a seasonal herb. The stems are sometimes tinged with red, erect, sometimes ascending, 4-15 cm long, usually branched or striate, with multi-cellular hairs, especially above, most leaf axils with a pair of divergent spines up to 2.5 cm long (Fig. 1.7). Leaves ovate to rhombic-ovate, lanceolate-oblong, or lanceolate, blades 1-12 cm long, 0.89-6 cm wide. Flowers are green, in axillary clusters in the lower part of the plant and in unbranched or branched spikes in the upper part, the lower clusters entirely pistillate as are the lower flowers of the spikes, the upper flowers in the spikes staminate. Fruits are ovoid, with a short inflated neck below the style base. Seeds are black, shiny, compressed, 0.8-1 mm long; inconspicuously reticulate (Moss, 1988).

Fig. 1.7: Flowering plant of *Amaranthus spinosus.*

A. spinosus is widespread in southern and central Africa. It spreads rapidly in cultivated land and grows on various soil types, under a wide range of environmental conditions. The plant grows as a weed around human settlements (Moss, 1988).

Traditional uses and biological studies

The leaves of *Amaranthus spinosus* are consumed as relish in rural staple diets, often mixed in a sauce or with other vegetables. The plant can be used fresh or it can also be harvested when coming into flower and dried for later use. According to Mathieu and Meissa (2007), as to the level of medicinal use, there is no distinction between the different species of *Amaranthus*. These authors reported that the root and leaves of *A. spinosus* are used for the treatment of ear infections, wounds, abscesses, headache as well as abdominal pains. The paste of root and seeds are used to heal broken bones in chickens, remove pus and

accelerate quick healing of boils (Manandhar, 1995). *A. spinosus* has also been used as a traditional medicine to treat diabetes (Katerere and Eloff, 2006).

A. spinosus has been reported to have a high concentration of antioxidant components (Amin *et al.* 2006). Antimicrobial peptide Ar-AMP from *Amaranthus* seeds were found to effectively inhibit the growth of different pathogenic fungi, especially that of *Fusarium culmorium* (Lipkin *et al.* 2005). *Amaranthus* have been reported to have fungistatic (transitory antifungal) activity against *Botrytis cinerea* (Wilson *et al.* 1997).

1.10 *Vigna unguiculata* L.

Family: Fabaceae

Synonyms: *Dolichos sinensis; Dolichos unguiculata*

Common names: cowpea, blackeyed-pea (English), monawa (Sotho), boontjies (Afrikaans), munyemba (Shona), isihlumaya (Zulu)

Botanical description and geographical distribution

V. unguiculata is a herbaceous plant generally 30-80 cm in height with indeterminate or (rarely) determinate growth (Fig. 1.8). It is erect and bushy to prostrate and creeping growth habits exist depending on cultivar and growing conditions. Stems have circular sections and are pock marked. They are sometimes slightly grooved. The texture is fibrous and hard, firm and not inflated when young. The leaves are trifoliate and the leaflets are oval, pointed (6-15 x 4-11 cm), and they are generally entire and sometimes lobed. The inflorescences

are auxiliary clusters, with an axis 20-25 cm long; flowers are in twos along this axis. Often a single pair gives two pods. Flower colour ranges from white, yellowish, pale blue, pink and violet. Flowers are produced in inflorescences that are compound racemes of several modified simple racemes borne on peduncles usually between 5-60 cm long (Pasquet, 1996).

Fig. 1.8: *Vigna unguiculata* plant indicating leaves and tender shoots.

V. unguiculata is widespread and is also domesticated in many parts of Africa. It is now widely cultivated throughout the tropics and subtropics (Moss, 1988).

Traditional uses and biological studies

V. unguiculata grains are primarily in demand for human consumption in West and Central Africa. They are used in many different dishes in whole grain or milled forms, sometimes eaten with oil and seasoning, but more common uses are mixtures of cowpea and cereals (for example, rice and beans) and as an

ingredient in soups and/ or stew (Langyintuo *et al.* 2003). Tender shoot tips and leaves of cowpea are consumed in spinach and condiment form (Fery, 2002). The root infusions of *V. unguiculata* are used to treat dysmenorrhoea and epilepsy, while the soup made of seeds is used to treat bilharziasis (Kritzinger *et al.* 2005). Ngouajio *et al.* (2003) reported that when cowpea residues were incorporated into the soil and used as cover crop and as surface mulch in bell pepper (*Capsicum annuum* L.) production, weed infestation was significantly reduced by up to 80-90%.

V. unguiculata has been reported to produce a proteinase inhibitor identified as cystatin which is thought to be vital for the plant's defense against a diverse set of pests (Aguiar *et al.* 2006). *In vitro* testing of cystatin was found to strongly inhibit papain and proteinases from midgets of bean weevils *Acanthoscelides obtectus* and *Zabrotes subfasciatus* (Aguiar *et al.* 2006). *In vitro* testing of three compounds extracted from the root of cowpea showed a strong inhibition of *Fusarium oxysporum* spore germination (Sundaresan *et al.* 1993). Even though processed cowpea seeds and leaves are increasingly consumed as human food and known for their health promoting benefits, the antifungal effects of cowpea bioactive compounds remain unexplored.

1.11 Phytochemical review

Because the number of known chemical substances occurring in plants is so large, this section deals with phytochemicals isolated specifically from *T. minuta, L. javanica, A. spinosus* and *V. unguiculata* by previous researchers. A range of

phytochemical compounds has been isolated from these four plant species, however, literature review showed that compounds that are found in the highest frequency are the flavonoids, tannins, and phenolic acids. Table 1.1 shows a summary of compounds previously identified in the test plants.

Table 1.1: Compounds identified in the test plants.

	Compounds					
Plant species	Flavonoids	Alkaloids	Tannnins	Terpenoids	Phenolic acids.	References
A. spinosus	✔	Traces.	✔	✔	✔	Hilou *et al.* 2006;; Zeashan *et al.* 2008, Stintzing et al. 2004
V. unguiculata	✔	Traces.	✔	✔	✔	Siddhuraj and Becker, 2006; Onyilagha *et al.* 2009
T. minuta	✔	✔	✔	✔	✔	Ickes *et al.* 2006; Gil *et al.* 2002; Tereschuk *et al.* 1997
L. javanica	✔	✔	✔	Traces	✔	Pascual *et al.* 2001, Manenzhe *et al.* 2004

✔ = detected

1.11.1 Flavonoids

Flavonoids are a group of polyphenolic compounds, which are widely distributed through-out the plant kingdom. One of the established functions of flavonoids is the production of colour particularly to flowers and fruits making them attractive to plant pollinators (Harborne and Williams, 2000). There is increased evidence that these flavonoids, particularly when they are located at the upper surface of the leaf or in the epidermal cells, have a role to play in the survival of plants (Grayer and Kokubun, 2001). Flavonoids have been reported to act as UV light filters, protecting the underlying photosynthetic tissues from damage (Harborne and

Williams, 2000). They also protect the plant against microbial invasion (Grayer and Kokubun, 2001).

Structurally, flavonoids occur as aglycones, glycosides and methylated derivatives (Cos *et al.* 1998). The flavonoid aglycone consists of a benzene ring (A) condensed with a six membered ring (C) which in the 2- position carries a phenyl ring (B) as a substituent. Six member ring condensed with the benzene ring is either a alpha-pyrone (flavonols and flavonones) or its dihydro-derivative (flavonols and flavonones). Flavonols differ from flavonones by hydroxyl group the 3-position and C_2-C_3 double bonds. Flavonoids are often hydroxylated in position 3, 5, 7, 2', 3', 4' and 5'. Methyl ethers and acetyl esters of the alcohol group are known to oocur in nature (Harbone and Williams, 2000).

Flavonoids also known to be potent antioxidants and have free radical scavenging activities (Gao *et al.* 2000). They have been shown to inhibit the growth of various cancer cell lines *in vitro*, and reduce tumour development in experimental animals (Guardia *et al.* 2001). The flavonoids quercetagetin and its derivatives has been isolated from *T. minuta* and reported to have inhibitory activity against bacteria, *viz. Escherichia coli, Staphylococcus aureus* and *Staphylococcus epidermidis* (Tereschuk *et al.* 1997). Luteolin has been isolated from *L. javanica* and shown to be both anti-tumour promoter and mutagen (Chowdhury *et al.* 2002). The flavonoids quercetin and isorhamnetin have been isolated from *V. unguiculata* and *A. spinosus* and have been shown to provide resistance to insect attack (Lattanzio *et al.* 2000).

1.11.2 Tannins

Tannins are a diverse group of polyphenolic compounds which have the ability to precipitate proteins (Hagerman *et al.* 1992). They are synthesized via the shikimate pathway, which is the same pathway that results in the formation of isoflavones, coumarins, stilbenoids and other phenolic metabolites (Katerere, 2001). Tannins are divided into two classes, condensed (often called proanthocyanidins, PA) and hydrolyzable, based on their chemical structures. Condensed tannins are oligomers or polymers of flavonoid units (i.e. flavan-3-ol) linked by carbon-carbon bonds not susceptible to cleavage by hydrolysis. Proanthocyanidins are derived from the acid catalysed oxidation reaction that produces red anthocyanidins upon heating proanthocyanidins in acidic alcohol solutions. The most common anthocyanidins produced are cyanidin and delphinidin. PAs may contain from 2-50 or greater flavonoid units. PA polymers have complex structures because the flavonoid units can differ for substituent and because of the variable sites for interflavan bonds. The best known PAs are procyanidins, based on catechin and /or epicatechin units, and oligomers up to the hexamer have been found in plants. Anthocyanidin pigments are responsible for the wide array of pink, scarlet, red, mauve, violet and blue colours in flowers, leaves, fruits, fruits juice and wines (Harborne, 1994).

Hydrolysable tannins (HTs) are molecules with a polyol (generally D-glucose) as a central core. The hydroxyl groups of these carbohydrates are partially or totally esterified with phenolic groups like gallic acid (gallotannins) or ellagic acid

(ellagitannins). Hydrolysable tannins are usually present in low amounts in plants. Hydrolysable tannins are hydrolysed by mild acids or mild bases to yield carbohydrate and phenolic acids. Hydrolysable tannins are also hydrolysed by hot water or enzymes (Harbone, 1994).

Scalbert (1991) reviewed the antimicrobial properties of tannins and reported that they can be toxic to filamentous fungi, yeasts, and bacteria. Condensed tannins have been shown to bind cell walls of ruminal bacteria, preventing growth and protease activity. Tannins have been considered to be at least partially responsible for the antibiotic activity of methanolic extracts of the bark of *Terminalia alata* (Taylor *et al.* 1996).

1.11.3 Terpenoids

Terpenoids are all based on the isoprene molecule $CH_2=C(CH_3)-CH=CH_2$ and their carbon skeletons are built up from the union of two or more of these C_5 units (Connolly and Hill, 1991). They are classified according to whether they contain two (C_{10}), three (C_{15}), four (C_{20}), six (C_{30}) or eight (C_{40}) units. Terpenoids range from essential oil components, volatile mono- and sesquiterpenes (C_{10} and C_{15}) through to the less volatile diterpenes (C_{20}) to the involatile triterpenoids and steroids (C_{30}) and carotenoids pigments (C_{40}) (Harborne, 1994). Chemically, terpenoids are generally lipid soluble and are located in the cytoplasm of the plant cell and leaf cuticle (McGarvey and Croteau, 1995). Essential oils sometimes occur in special granular cells on the leaf surface, whilst carotenoids are especially associated with the chloroplast (Harborne, 1994).

A considerable number of different functions have been ascribed to plant terpenoids. The growth regulating properties are very well documented, two of the major classes of growth regulators are the sesquiterpenoids, abscisins and the diterpenoid-based gibberellins. The important contribution of carotenoids to plant colour is well known and it is certain that these C_{10} terpenoids are also involved as accessory pigments in photosynthesis. The mono- and sesquiterpenes provide plants with their distinctive smell (Harbone, 1994).

1.11.4 Alkaloids

Alkaloids have been defined as cyclic nitrogen-containing compounds of limited occurrence produced by living organisms (Pelletier, 1996). Alkaloids are basic compounds containing one or more heterocyclic nitrogen atoms. Most alkaloids are derived from amino acid precursors. Classification of alkaloids has been reported as problematic, with some authors prefering a classification based on chemical structure (e.g. pyridine, tropane, pyrrolizidine alkaloids), while others base theirs on biosynthetic origin (Harbone 1994). Alkaloids have been found to have microbiocidal effects against *Giardia* and *Entamoeba* species (Ghoshal *et al.* 1996).

1.12 Aim of the study

The aim of this study was:

- To investigate the antifungal activity of extracts of *Lippia javanica, Tagetes minuta, Amaranthus spinosus* and *Vigna unguiculata* against isolates of four mycotoxigenic pre- and post harvest fungi.

1.13 Broad objective of the study

The broad objective of the study was to investigate and identify compounds from these selected plants that can inhibit growth of agriculturally important fungi and modulate production of mycotoxins.

1.14 Specific objectives of the study

The specific objectives of the study were:

- To investigate the antifungal activity of organic and aqueous extracts obtained from *L. javanica, T. minuta, A. spinosus* and *V. unguiculata.*
- To determine the amount of toxin produced by mycotoxigenic fungi in the presence of the plant extracts.
- To determine the chemistry of each of the selected plant extracts with particular emphasis of determining and identifying the presence of phenolic compounds with antioxidant properties in the plant extracts.

1.15 Hypothesis

Extracts from medicinal/dietary plants possess compounds that have a potential to be used as alternative and safer fungicides/fungistats.

CHAPTER 2

PLANT COLLECTION, EXTRACTION AND ANALYSIS

2.1 Introduction

In phytochemical studies whole plant material or different parts such as roots and aerial parts are normally collected, dried and used as sources of secondary plant components. The collected plant material is always accurately identified. Following this, generally, different studies have always used different solvents such as acetone, hexane, methanol and others to extract bioactive compounds (Eloff, 1998a). In the present study, the collected plant material was extracted with solvents of different polarities, starting with the least polar to the most polar. The aim was to extract a broad spectrum of compounds. The compounds are normally chromatogramed on a Thin Layer Chromatography (TLC) plate (Bague and Kline, 1972). TLC allows localizing compounds in a crude extract on the chromatogram. This makes it possible to separate compounds. The present study was carried out using this particular method to identify compounds with possible antifungal activity.

2.2 Materials and Methods

2.2.1 Plant collection and preparation

T. minuta and *L. javanica* were collected from Onderstepoort, Pretoria, *A. spinosus* and *V. unguiculata* were collected from Moruleng village of Rustenburg,

North West Province. The identification of the plants was done with the assistance of a botanist at University of Western Cape Herbarium where the specimens were subsequently deposited. The following specimen numbers were allocated to the plants; *T. minuta*, UWC 5492; *A. spinosus*, UWC 5493; *V. unguiculata*, UWC 5494 and *L. javanica* UWC 5495. The plants were collected in March-April 2006 form the wild and allowed to dry at room temperature. The aerial parts of the plants were ground into powder using a Romer Labs Series II Grinding / Subsampling mill, USA and stored in a cold room until used.

2.2.2 Exhaustive extraction of the plant material

For each plant species, 80 g of the powdered leaves was sequentially extracted with 600 ml of each of the following analytical grade solvents: hexane (Hex), dichloromethane (DCM), methanol (MeOH) and water (H_2O) in a centrifuge tube by vigorously mixing with a Polytron (Kinematica PT 3100 Lucerne, Switzerland) for 5 minutes at 14000-18000 rpm. The extracts were then centrifuged at 4000 rpm for 10 minutes in a Sorvall Instruments RC-3B Refrigerated Centrifuge (Newtown, CT) and filtered through Whatman no. 1 filter paper. To increase yield, extraction with each solvent was repeated twice. The organic extracts were dried on a rotary evaporator (Buchi, Germany) at temperature of between 55-60°C, whereas the aqueous extracts were freeze-dried. Once concentrated to a small volume, the extracts were placed in pre-weighed beakers and allowed to dry completely in front of a cool stream of air and the final weight was recorded. The dried extracts were stored in dessicator in a cold room at 4°C until used.

2.2.3 Analysis of plant extracts by thin layer chromatography (TLC)

Hexane, dichloromethane, methanol and water extracts were solubilised in 1 ml acetone. The chemical profile of the extracts was determined by using aluminium backed thin layer chromatography plates (Merck, Darmstadt, Germany). In each case 50 µg was chromatographed. The following three solvent systems were used to develop the plates:

- Chloroform/ethyl acetate/formic acid (CEF, 10:8:2)
- Benzene/ethanol/ammonia (BEA, 18:2:0.2)
- Ethyl acetate/methanol/water (EMW, 10:1.35:1)

Development of the chromatogram was done in closed tanks in which the atmosphere had been saturated with eluent vapour by wetting a filter paper lining. Samples were applied rapidly and placed in the TLC tanks immediately to minimize the possibility of oxidation or photo-oxidation of constituents. Once developed, the separated components were visualized under visible and ultraviolet light (254 and 360 nm, Camac Universal UV lamp TL-600, USA). The TLC plates were subsequently sprayed with vanillin sulphuric acid spray reagent (2 mg of vanillin in 28 ml of methanol plus 1 ml of concentrated sulphuric acid) and heated for 4-5 minutes at 100°C until the coloured bands showed clearly on the plates.

2.3 Results

2.3.1 Serial exhaustive extraction of plant material

The amounts of dried plant material extracted by each of the solvents used in this study are shown in Table 2.1. The highest extraction yields were obtained with H_2O and MeOH while Hex and DCM gave similar but lower values. As can be seen from the table, the lowest total yields were obtained from *A. spinosus* irrespective of the solvent used.

Table 2.1: Extraction yields of weedy plants using different solvents.

Solvent	Extraction yields (%)			
	Tagetes minuta	*Lippia javanica*	*Vigna unguiculata*	*Amaranthus spinosus*
Hex	13.1	12.0	11.3	6.0
DCM	16.1	13.2	11.9	15.9
MeOH	31.8	30.7	30.1	19.8
H_2O	35.5	36.0	32.1	40.6
Total yield	96.5	91.9	85.4	82.3

Each plant was extracted 3 times with each solvent. Hex: hexane, DCM: dichloromethane, MeOH: methanol, H_2O: water.

2.3.2 TLC analysis of the plant extracts

The results obtained from this assay indicate that separation was more effective i.e. the greater number of bands, in benzene/ethanol/ammonia (BEA) solvent

system compared to the other solvent systems. The average number of bands separated using the different extracts varied from 3.7 (Hex) to 5.7 (DCM) (Table 2.2). Dichloromethane extracted the highest number of compounds that reacted with vanillin spray reagent on the TLC plates, based on the number of visible bands on the plates. No bands could be identified from the water extracts.

Table 2.2: Average number of compounds visible on chromatograms of plant extracts using three solvent systems (chromatograms treated with vanillin spray reagent).

Extractant	Number of compounds extracted			
	BEA	CEF	EMW	Average
Hex	4	4	3	3.7
DCM	7	6	4	5.7
MeOH	7	5	3	5

BEA: Benzene/ethanol/ammonia ; CEF: Chloroform/ethyl acetate/formic acid
EMW: Ethyl acetate/methanol/water; Hex: hexane; DCM: dichloromethane; MeOH: methanol

2.4 Discussion

As can be seen from the results, H_2O and MeOH extracted the highest quantity of material. It would therefore appear that the bulk of the material in the plant species collected is polar. When subjected to the TLC assay, the material showed that the number of compounds separated was less than those separated in DCM (Table 2.2). DCM is known to extract compounds such as terpenes, flavonoids, aglycones, coumarins and phenolic acids (Sarr *et al*, 2009).

Generally, hexane extracted the least amount of material and number of compounds in all the plant species. This could be expected since hexane is a non-polar solvent and will only extract non-polar compounds such as oils and fatty acids (Flores, 2009).

From the results obtained, the use of different solvents made it possible to extract preferentially, compounds that were subsequently investigated for antifungal activity. An extract with few compounds and high antifungal activity would be a logical choice for isolating potential antifungal compounds. The advantage of having an extract with a higher number of compounds is that there is a better chance that bioactive compounds will be extracted if a specific class of chemical components is not targeted. Though H_2O and MeOH yielded the highest amount of extracted material, and the DCM extract showed the highest number of compounds on TLC plates, no specific plant or solvent was chosen for the large scale screening in this study. Extracts from all four study plants were subsequently investigated for antifungal activity.

CHAPTER 3

BIOLOGICAL ASSAYS FOR PRELIMINARY SCREENING

3.1 Introduction

A number of widely-used medicinal and dietary plants have been shown to contain compounds that exhibit antifungal properties. Studies that have been carried out on some of these plants resulted in a development of formulations for food, cosmetic and other applications (Miliauskas *et al.* 2004). However, scientific information on antifungal properties of most plants, particularly those that are less widely used or considered as famine food are still rather scarce. Assessing the antifungal properties of these plants may provide untapped resources of useful and new sources for natural antifungals. The aim of the present study was to conduct preliminary screening of the extracts of *T. minuta, L. javanica, A. spinosus* and *V. unguiculata* for antifungal activity.

3.2 Materials and Methods

3.2.1 Fungal isolates

The fungal isolates were prepared according to the standard operating procedure (SOP) used at the Programme on Mycotoxin and Experimental Carcinogenesis (PROMEC) Unit of the Medical Research Council of South Africa. Isolates of four fungal species, *Fusarium verticillioides* (MRC 826, 8559 and 8267); *F. proliferatum* (MRC 6908, 7140 and 2301), *Aspergillus flavus* (MRC 3951, 3953

and 3954) and *A. parasiticus* (MRC 0200, 2528 and 0352) kept in the PROMEC Unit culture collection of the South African Medical Research Council (MRC) were used. These fungi were chosen on the basis that they are important crop pathogens in Africa and are either high or low producers of fumonisins and/or aflatoxins as shown in Table 3.1. The *Fusarium verticillioides* strains, originally isolated from maize, produced high levels of fumonisin B_1 (5.6-12.1 g/kg) while *F. proliferatum* strains from maize produced similar levels. Isolates from rice and asparagus produced lower levels (2.0-2.8 g/kg) (Table 3.1). AFB_1 production by the *A. flavus* and *A. parasiticus* isolates in maize cultures varies between extremely high (12.1 g/kg) to very low (0.04 mg/kg). The *Fusarium* isolates were grown on Carnation Leaf Agar (CLA) slants and *Aspergillus* isolates were grown on Potato Dextrose Agar (PDA) slants for 7-14 days at 100% relative humidity and temperature of 25°C. The slants were stored in a cold room at 4°C until used. Fungal suspensions were prepared by dislodging the conidia in a 20 ml aqueous solution of 0.05% Tween 20 per slant. Conidium suspensions were standardized to a 0.5 McFarland concentration and were added (12.5 ml) to 37.5 ml Potato Dextrose Broth (PDB).

Table 3.1: Fungal isolates used with the respective FB_1 and AFB_1 production profiles on maize

Fungal isolate*. / MRC number	Origin of isolate	FB_1 level (g/kg)
F. verticillioides		
MRC 826	Maize	9.1
MRC 8267	Maize	12.1
MRC 8559	Maize	5.6
F. proliferatum		
MRC 6908	Rice	2.8
MRC 2301	Maize	6.5
MRC 7140	Asparagus	2.0
A. flavus		AFB_1 level (mg/kg)
MRC 3951	Maize	18.7
MRC 3954	Maize	3.5
MRC 3953	Maize	0.04
A. parasiticus		
MRC 200	Maize	4.9
MRC 352	Peanut	73.0
MRC 2528	Unknown	11.8

*Source: Medical Research Council (MRC), PROMEC Unit culture collection database.

3.2.2 Preparation of 0.5 McFarland standard

The McFarland standard is used as a reference standard for standardizing the number of colony forming units in a fungal suspension. Barium chloride ($BaCl_2$) (1.175 g) was weighed out and made up to 100 ml solution by adding water in a 100 ml volumetric flask. In a separate flask 1 ml of concentrated sulphuric acid (H_2SO_4) was added to 99 ml of water to make a 1:100 ml solution. From this solution, 0.5 ml was discarded and 0.5 ml of the $BaCl_2$ solution was added to make a 0.5 McFarland standard.

3.2.3 Determination of minimum inhibitory concentration (MIC) of plant extracts on fungi

3.2.3.1 Agar Diffusion Assay

In this assay, the method of Rex *et al.* (1993) was used to determine the minimum inhibition concentration (MIC) of the plant extracts on isolates of the four fungi. The plant extracts (200 mg) were solubilized in 1 ml acetone because this solvent was found not to be harmful to fungi or bacteria (Eloff, 1998a) and dissolved in water to a final concentration of 10 mg/ml. This concentration was further diluted to obtain plants extracts of concentrations 8; 4; 2 and 1 mg/ml. One millilitre (1 ml) of each concentration was homogenously mixed with 9 ml of Potato Dextrose Agar (PDA) in a petri dish (60 mm in diameter). The plates were left to set for an hour and thereafter inoculated with 1 ml of a 0.5 McFarland standardized fungal suspension by evenly streaking the suspension on the agar using a sterile disposable quad loop. Amphotericin B, an antifungal agent used to

treat human pathogens and Cantus® WG, an agricultural fungicide containing boscalid as the active ingredient, were used as positive controls. All plates were covered and incubated at 35°C and 100% relative humidity. The plates were examined after 24 hours and every 24 hours thereafter up to 72 hours. The MIC was recorded as the lowest concentration of the plant extracts that inhibited fungal growth at each time interval. Clear zones on the agar indicated inhibition of fungal grow.

3.2.3.2 Microtitre Assay

A serial microdilution technique (Eloff, 1998b, Kuhajek *et al.* 2003) was used to determine the minimum inhibitory concentration (MIC) for plant extracts against the standardized conidial fungal suspensions using 96 well flat bottom plate. Each plant extract (100 mg) was solubilised/suspended in acetone (1 ml) and diluted in water (9 ml) to obtain a final concentration of 10 mg/ml. Water (100 µl) was added in all the 96 wells using a multichannel micropipette (Labnet International, Inc). The solubilized/suspended plant extracts (100 µl) were added in each of the first row of 12 wells and serially diluted to reduce the concentration of each well by 50%. To each of the diluted wells, 100 µl of standardized conidium suspensions (12.5 ml) prepared in 37.5 ml Potato Dextrose Broth (PDB) was added. The final concentrations were 2.5, 0.64, 0.32, 0.08, 0.04 and 0.02 mg/ml. Amphotericin B and Cantus® WG (boscalid) were used as positive controls. Growth controls of PDB with the respective fungal conidium suspensions without the plant extracts were also included. As an indicator of

growth, 40 µl of 0.2 mg/ml of *p*-iodonitrotetrazolium (INT, Sigma-Aldrich, Germany) was added to each of the microplate wells. The covered microplates were incubated at 25°C and 100% relative humidity initially for 48 hrs for MIC determination, and up to 120 hours for the determination of loss of activity (stability). Extracts were considered to be stable if they can maintain MIC values that ranged from 0.6-0.02 mg/ml. The plates were visually examined every 24 hours. The MIC is here recorded as the lowest concentration of the extract that inhibited fungal growth at each time interval. Fungal growth was indicated by the red colour of the INT reduced to formazan (Eloff, 1998b).

3.2.3.3 Bioautography Assay

Bioautography is a method that is used to localize antimicrobial activity on a chromatogram, where the microorganism grows directly on the TLC plate. In this study, the bioautography procedure described by Begue and Kline (1972) was used. Thin Layer Chromatography aluminium backed silica gel plates were loaded with 10 µl of solubilised plant extracts and developed using the eluents described earlier in section 2.2.3. The plates were dried for 5 days in a stream of air to remove all traces of the eluents. Once dry, the plates were sprayed with a standardized fungal suspension of actively growing cultures of isolates of the four fungi prepared in section 3.2.1. The plates were incubated overnight at 35°C and 100% relative humidity. This was followed by the spraying of the plates with a solution of INT, a fungal growth indicator, to detect biological activity on the chromatograms. INT was prepared by weighing out 50 mg of INT powder

dissolved in 25 ml of distilled water to make a stock solution. From the stock solution 2 ml of INT was added to 18 ml of distilled water to make a working solution of 0.2-mg/ml concentration. The INT solution was always prepared on the day of use. Clear zones on the chromatograms indicated inhibition of fungal growth.

3.3 Results

3.3.1 Agar diffusion assay

The results obtained in this assay indicated that none of the plant extracts inhibited the growth of the fungi tested when compared to the positive controls by visual inspection.

3.3.2 Microtitre assay

None of the plant extracts showed growth inhibitory effects against any of the *Aspergillus* isolates tested. However, conidium (spore) formation was enhanced and was most pronounced in the case of the DCM extract followed by Hex and MeOH extracts as shown in Fig. 3.1. The exact opposite was observed in the presence of Amphotericin B and Cantus® as shown in Fig. 3.2. In general, the DCM extract was more effective in inducing conidia formation followed by Hex and MeOH. No effect was observed with the H_20 extracts. Amphotericin B and Cantus, the positive controls used, did not have an effect on fungal sporulation.

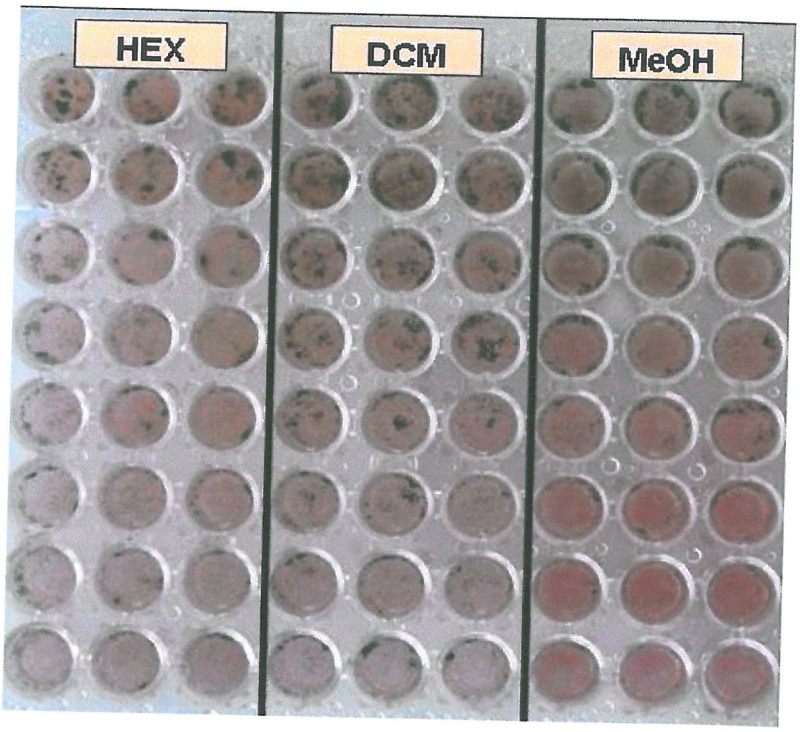


Fig. 3.1: Activity of plant extracts against *Aspergillus flavus* (MRC 3953). HEX: hexane, DCM: dichloromethane, MeOH: methanol.

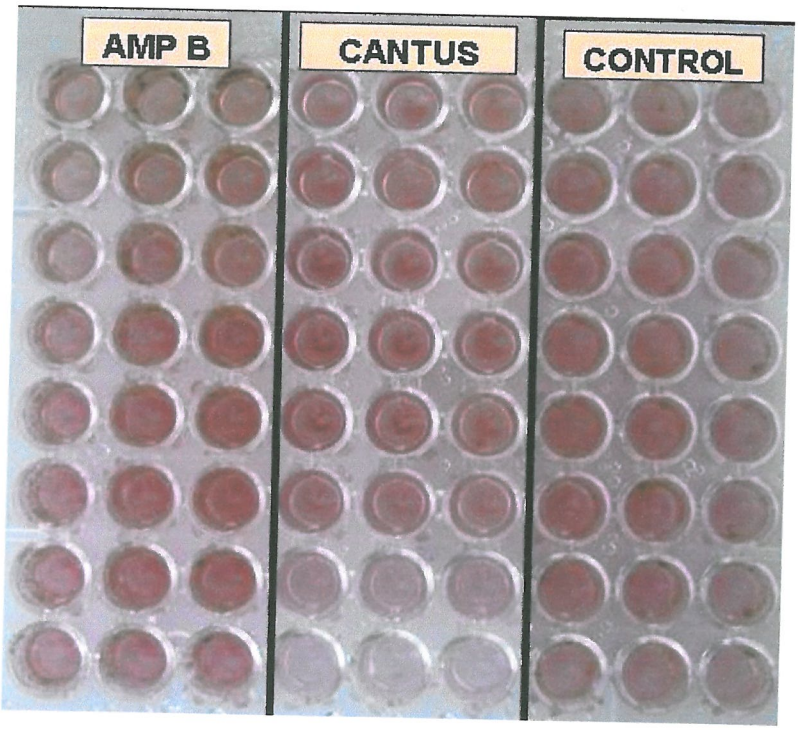


Fig. 3.2: Activity of controls against *Aspergillus flavus* (MRC 3953). Amp B: Amphotericin B.

There is no validated criteria for the MIC end points for *in vitro* testing of plant extracts, however (Souza *et al.* 2007) working on essential oils proposed classification for plant materials based on MIC results after 48 hours as follows: strong inhibitors: MIC lower than 0.5 mg/ml; moderate inhibitors: MIC between 0.6 and 1.5 mg/ml and weak inhibitors: MIC above 1.6 mg/ml. Based on the above classifications, and as can be seen in Table 3.2, the MeOH and Hex extracts of *V. unguiculata* and *A. spinosus* exhibited the broadest spectra of antifungal activity against the isolates of *Fusarium* species. Other plant extracts used exhibited moderate to weak activity and were selective in inhibiting the various fungal isolates. The MeOH extract of *T. minuta* exhibited the highest activity against *F. verticillioides* strains MRC 8559 and MRC 8267. The Hex extract showed the highest activity against two strains of *F. proliferatum,* MRC 2301 and MRC 7140. The DCM extract also exhibited the highest activity against MRC 6908 and MRC 7140. It is also interesting to note that the strain MRC 7140 was sensitive to both the Hex and DCM extracts.

The Hex extract of *L. javanica* showed the highest activity against *F. proliferatum* strains MRC 2301 and MRC 7140 and *F. verticilliodes* strain MRC 8559. The DCM extract showed the highest activity against one strain of *F. proliferatum* MRC 2301. The MeOH extract of *V. unguiculata* exhibited the highest activity against one strain of *F. proliferatum* MRC 6908 and two strains of *F. verticilliodes*, MRC 826 and MRC 8559. The Hex extract also showed the highest activity against MRC 7140 and MRC 8559. In the case of *A. spinosus*, the

highest activity was observed with the MeOH and Hex extracts. Both these extracts showed activity against MRC 7140 and MRC 8559. In general, extracts from the solvents used had at least one highly active constituent as seen in Table 3.2. Irrespective of the solvent system used, some extracts exhibited activity similar to that of Amphotericin B, the positive control used. Of all the fungal strains used, the most sensitive to the different plants extracts was MRC 7140 and MRC 8559.

Table 3.2: MIC values of plant extracts against *Fusarium proliferatum* and *F. verticilliodes* after 48 hrs.

Plants	Extracts	MIC values (mg/ml)					
		F. proliferatum			*F. verticilliodes*		
		*6908	*2301	*7140	*826	*8559	*8267
Tagetes minuta	MeOH	2.50	0.08	2.50	2.50	0.02	0.02
	Hex	2.50	0.02	0.02	0.04	0.32	0.32
	DCM	0.02	0.16	0.02	0.08	0.32	0.32
Lippia javanica	MeOH	0.32	2.50	2.50	0.32	0.64	0.32
	Hex	0.64	0.02	0.02	0.08	0.02	1.25
	DCM	0.32	0.02	0.08	0.08	0.16	0.32
Vigna unguiculata	MeOH	0.02	0.08	0.32	0.02	0.02	0.32
	Hex	0.32	0.08	0.02	0.04	0.02	0.16
	DCM	0.04	0.32	0.32	0.02	0.16	0.08
Amaranthus spinosus	MeOH	0.16	0.04	0.02	0.16	0.02	0.16
	Hex	0.04	0.04	0.02	0.04	0.02	0.16
	DCM	0.04	1.25	0.32	0.64	0.16	0.04
Amphotericin B[#]			0.04			0.02	
Cantus[#]			0.04			0.04	

MIC: minimum inhibitory concentration (values are means of triplicate determinations), MeOH: methanol, Hex: hexane, DCM: dichloromethane,

*MRC isolates. Values are means of triplicate determinations

[#]MIC of controls was the same for all strains shown.

The fungal isolates showed varying degrees of sensitivity and tolerance to the plant extracts. The results showed that there was no uniform response within or between fungal isolates of the same species in terms of susceptibility to antifungal activity in the extracts. *F. proliferatum* strain MRC 2301 was more sensitive to the MeOH extract of *T. minuta* than the other two strains of this species, while strains MRC 2301 and MRC 7140 were more sensitive to the Hex extract. All three strains exhibited a similar sensitivity to the DCM extract. When considering the MeOH extract of *T. minuta*, *F. verticillioides* strains MRC 8556 and MRC 8267 were more sensitive than strain MRC 826, while the latter was more sensitive to the Hex and DCM extracts. With respect to *T. minuta* and *L. javanica* the DCM extracts tended to exhibit the highest activity, although there were exceptions.

Tables 3.3 and 3.4 show results obtained from an investigation carried out to determine the stability of the plant extracts against the test fungi. The results obtained in the presence of Amphotericin B and Cantus appear to suggest that after 24 hrs the inhibitory effect decreases by more than 80 times. The methanol extract of *T. minuta* exhibited growth inhibitory activity against *F. proliferatum* isolate MRC 2301. The same extract, however, was only stable up to 72 hrs against *F. verticilliodes* strain MRC 8559. The hexane extract maintained its stability against MRC 2301 and MRC 7140 and against all *F. verticilliodes* strains through out the incubation time period. The DCM extract was only stable for 72 hrs against all strains of the *Fusarium* species tested. All extracts of *L. javanica* appear to be unstable, losing activity after 72 hrs. In general it would appear that

the MeOH and Hex extracts from *V. unguiculata* and *A. spinosus* not only exhibited the highest inhibitory effect but also showed the highest stability over the 120 hrs incubation period against all *Fusarium* strains. Once again the DCM extract of *V. unguiculata* was only stable against one strain of *F. proliferatum*, MRC 6908 and two strains of *F. verticilliodes*, MRC 826 and MRC 8559. A similar response was observed with the DCM extract of *A. spinosus* which only maintained stability against *F. proliferatum* strain MRC 6908. Generally, it would appear that the DCM extracts are not stable over the extended experimental time period. The water extracts exhibited no activity at the highest concentration (2.5 mg/ml) used.

The polarity of the extraction solvents used sequentially decrease in the order of MeOH>DCM>Hex. Generally, it appears that the inhibitory effect of the plant extracts varies depending on the specific plant and solvent used as well as the fungal isolate with no specific trend related to the polarity of the solvent.

Table 3.3: MIC values of plant extracts against *Fusarium proliferatum*.

Plants	Extracts	MIC (mg/ml)											
		48 hrs			72 hrs			96 hrs			120 hrs		
		**6908*	**2301*	**7140*	*6908*	*2301*	*7140*	*6908*	*2301*	*7140*	*6908*	*2301*	*7140*
Tagetes minuta	MeOH	2.50	0.08	2.50	2.50	0.64	2.50	2.50	0.64	2.50	2.50	0.64	2.50
	Hex	2.50	0.02	0.02	2.50	0.32	0.16	2.50	0.64	0.32	2.50	0.64	0.32
	DCM	0.02	0.16	0.02	0.02	0.64	0.64	0.16	2.50	2.50	2.50	2.50	2.50
Lippia javanica	MeOH	0.32	2.50	2.50	0.64	2.50	2.50	0.64	2.50	2.50	1.25	2.50	2.50
	Hex	0.64	0.02	0.02	1.25	0.32	0.64	2.50	2.50	1.25	2.50	2.50	2.50
	DCM	0.32	0.02	0.08	0.32	2.50	1.25	1.25	2.50	2.50	1.25	2.50	2.50
Vigna unguiculata	MeOH	0.02	0.08	0.32	0.08	0.08	0.32	0.16	0.32	0.32	0.32	0.32	0.32
	Hex	0.32	0.08	0.02	0.32	0.08	0.16	0.32	0.64	0.16	0.64	0.64	0.32
	DCM	0.04	0.32	0.32	0.16	1.25	1.25	0.16	2.50	2.50	0.32	2.50	2.50
Amaranthus spinosus	MeOH	0.16	0.04	0.02	0.16	0.04	0.02	0.16	0.04	0.04	0.32	0.16	0.16
	Hex	0.04	0.04	0.02	0.04	0.08	0.08	0.04	0.16	0.08	0.08	0.16	0.16
	DCM	0.04	1.25	0.32	0.08	2.50	2.50	0.08	2.50	2.50	0.08	2.50	2.50
Amphotericin B[#]			0.04			2.50			2.50			2.50	
Cantus[#]			0.04			2.50			2.50			2.50	

MIC: minimum inhibitory concentration (values are means of triplicate determinations)

MeOH: methanol, Hex: hexane, DCM: dichloromethane

*MRC isolates. Values are means of triplicate determinations

[#]MIC of controls was the same for all strains shown

Table 3.4: MIC values of plant extracts against *Fusarium verticillioides.*

Plants	Extracts	MIC (mg/ml)											
		48 hrs			72 hrs			96 hrs			120 hrs		
		*826	*8559	*8267	826	8559	8267	826	8559	8267	826	8559	8267
Tagetes minuta	MeOH	2.50	0.02	0.02	2.50	0.64	2.50	2.50	2.50	2.50	2.50	2.50	2.50
	Hex	0.04	0.32	0.32	0.16	0.32	0.32	0.64	0.64	0.64	0.64	0.64	0.64
	DCM	0.08	0.32	0.32	0.08	0.32	0.64	0.64	2.50	2.50	1.25	2.50	2.50
Lippia javanica	MeOH	0.32	0.64	0.32	0.32	0.64	0.64	0.64	2.50	2.50	1.25	2.50	2.50
	Hex	0.08	0.02	1.25	0.32	0.02	2.50	0.64	2.50	2.50	1.25	2.50	2.50
	DCM	0.08	0.16	0.32	0.08	0.64	0.64	0.64	2.50	2.50	1.25	2.50	2.50
Vigna unguiculata	MeOH	0.02	0.02	0.32	0.08	0.02	0.32	0.08	0.64	0.32	0.08	0.64	0.32
	Hex	0.04	0.02	0.16	0.08	0.02	0.16	0.16	0.64	0.64	0.16	0.64	0.64
	DCM	0.02	0.16	0.08	0.04	0.16	0.16	0.08	0.32	2.50	0.32	0.32	2.50
Amaranthus spinosus	MeOH	0.16	0.02	0.16	0.16	0.02	0.32	0.32	0.04	0.32	0.32	0.32	0.64
	Hex	0.04	0.02	0.16	0.16	0.04	0.32	0.32	0.16	0.64	0.64	0.32	0.64
	DCM	0.64	0.16	0.04	0.64	0.16	2.50	1.25	1.25	2.50	1.25	1.25	2.50
Amphotericin B[#]			0.02			2.50			2.50			2.50	
Cantus[#]			0.04			2.50			2.50			2.50	

MIC: minimum inhibitory concentration (values are means of triplicate determinations)

MeOH: methanol, Hex: hexane, DCM: dichloromethane.

*MRC isolates. Values are means of triplicate determinations

[#]MIC of controls was the same for all strains shown

3.3.3 Bioautography assay

This method did not work - the fungal growth on TLC plates was poor and it was difficult to detect inhibition zones.

3.4 Discussion

Both Amphotericin B and Cantus appear to be unstable beyond 48 hrs. This finding is not surprising as Amphotericin B have been previously reported to be unstable against fungal cultures (Cheung *et al.* 1975). Amphotericin B has been reported to be stable in the intravenous solution 5% dextrose in water, even when exposed to fluorescent light, for up to 24 hrs at room temperature (Block and Bennett, 1973; Shadomy *et al.* 1973). However, reducing agents, pH, and temperature of incubation have all been shown to affect its antifungal activity (Cheung *et al.* 1975). Other authors have also reported a pronounced decay of Amphotericin B in various media when the incubation period of was increased by 72 to 96 hrs (Brandsber and French, 1972). No data could be found in the literature to show that Cantus is also unstable.

Lack of activity by the plant extracts against fungal growth observed when using the agar diffusion assay can be explained by a number of possible reasons. Firstly, it is worth noting that testing for antifungal activity using plants extracts on fungi of agricultural importance has not been extensively explored. Hence there are not standardised or validated methods of testing plants extracts on fungal growth. The agar diffusion assay which is normally used for bacterial growth was

tried and proved to be of little or no use in as far as the fungi used in the study is concerned. Even though this has been previously observed (Pauli, 2006), it was worth trying since different fungi have different charecteristics of growth. It would therefore seem unreasonable to conclude that the agar diffusion assay is not appropriate for fungal growth.

Secondly, it could be possible that there was a problem with the solubility of the plants extracts in agar due to some physical-chemical properties of the constituents in the extracts and the agar. The study of these physical-chemical properties of these constituents was beyond the scope of the current study. In this study all the dried plant extracts were solubilised in acetone and dissolved in water. It could be possible that once the solubilised plant extracts were mixed with agar, when acetone evaporated, resulted in the precipitation of the plant constituents, influencing the rate at which constituents is absorbed or becomes available at the target place by diffusion. This assumption is based on fact that the agar diffusion assay is limited to substances with considerable water solubility (Scorzoni *et al.* 2007).

Lastly, it is possible the fungi used had limited submerged growth. This is in accordance with an explanation proferred by an experienced mycologist (personal communication). It could be possible that during growth they did not fully come into contact with the extracts in the agar medium or was unable to absorb the plant extracts to have an effect.

Although the plant extracts did not show any activity when using the agar diffusion assay, the extracts were observed to be active when the microtitre assay was used. As seen from the results the activity of the plant extracts differed when tested against different fungal isolates of the same species. This agrees with what has been previously reported about fungal sensitivity to antifungal agents (Souza *et al.* 2007; Mazzola *et al.* 1995). These kinds of differences in sensitivity among fungi against antifungal compounds in plants extracts may be explained by the differences in cell wall composition and/or chromosomal alteration that can be transferred among fungal isolates (Mazzola *et al.* 1995). In this study it is possible that a resistance mechanism is operating in isolates that exhibited reduced sensitivity to the plant extracts. According to Mazzola *et al.* (1995), in bacteria if resistance to a xenobiotic is conferred by way of a detoxification mechanism, co-existence of sensitive and resistant strains at a particular microsite may have the potential to decrease the efficacy of that substance. Whether this occurs with fungi or not would be interesting to investigate. The sensitivity of a specific fungal isolates to a specific extract is also of interest as discussed above. This would imply that the diverse fungal genetics and the selectivity of specific plant constituents are important when considering the antifungal properties. Care should therefore be exercised as this could result in the selection of a specific resistant genotype that can either be less or more mycotoxigenic.

The difference in inhibitory activity of the plant extracts observed in this study could also be explained by the diversity of molecules extracted with the different

solvents. As previously stated in the introduction (Table 1.2), MeOH tends to extract compounds such as polyphenols (tannins and flavonoids), glycosides, and to some extent glycosylated terpenoids (Rauha *et al.* 2000) that can be assumed to have contributed to the inhibitory effect of the extract. Hexane extracts mainly fats and fatty acid that are presumably more stable (Gomez *et al.* 1996), therefore it is not suprising that in the stability study activity would still be observed. The results obtained from the stability study implied that constituents that had been extracted by the hexane remained stable. Using the cutoff point of 0.6 mg/ml, *V. unguiculata* and *A. spinosus* gave extracts with the most stability. It would appear that of the three extractants used in this study, the Hex extracts of *T. minuta* as well as both the Hex and MeOH extracts of *V. unguiculata* and *A. spinosus* would be a logical choice for further development since these extracts exhibited the highest activity and stability over the 120 hrs incubation period.

In the case of the *Aspergillus* species tested, the results obtained when exposing the fungi to the plant extracts are in agreement with the previously reported findings by Paterson, (2007). In that study, spore formation was reported to occur when fungi were under stress due to the application of fungicides, nitrogen fertilizers. It is likely that the enhanced spore formation observed with *Aspergillus* isolates in the present study was in reaction to treatment with the plants extracts. It is reasonable to assume that the plants extracts used in this study contain fungistatic agents. These are agents that are known to reduce the replication of fungi (Paterson, 2007). Again as argued earlier, it would be logical to pursue

further investigation studies that will lead to the development of fungistatic agents from this plant species.

The bioautography assay appeared not to be a good guide for any further phytochemical studies that would lead to the development of antifungal agents using the *Apergillus* and *Fusarium* species. Instead identification in the plant extracts was done using Liquid Chromatography/ Mass Spectrophotometry fingerprinting. This was done after investigating the effect of the plants extracts on toxin production by the test fungi.

CHAPTER 4

THE EFFECT OF PLANT EXTRACTS ON MYCOTOXIN PRODUCTION

4.1 Introduction

Food and feed are often infected before harvest and in storage by fungi that produce mycotoxins (Sánchez *et al.* 2005). The most effective way to control the presence of mycotoxins in feed and food is to prevent their formation in the field or during storage. Fungal growth and mycotoxin production risk can be lowered by the application of synthetic fungicides, however, their effectiveness is marred by setbacks that result from their widespread use (see section 1.5). The present study was undertaken to investigate the effect of extracts of *T. minuta, L. javanica, A. spinosus* and *V. unguiculata* on toxin profile of isolates of *F. verticilliodes*. For the purpose of this study only *F. verticilliodes* was selected because it is the most prevalent species in maize and is also a prolific fumonisin producer.

4.2 Materials and Methods

4.2.1 Preservation of fungal isolates

4.2.1.1 Lyophilization of fungal strains

The culture material was lyophilized using the standardized freeze dry method used by the PROMEC Unit of MRC and stored at 4°C until used.

4.2.1.2 Preparation of maize patty cultures and plant extracts

Maize patty cultures were prepared according to the method of Alberts *et al.* (1993). Commercial whole yellow maize kernels (A grade, regarded to be safe for human consumption) were finely ground using a Romer Labs Series II Mills / Subsampling mill, USA. Thirty grams (30 g) of the ground maize meal was weighed into 90 mm diameter pyrex Petri dishes using a Mettler PC 4400 Delta Range® weighing balance and 20 ml of distilled water was added to each Petri dish. The maize patties in the Petri dishes were autoclaved for 1 hr at 121°C. The maize patties were allowed to stand overnight and autoclaved once more for 1 hr. The plant extracts were suspended in acetone (1 ml) and dissolved in water to a concentration of 2.5 mg/ml. The plant extracts were sterilized by filtering them through a polycarbonated Sartorius filtering system (Goettingen, Germany) before they were used. The sterile plant extracts were sequentially diluted in 1% acetone to give concentrations of 2.5, 0.625, 0.313, 0.16 and 0.08 mg/ml. Ten milliliters (10 ml) of these concentrations of each plant extracts was added to the maize patties (10 maize patties per concentration) and incubated overnight at 25°C to enable the extracts to be absorbed into the maize patties. Controls were prepared by spiking standards into maize patties.

4.2.1.3 Inoculation and harvesting of maize patty cultures

The contents of the lyophilized vials of fungal strains being tested were reconstituted with 2 ml of sterile water to form conidial suspensions of which 1 ml was transferred into Erlenmeyer flask containing 150 ml sterile water. Each patty

was inoculated with 1 ml of this diluted spore suspension. Amphotericin B and Cantus® were used as positive controls. The negative controls were made up as maize patties (30 g) inoculated with the fungal suspension but without treatment. The maize patties were incubated in the dark for 21 days at 25°C. Thereafter, the patty cultures were harvested by scraping the patties onto a plate and placing in an oven and allowed to dry overnight at ± 50-55°C. The patties were ground using the laboratory mill (Falling Number AB, Stockholm, Sweden) to a fine meal and packaged into sterile labeled sample envelopes. The samples were stored at 4°C until mycotoxin analysis. The mill was thoroughly cleaned between each sample. Gloves and masks were worn for personal safety measures when harvesting the patties.

4.2.2 Mycotoxin analysis

4.2.2.1 Extraction of fumonisins from maize patties

Fumonisin extraction was done using the method of Shephard *et al.* (1990) with modifications. In brief, 20 g of ground maize patties were extracted by blending for 3 min with methanol : water (3:1;100 ml) in a Sorvall (Newtown, CT, USA) Omni-mixer followed by centrifugation at 4000 rpm at 4°C for 10 min. The supernatant was filtered out through Whatman no. 4 filter paper and adjusted to pH 6.00-6.50 using either sodium hydroxide or hydrochloric acid. An aliquot (10 ml) was loaded onto solid phase extraction cartridges containing silica based strong anion-exchange (SAX) media (Varian, Habor City, CA) fitted onto the solid phase extraction manifold. The columns were conditioned with methanol (5 ml)

followed by methanol : water (3:1;5 ml) and eluted at a rate of 1 ml/min. Subsequently the cartridge was washed successively with methanol : water (3:1,5 ml) and methanol (3 ml), whereafter the toxins were eluted with acetic acid:methanol (1:99;10 ml) into 20 ml scintillation vials under gravity. The eluates were transferred into 4 ml glass vials evaporated to dryness under a stream of nitrogen at 60°C. The extracts were stored in a refrigerator at 4°C until used. The extracts were reconstituted in methanol (200 µl) and 25 µl of this solution was derivatized and injected onto the High Performance Liquid Chromatography (HPLC).

4.2.2.2 Preparation of HPLC mobile phase

Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$; 15.6 g) was weighed out and made up to 1L with distilled water in a volumetric flask to make a 0.1M solution. Methanol and 0.1M $NaH_2PO_4.2H_2O$ solution (79:21) were measured and mixed well to make a mobile phase. O-phosphoric acid was used to adjust the pH of the mobile phase to 3.35. The mobile phase was filtered through 0.45 µm X 47mm membrane filter (Advantec, MFS, Inc.) under vacuum at 1 ml / min flow rate.

4.2.2.3 Preparation of *o*-phthaldialdehyde

Since the structures of fumonisins do not contain any UV chromophore or fluorescence characteristics (Ndube *et al.* 2009), HPLC with fluorescence detection of fumonisin was used. The HPLC method used in this study utilizes

precolumn derivatization with *o*-phthaldialdehyde (OPA). OPA in the presence of mercaptan reacts rapidly with primary amino acids to form intensely fluorescent derivatives. These derivatives are analyzed with good sensitivity and selectivity by high-performance liquid chromatography employing 3-microns particle size reversed-phase columns (Jones and Gilligan, 1983).

Forty milligrams (40 mg) of OPA was dissolved in 1 ml of methanol, vortexed and then diluted with 5 ml of 0.1M disodium tetraborate (3.8 g $Na_2B_4O_7.10H_2O$ in 100 ml distilled water). Fifty microlitres (50 µl) of 2-mercaptoethanol was added to the OPA solution and vortexed. The solution was stored at room temperature in a capped amber or aluminium foil-covered vial.

4.2.2.4 Derivatizing of samples and standards for HPLC analysis

Derivatization was done according to the method of Sydenham *et al.* (1996). Standards (25 µl) were derivatized with 225 µl OPA reagent and 10 µl injected onto the HPLC. Three injections of the standard were made prior to injecting samples to ensure repeatability. The samples were reconstituted in 200 µl of methanol and 25 µl of the solution derivitized with OPA (225 µl). 10 µl of the derivatized solution was injected onto the HPLC within 90 s after mixing. The fumonisin levels were quantified using the HPLC and calculated based on a standard curve. The fumonisin analoques were detected on the basis of their retention times (Rheeder *et al.* 2002).

4.3 Statistical analysis

Experimental data to determine the inhibitory concentration (IC_{50}) and coefficient of correlation (R^2) was analyzed using the Student t-test with Prism version 5.0 software.

4.4 Results

The effect of the plant extracts on fumonisin B_1 production is shown in Tables 4.1, 4.2, 4.3 and 4.4. An inhibitory effect on fumonisin B_1 production was observed at different concentrations of the plant extracts used in this study and was found to be dose dependent, i.e. as the concentration of the plant extracts increases the inhibition effect increases (figs 4.1). Generally, at higher concentrations of 0.6 mg/ml all plant extracts showed apparent inhibition of fungal growth (fig 4.1) and no toxin levels were detected (limit of detection is 5 ng/g).

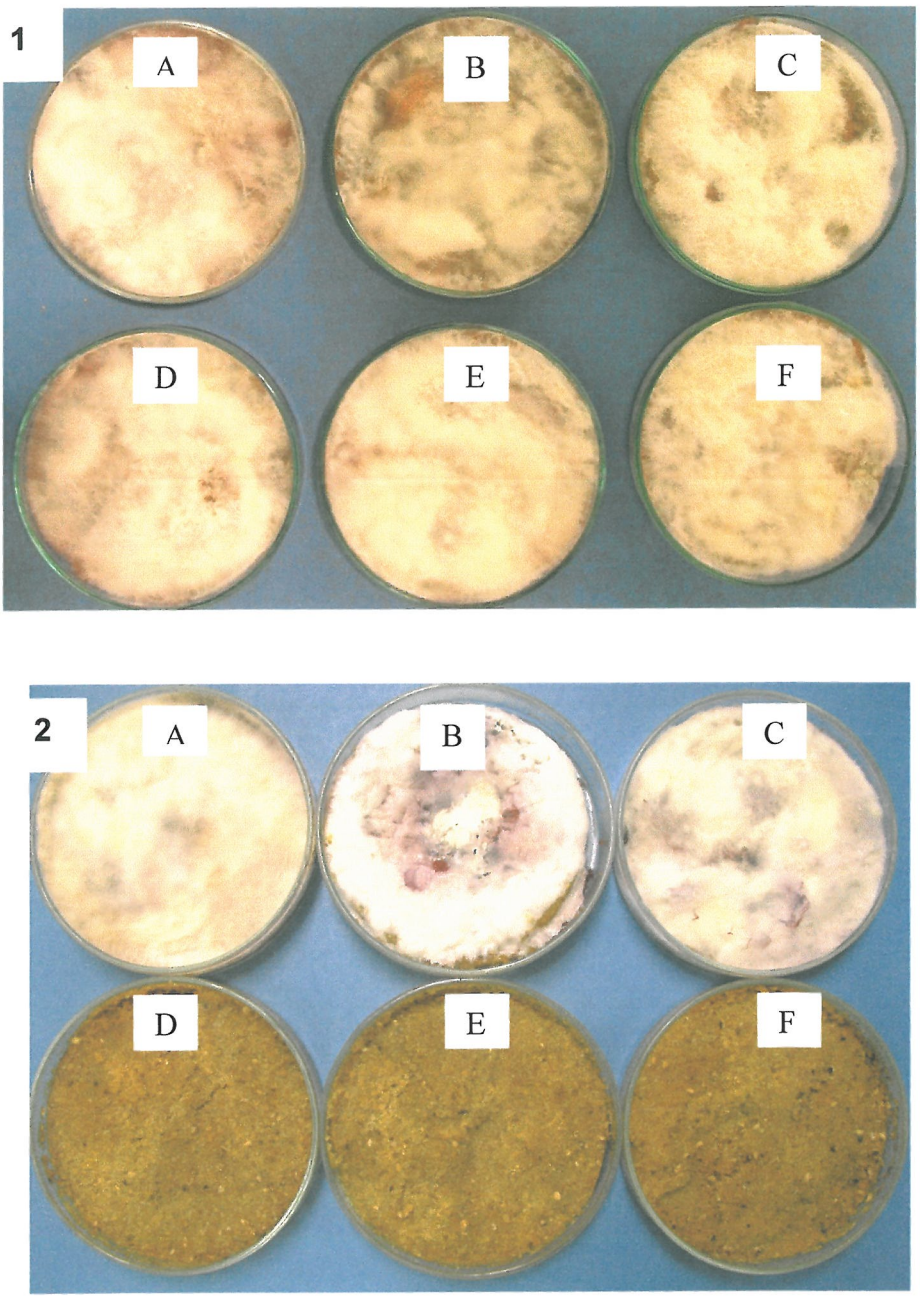


Fig 4.1: Effects of plant extracts at (1) concentrations of 0.02 mg/l; (2) concentrations of 0.6 mg/ml and controls against MRC 8267. A = Amphotericin B, B = Cantus, C = growth control, D = Hex extract, E = DCM extract, F = MeOH extract.

Fumonisin B_1 production by isolates of *F. verticillioides* was completely inhibited by the DCM extract of *T. minuta*, except at concentrations of 0.08 mg/ml. A similar pattern of inhibition was observed on the MeOH and Hex extracts of *T.*

minuta, except for the Hex extract at a concentration of 0.16 mg/ml against MRC 8267 and for the MeOH extracts at the same concentration against MRC 826 and MRC 8559.

Extracts of *L. javanica* exhibited different degrees of activity against fumonisin B_1 production. The DCM extract was generally less inhibitory on fumonisin B_1 production by all isolates of *F. verticillioides*. The MeOH and Hex extracts were the most active, inhibiting toxin production at concentrations of 0.16 mg/ml against all isolates of *F. verticillioides.*

When considering extracts of *V. unguiculata*, the DCM and MeOH extracts exhibited activity at concentrations of 0.16 mg/ml against all isolates of *F. verticillioides*, while complete inhibition was observed by the Hex extract except against MRC 826.

DCM extracts of *A. spinosus* inhibited FB_1 production at concentrations above 0.16 mg/ml against all isolates of *F. verticillioides*. Complete inhibition of FB_1 production was however observed on the MeOH extracts against MRC 8267 and MRC 8559. The Hex extracts completely inhibited the production of toxins by MRC 826. The same extracts however, showed activity against MRC 8267 and MRC 8559 except at lower concentrations of 0.08 mg/ml. In general, all plant extracts exhibited activity on FB_1 production by isolates of *F. verticillioides*, however, it would appear that the different extracts from *V. unguiculata* and *A. spinosus* exhibited the highest toxin inhibitory effects. The effect of the plant extracts at concentrations of 0.08 mg/ml was similar to that of Cantus®, the

agricultural fungicide used. Amphotericin B exhibited the least activity against all isolates of *F. verticillioides*.

Table 4.1: Effects of plant extracts on FB_1 production (g/kg) by *F. verticillioides* (MRC 826).

Extractants	Extracts concentration (mg/ml)	FB_1 concentrations* (g/kg) produced			
		T. minuta	*L. javanica*	*V. unguiculata*	*A. spinosus*
DCM	0.313	ND	0.8 ± 0.03	ND	ND
	0.16	ND	0.8 ± 0.04	ND	1.1 ± 0.1
	0.08	0.1 ± 0.02	0.9 ± 0.1	1.5 ± 0.1	1.5 ± 0.1
MeOH	0.313	ND	ND	ND	ND
	0.16	0.7 ± 0.04	1.1 ± 0.1	ND	ND
	0.08	0.9 ± 0.1	0.7 ± 0.04	0.7 ± 0.1	1.1 ± 0.1
Hex	0.313	ND	ND	ND	ND
	0.16	ND	ND	ND	ND
	0.08	0.2 ± 0.02	1.2 ± 0.2	0.9 ± 0.1	ND

DCM: dichloromethane, MeOH: methanol, Hex: hexane, ND: not detected [limit of detection (LOD) = 5ng/g]; *Mean FB_1 levels (n=3).

Table 4.2: Effects of plant extracts on FB_1 production (g/kg) by *F. verticillioides* (MRC 8267).

Extractants	Extracts concentration (mg/ml)	FB_1 concentrations* (g/kg) produced			
		T. minuta	*L. javanica*	*V. unguiculata*	*A. spinosus*
DCM	0.313	ND	1.0 ± 0.1	ND	ND
	0.16	ND	1.1 ± 0.01	ND	1.1 ± 0.1
	0.08	1.6 ± 0.2	1.1 ± 0.1	1.2 ± 0.2	1.4 ± 0.1
MeOH	0.313	ND	ND	ND	ND
	0.16	ND	ND	ND	ND
	0.08	0.7 ± 0.1	0.7 ± 0.04	1.3 ± 0.2	ND
Hex	0.313	ND	ND	ND	ND
	0.16	0.6 ± 0.2	ND	ND	ND
	0.08	1.4 ± 0.1	0.1 ± 0.02	ND	0.2 ± 0.05

DCM: dichloromethane, MeOH: methanol, Hex: hexane, ND: not detected [limit of detection (LOD) = 5ng/g]; *Mean FB_1 levels (n=3).

Table 4.3: Effects of plant extracts on FB_1 production (g/kg) by *F. verticillioides* (MRC .8559).

Extractants	Extracts concentration (mg/ml)	FB_1 concentrations* (g/kg) produced			
		T. minuta	*L. javanica*	*V. unguiculata*	*A. spinosus*
DCM	0.313	ND	0.7 ± 0.1	ND	ND
	0.16	ND	0.9 ± 0.1	ND	0.7 ± 0.2
	0.08	0.2 ± 0.02	1.0 ± 0.1	0.5 ± 0.04	1.2 ± 0.2
MeOH	0.313	ND	ND	ND	ND
	0.16	0.7 ± 0.4	ND	ND	ND
	0.08	1.5 ± 0.2	0.3 ± 0.04	1.5 ± 0.2	ND
Hex	0.313	ND	ND	ND	ND
	0.16	ND	ND	ND	ND
	0.08	ND	0.5 ± 0.04	ND	1.6 ± 0.3

DCM: dichloromethane, MeOH: methanol, Hex: hexane, ND: not detected [limit of detection (LOD) = 5ng/g]; *Mean FB_1 levels (n=3).

Table 4.4: Effects of Amphotericin B, Cantus and growth control on FB_1 production (g/kg) by *F. verticillioides* strains.

Control sets	Controls	Concentration (mg/ml)	FB_1 concentrations* (g/kg) produced		
			MRC 826	MRC 8267	MRC 8559
For DCM experiment	Growth control	-	5.0 ± 0.8	7.5 ± 2.0	6.0 ± 0.6
	Amphotericin B	0.8	3.0 ± 1.0	3.6 ± 1.0	3.3 ± 0.9
	Cantus	0.8	0.8 ± 0.4	1.1 ± 0.6	1.0 ± 0.6
For MeOH experiment	Growth control	-	4.8 ± 1.0	6.1 ± 1.0	6.3 ± 0.8
	Amphotericin B	0.8	2.7 ± 1.3	2.5 ± 0.5	3.5 ± 0.9
	Cantus	0.8	1.0 ± 0.5	1.2 ± 0.4	1.4 ± 0.4
For Hex experiment	Growth control	-	6.2 ± 1.0	5.9 ± 2.3	4.9 ± 0.8
	Amphotericin B	0.8	3.7 ± 1.5	2.6 ± 1.9	3.6 ± 1.1
	Cantus	0.8	0.9 ± 0.5	1.6 ± 1.4	1.3 ± 0.2

DCM: dichloromethane, MeOH: methanol, Hex: hexane, *Mean FB_1 levels, (n=12/ control levels), (LOD) = 5ng/g]; *Mean FB_1 levels (n=3).

Based on the good inhibitory effect of the plant extracts on mycotoxin production, it was necessary to determine the inhibition concentration (IC_{50}). To determine IC_{50}, assays were carried out below the lowest concentration of 0.08 mg/ml, i.e. 0.04, 0.02 and 0.01 mg/ml of the plant extracts. IC_{50} is the concentration of a material which inhibits toxin production by 50%. The IC_{50} was determined using the DCM and MeOH extracts of *V. unguiculata* against MRC 826 and MRC 8267. *V. unguiculata* was chosen on the basis that it was the most active against toxin production by isolates of *F. verticilliodes*. The IC_{50} values for DCM extract ranged from 0.02-0.04 mg/ml against MRC 826 and 0.03-0.04 mg/ml against MRC 8267, while the MeOH extracts ranged from 0.01-0.3 mg/ml against MRC 826 and 0.02-0.05 mg/ml against MRC 8267 (Table 4.5).

Table 4.5: IC_{50} of the DCM and MeOH extracts of *V. unguiculata* against MRC 826 and MRC 8267.

Extract	IC_{50} (mg/ml)	
	MRC 826	MRC 8267
V. unguiculata DCM	0.02 - 0.04 (R^2 = 0.9)	0.03 - 0.04 (R^2 = 1.0)
V. unguiculata MeOH	0.01 - 0.30 (R^2 = 0.9)	0.02 - 0.05 (R^2 = 0.9)

R: coefficient of correlation

4.5 Discussion

The findings of the present study highlight the potential of plant extracts in the protection of food commodities against fungal contamination. The broad spectrum of

activity exhibited by some plant extracts used in the current study was comparatively lower than that of the synthetic fungicides tested. The plant extracts successfully reduced toxin levels to levels well within the maximum tolerable limits, i.e. 1 g/kg in maize intended for direct human consumption as set by the European Union (EU) in the Commission regulation (EC) NO. 1126/ 2007 (EU, 2007). Given that the occurrence of fungal contamination and subsequent mycotoxin poisoning occurs mainly in localized hot spots, the large reductions demonstrated in this study would help to reduce the risk posed by mycotoxins. Mycotoxin production has been reported to be influenced by stresses such as the application of fungicides to crops (Paterson 2007). Fungicides and fungistats can effectively inhibit the growth of fungi, however, where control is only partial and allows some growth, mycotoxin production is stimulated (Magan *et al.* 2002). Complete inhibition of fungal growth by some plant extracts in this study is good in terms of the possibility of mycotoxin production. Anti-mycotoxigenic activity of plant extracts has been previously reported (Joseph *et al.* 2005; Atanda *et al.* 2007; Soliman and Badeaa, 2002; Rasooli and Owlia, 2005; Lopez-Malo *et al.* 2005; Hua *et al.* 1999). Most of these studies however, focused mainly on the essential oils of herbs, spices and a few plants. Nothing has been reported on the plant species used in this study, except for the antimycotoxigenic activities of essential oils of *T. minuta* against *Aspergillus parasiticus* (Rasooli and Owlia, 2005). This is the first study to report on the antimycotoxigenic activity of extracts of *L. javanica, T. minuta, A. spinosus* and *V. unguiculata*.

The mechanism of inhibition of toxin production by the plant extracts in this study is still unclear, but we can speculate that the observed activity was due to an enhanced oxidation of phenolic compounds from the plant extracts. Phenolic oxidation has been reported to produce semiquinones, quinones and free radicals which play an important role in the resistance of plants to microbial infections and adverse climatic conditions (Ruuhola *et al.* 2007). Once formed, these quinones undergo polymerization reactions, leading to the production of dark, insoluble polymers referred to as melanins, which exhibit antifungal activity (Mayer, 2006; Mahanil *et al.* 2008). Quinones are aromatic rings with two ketone substitutions. They are ubiquitous in nature and are characteristically highly reactive (Schmidt, 1988). In addition to providing a source of stable free radicals, quinones are known to complex irreversibly with nucleophilic amino acids in proteins (Stern *et al.* 1996), often leading to inactivation of the protein and loss of function. For that reason, the potential range of quinone antimicrobial effects is great. Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes. Quinones may also render substrates unavailable to the microorganism (Duke, 1985).

Based on the strong activity of the plant extracts tested in this study, the aim of the next chapter was focused on determination of the antioxidant and phenolic content of the plant extracts. We speculated based on the literature that activity was due to the present of phenolic compounds.

CHAPTER 5

ANTIOXIDANT ACTIVITY AND PHENOLIC CONTENT IN PLANT EXTRACTS

5.1 Introduction

Previous studies have shown that more than 90% of the antioxidant capacity of plant extracts is derived from the contribution of phenolic compounds some of which have also been found to be antifungal (Javanmardi *et al.* 2003). However, it is also worth noting that antioxidant capacity is not limited to phenolics. The activity may also come from other compounds that are not phenolic compounds. Phenolic compounds have been used for many years as disease preventing agents and have also been reported to have antifungal activities (Manson *et al* 1997). The present study was undertaken to investigate the total phenolic content and related antioxidant activities of extracts of *A.spinosus, V. unguiculata, L. javanica* and *T. minuta*. We also wanted to find out if there was any correlation between biological activity seen in chapter 3 and the presence of phenolic compounds / anti-oxidant activity.

5.2 Materials and methods

5.2.1 The 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method

Anti-oxidant activity of the plant extracts was determined using the method described by Re *et al.* (1999) with slight modifications using trolox as a standard. This method measures the hydrogen atom (or one electron) donating activity and hence provides a measure of free radical scavenging antioxidant activity (Kirby and

Schmidt, 1997). The principle of the reaction is that the anti-oxidant compounds react with $ABTS^+$ radical. During the reaction, the solution becomes clear or decreases in colour intensity, thus decreasing the absorbance. The ABTS solution was prepared at a concentration of 7 mM (0.01920g ABTS) and the potassium persulfate ($K_2S_2O_8$) solution at a concentration of 2.45 mM (0.18923g $K_2S_2O_8$) using distilled water (5 ml). The radical cation $ABTS^+$ was chemically generated with $K_2S_2O_8$ and the ABTS solution. Hence 88 µl of the $K_2S_2O_8$ solution was added to 5 ml of ABTS solution, mixed well, covered with foil and kept at room temperature for 12-16 hours before use in the experiment. The $ABTS^+$ radical (stable for 2-3 days) was diluted with ethanol and maintained on ice throughout the experiment. The experiment was preformed only if the absorbance of the radical was 0.70±0.02 at 734 nm using a UVIKON 923, double beam UV/VIS spectrophotometer (Bio-tek Kontron Instrument, A.D.F, South Africa).

Working solutions of plant extracts were prepared at starting concentrations of 5 mg/ml for DCM and MeOH extracts in dimethy sulfoxide. The Hex extracts were prepared at starting concentrations of 20 mg/ml because lower concentrations gave readings outside the standard range.

From the working solutions of the plant extracts, two further dilutions were prepared in DMSO to obtain final concentrations of 5, 2.5 and 1 mg/ml for the DCM and MeOH extracts and 20; 10 and 4 mg/ml for the Hex extracts. To a sample of 50 µl of each concentration, in a test tube, a volume of 1 ml of the $ABTS^+$ radical was added and kept at 37°C in a water bath for four minutes before being transferred into a

cuvette and the absorbance was measured at 734 nm. Each sample was tested in triplicate. The colour interference of the plant extracts was not taken into consideration because there was very little variation.

5.2.2 The Ferric Reducing Ability of Plasma (FRAP) method

The FRAP assay was carried out according to the method of Benzie and Strain (1996) using trolox as a standard. This method measures the ability of a compound to reduce Fe^{3+} to Fe^{2+}. The FRAP assay uses the antioxidants as reductants in a redox-linked colorimetric method, in which Fe^{3+}-TPTZ is reduced to Fe^{2+l}-TPTZ by antioxidants. Fe^{2+}-TPTZ has an intense indigo/blue colour. FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue colored Fe^{2+}-TPTZ compound from colourless oxidized Fe^{3+} form by the action of electron donating antioxidants (Bakasso *et al.* 2008).

The FRAP reagent included the preparation of 300 mM sodium acetate buffer solution, pH 3.6 [3.1 g sodium acetate and 16 ml glacial acetic acid per litre of buffer solution]; 10 mM/L TPTZ in 40 mM HCl (0.06248 g TPTZ in 50 ml of 40 mM HCl); 20 mM $FeCl_3.6H_2O$ (0.1622 g $FeCl_3$ in 50 ml H_2O); 5 mM Trolox stock solution (0.01251 g Trolox in 10 ml MeOH). The working FRAP reagent was prepared by mixing 150 ml of the 300 mM sodium acetate buffer solution, 15 ml of the 10 mM TPTZ in 40 mM HCl and 15 ml of the 20 mM $FeCl_3.6H_2O$ solution. The FRAP reagent was only mixed once the standards and extracts (in triplicate) were ready for the 4 min incubation. Plant extracts were prepared as in section 2.1.2. To a sample of 0.1 µl of

each concentration, in a test tube, a volume of 3 ml of the FRAP reagent was added and kept at 37°C in a water bath for four minutes before being transferred into a cuvette and the absorbance was measured at 593 nm.

5.2.3 Investigation of total phenolic content

Total phenolic concentration in the plant extracts was determined spectrophotometrically by the Folin-Ciocalteu assay (Gao *et al.,* 2000). The method is based on the reduction of clear phosphotungstic acid in alkaline solution to phosphotungstic blue. The absorbance of formed phosphotungstic blue is proportional to the number of aromatic phenolic groups and is used for their quantification, with gallic acid as the standard (Horzic *et al.* 2009).

Anhydrous sodium carbonate was prepared at a concentration of 75 g/L. The solution was stirred and heated until the sodium carbonate was completely dissolved before the solution was filtered using Whatman no 1 filter paper. The Folin-Ciocalteu stock solution was prepared at a concentration of 10% (v/v) with distilled water. The correlation between the antioxidant activity and total phenolic content was determined using a linear regression. To determine the total phenolic content, the mean of three readings was recorded and the quantification expressed in mg/ g of gallic acid equivalent dry sample (mg/g GAE). A 1 ml volume plant extract was mixed with 5 ml of Folin-Ciocalteu reagent followed by the addition of 4 ml of anhydrous sodium carbonate and incubation at room temperature in the dark for 2

hours. The absorbance of the reaction mixture was determined twice at 765 nm against a sample containing only DMSO.

5.3 Statistics

Statistical analysis of phenolic content and antioxidant activity to determine the coefficient of correlation (R^2) was analyzed using the Student t-test with Prism version 5.0 software. Correlation between the anti-oxidant activity and the total phenolic content was determined using Strata software.

5.4 Results

The results of the three assays FRAP, ABTS and total polyphenolic content for antioxidant activity of the plant extracts are given in Table 5.1. The best activities using the FRAP assay were found with extracts of *V. unguiculata* (0.06- 0.13 mmol trolox/mg) and *T. minuta* (0.04-0.27 mmol trolox/mg). Extracts of *A. spinosus* and *L. javanica* had similar activities (0.01-0.09 mmol trolox/mg) except for the MeOH extracts (0.11 and 0.27 mmol trolox/mg respectively).

Using the ABTS assay highest antioxidant activity was obtained from the MeOH extracts (0.33-0.69 µmol/mg) of the four plant species, followed by the DCM (0.15-0.37 µmol/mg), while Hex extracts had the lowest activities (0.03-0.09 µmol/mg). As can be seen from the table, the Hex extracts of all the plants had the lowest activity in both the FRAP and ABTS assays while the MeOH extracts showed the highest antioxidant activity, followed by the DCM extracts.

Table 5.1: Antioxidant activity and total phenolic content of the plant extracts.

Plant species	FRAP (mmol trolox/mg)			ABTS (µmol/mg)			Phenolic Content (mg GAE/ml)		
	Hex	DCM	MeOH	Hex	DCM	MeOH	Hex	DCM	MeOH
V. unguiculata	0.06	0.10	0.13	0.07	0.19	0.36	0.28	0.43	0.82
A. spinosus	0.01	0.09	0.11	0.03	0.15	0.33	0.11	0.42	0.64
L. javanica	0.01	0.09	0.27	0.05	0.37	0.74	0.24	0.43	1.63
T. minuta	0.04	0.14	0.27	0.09	0.21	0.69	0.86	1.07	1.42

Hex: hexane, DCM: dichloromethane, MeOH: methanol

The phenolic content estimated in the plant extracts ranged from 0.11-1.63 mg/g GAE dry weight (Table 5.1). The MeOH extracts of all the plant species had the highest phenolic content (0.64-1.63 mg GAE/ml) and this correlated to the highest antioxidant activity, while the Hex extracts had the lowest total phenolic content (0.11-0.28 mg GAE/ml), except for the *T. minuta* extract (0.86 mg GAE/ml) As seen from Table 5.2, strong statistical correlations ware found between the FRAP MeOH/ ABTS MeOH assays (R^2 = 0.99, $p<0.05$) and the FRAP Hex/ ABTS Hex (R^2 = 0.74, $p>0.05$) while no correlation was found between the FRAP DCM/ ABTS DCM (R^2 = -0.20, $p>0.05$) assays. A strong correlation was also obtained between the different total phenolic contents (TPP) and antioxidant activities (R^2 = 0.98, $p>0.05$ for FRAP MeOH/ TPP MeOH; R^2 = 0.88, $p>0.05$ for ABTS Hex/ TPP Hex and R^2 = 0.99, $p<0.05$ for ABTS MeOH/ TPP MeOH; R^2 = 0.98, $p>0.05$ for FRAP DCM/ TPP DCM).

Table 5.2: Statistical analysis of phenolic content and antioxidant activity.

	FRAP Hex	FRAP DCM	FRAP MeOH	ABTS Hex	ABTS DCM	ABTS MeOH	Phen Hex	Phen DCM
FRAP Hex	1.0							
FRAP DCM	R = 0.46	1.0						
	p> 0.54							
FRAP MeOH	-0.16	0.50	1.0					
	0.84	0.50						
ABTS Hex	0.74	0.87	0.51	1.0				
	0.26	0.13	0.50					
ABTS DCM	-0.37	-0.20	0.73	-0.0	1.0			
	0.63	0.80	0.273	1.0				
ABTS MeOH	-0.23	0.41	0.99	0.42	0.79	1.0		
	0.77	0.59	0.01	0.58	0.21			
Phen Hex	0.41	0.99	0.63	0.89	-0.04	0.55	1.0	
	0.59	0.01	0.37	0.11	0.96	0.45		
Phen DCM	0.28	0.98	0.58	0.78	-0.13	0.50	0.98	1.0
	0.72	0.02	0.42	0.22	0.87	0.50	0.02	
Phen MeOH	-0.19	0.34	0.98	0.42	0.85	0.99	0.42	1.0
	0.81	0.66	0.02	0.58	0.16	0.01	0.58	

R = coefficient of correlation, p = significant level

5.5 Discussion

In the present study the highest phenolic content and the best antioxidant activities using two different methods were observed with the MeOH extracts of all the plant species. The antioxidant activity and the total phenolic content of the plant extracts increased with increasing solvent polarity. It would be reasonable to assume that the phenolic compounds contained in the MeOH were responsible for the antioxidant activity. This agrees with results obtained from several other studies that have conclusively shown a close relationship between total phenolic content and antioxidant activity (Velioglu *et al.* 1998; Deighton *et al.* 2000; Surveswaran *et al.* 2007). The observed antioxidant activities have been attributed, in part, to the flavonoids (e.g. apigenin, rutin, quercetin, kaempferol and isorhamnetin) (Mucsi *et al.* 1992; Lattanzio *et al.* 2000; Pascual *et al.* 2001), terpenoids (Oigiangbe and Onigbinde, 1996; Manenzhe *et al.* 2004) and phenolic acids (Ickes *et al.* 2006; Gil *et al.* 2002). These compounds are known to be present in the plant species.

In some plants the flavanoids, terpenoids and phenolic compounds have been characterised. The antioxidant activity of an extract cannot be explained based only on their phenolic content without further characterization as has been reported by Heinonen *et al.* 1998; Muchuweti *et al.* 2006. Their studies have indicated that the antioxidant activity of phenolic compounds is related to their structure. It has been reported that phenolic compounds with *ortho*- and *para*-dihydroxylation or a hydroxyl and a methoxy group are more effective than simple phenolics (Frankel *et al.* 1995). It has been observed that these compounds exhibit strong activity when extracted using polar solvents (Duan *et al.* 1998; Tepe *et al.* 2005) which is what has been

observed in the present study. Briefly, it can be explained how this comes about. For example, if an electron donating group, especially a hydroxyl group (-OH), is located on the *o*- or *p*- positions of the phenolic compound, it makes the compound polar and therefore antioxidant activity is increased (Duan *et al.* 1998).

Effective antioxidants are radical scavengers that break down radical chain reactions. According to Salah *et al.* (1995), there are three chemical criteria for effective radical scavenging by polyphenols. These are the presence of the *o*-dihydroxy structure in the B ring which confers higher stability to the radical form and participates in electron delocalization, a 2, 3 double bond in conjugation with the 4-*oxo* function in the C ring contributes through participation in electron delocalization from the B ring, and the 3- and 5-OH groups with 4-*oxo* function in the A and C rings for maximum radical scavenging potential. As will be seen in the next chapter flavonoids seem to be present in higher quantities in the test plants, and seem to satisfy the above determinants. It is reasonable then to conclude that they are the ones responsible for the antioxidant activity. As was seen in chapter three, the MeOH extracts exhibited higher antifungal activity. MeOH extracts from all the plant species contained compounds that were responsible for the both antifungal and antioxidant activities. It can be hypothesized that the scavenging activity may result in antifungal activity. The mechanism involved in both scavenging activity, antifungal and antimycotoxigenic activities would require further elucidation and this can be gained thorough a understanding and investigation into the phytochemistry of the plant extracts.

CHAPTER 6

PHYTOCHEMICAL INVESTIGATION

6.1 Introduction

Phytochemical studies usually involve a combination of the use of different analytical instruments, including nuclear magnetic resonance (NMR), X-ray crystallography, mass spectrometry (MS), ultraviolet (UV) and infrared (IR). NMR spectroscopy is the gold standard for the characterization of the structures of pure isolated unknown compounds (Lee *et al.* 2005). However, for the gross analysis (fingerprinting) of complex mixtures, liquid chromatographic separation followed by spectroscopic measurement is suitable. Liquid chromatography / mass spectrometry (LC/MS) in particular has recently emerged as the preferred method of choice as it is more comprehensive and independent of physico-chemical factors, e.g. chromophoric properties (Brandt *et al.* 2004). In this phytochemical study, screening and identification of compounds present in extracts of *Lippia javanica, Tagetes minuta, Amaranthus spinosus* and *Vigna unguiculata* was carried out using LC-MS with a view of generating metabolomic fingerprints. The present study compared the total ion chromatograms (TIC), UV spectra and the LC-MS-MS fragmentation of the four most prominent abundant compounds in the plant species. For ease of reference, compounds of interest in this study were denoted LA-1, LA-2, LA-3, LA-4, TA-1 and UB-2.

6.2 Materials and methods

The plant extracts were prepared as in section 2.1.2. Mass spectrometer experiments were done at Stellenbosch University, Central Analytical Facility (CAF). Dr Marietjie Stander is thanked for running the experiments and for assistance with data interpretation. Extracts were introduced into a Waters (Milford, Massachusetts, USA) Time of Flight (TOF) Synapt G2 mass spectrometer by injecting into a Waters UPLC front-end. The chromatographic separation was achieved on a Waters BEH C_{18} column (2.1 x 50 mm) at a flow rate of 0.4 ml / min using mobile phase A (Water + 0.1% formic acid) and mobile phase B (acetonitrile + 0.1% formic acid). Gradient elution was done as follows: 0 - 0.5 min 100% A, 0.5 – 17 min linear gradient to 100% B, then return to original conditions in the last 2 min. The TOF MS/MS system was operated in electrospray positive ion mode with capillary voltage of 3 kV and cone voltage 15V. Mass spectra were acquired in a *m/z* range of 100 - 2000. All experiments were conducted under automatic gain conditions. Tandem MS was used on the most abundant peaks in at attempt to elucidate putative structures of the compounds.

6.3 Results and discussion

The metabolomic fingerprints showed in general a great diversity of compounds eluting between 0.5 min to 16 min. However, in general and across all solvents, *L. javanica* and *T. minuta* had more compound peaks than *A. spinosus* and *V. unguiculata* (Figs. 6.1, 6.2 and 6.3). Most compounds in the range 3 -12 min were low in molecular weight <529 amu and also UV active. This led us to believe that these compounds where phenolics quite possibly flavonoids and phenolic acids. Above 12

min, there were compounds of higher molecular weight and poor chromophoric properties. It could be postulated that these compounds might be terpenoids or saturated aliphatic.

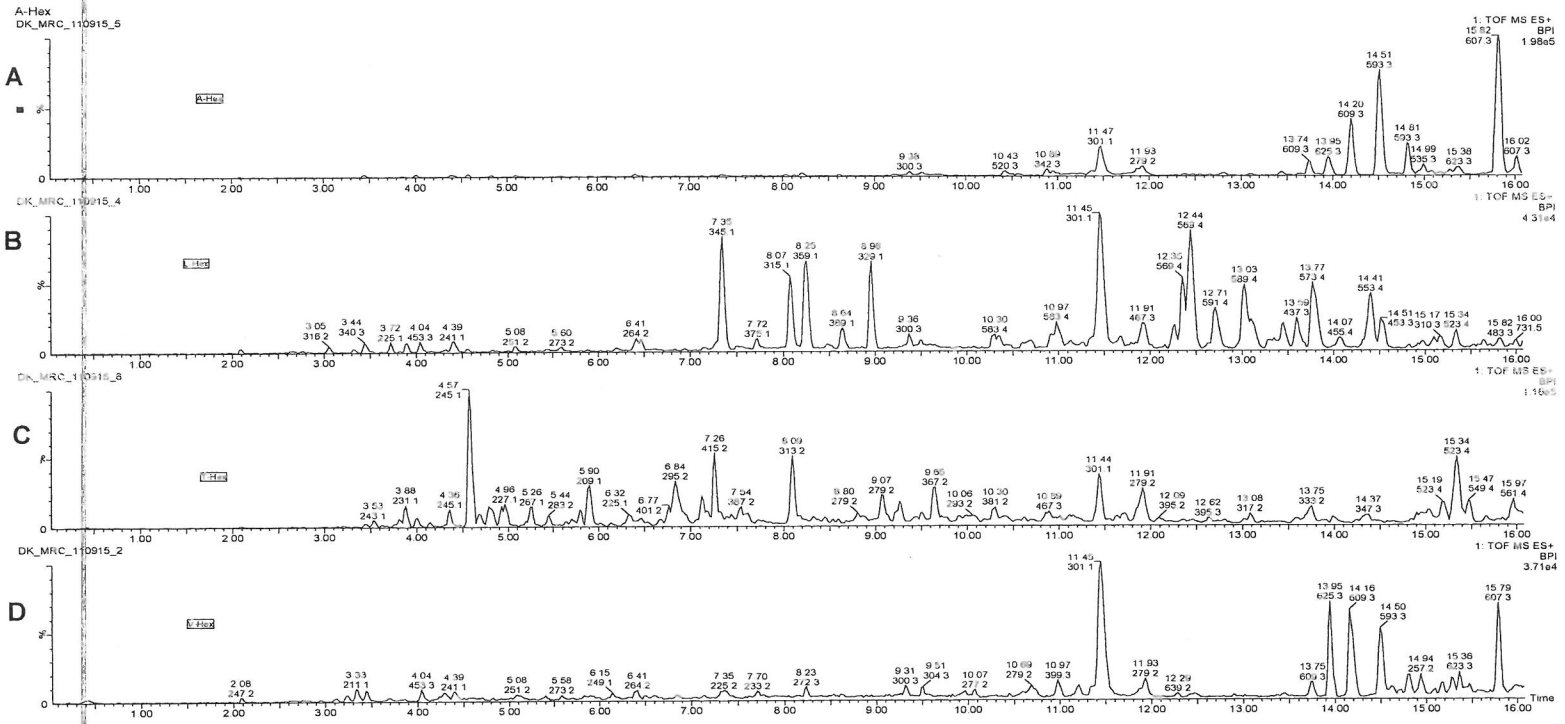


Fig. 6.1: Chromatograms of the Hex extracts – A= *A. spinosus, B= L. javanica, C= T. minuta,D= V. unguiculata.*

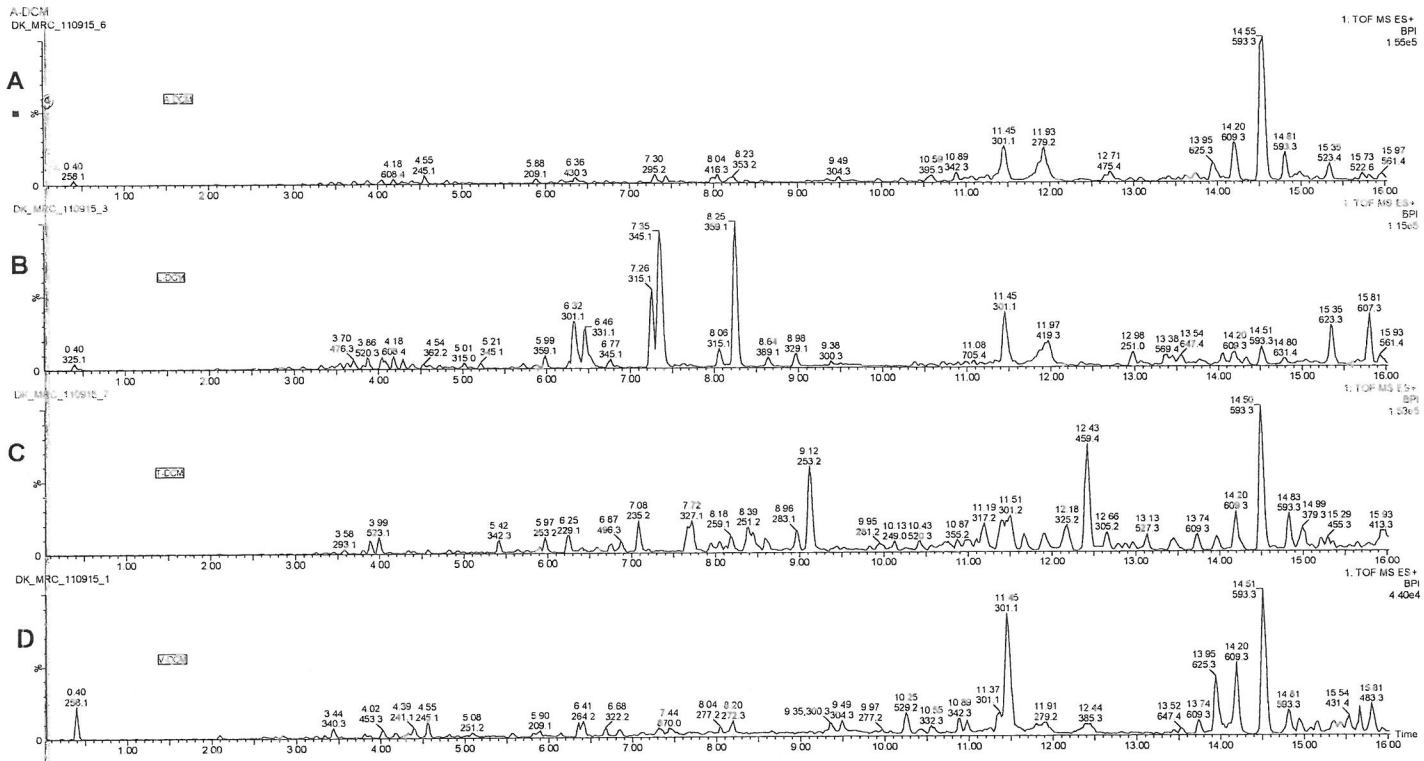


Fig. 6.2: Chromatograms of the DCM extracts – A= *A. spinosus, B= L. javanica, C= T. minuta D= V. unguiculata.*

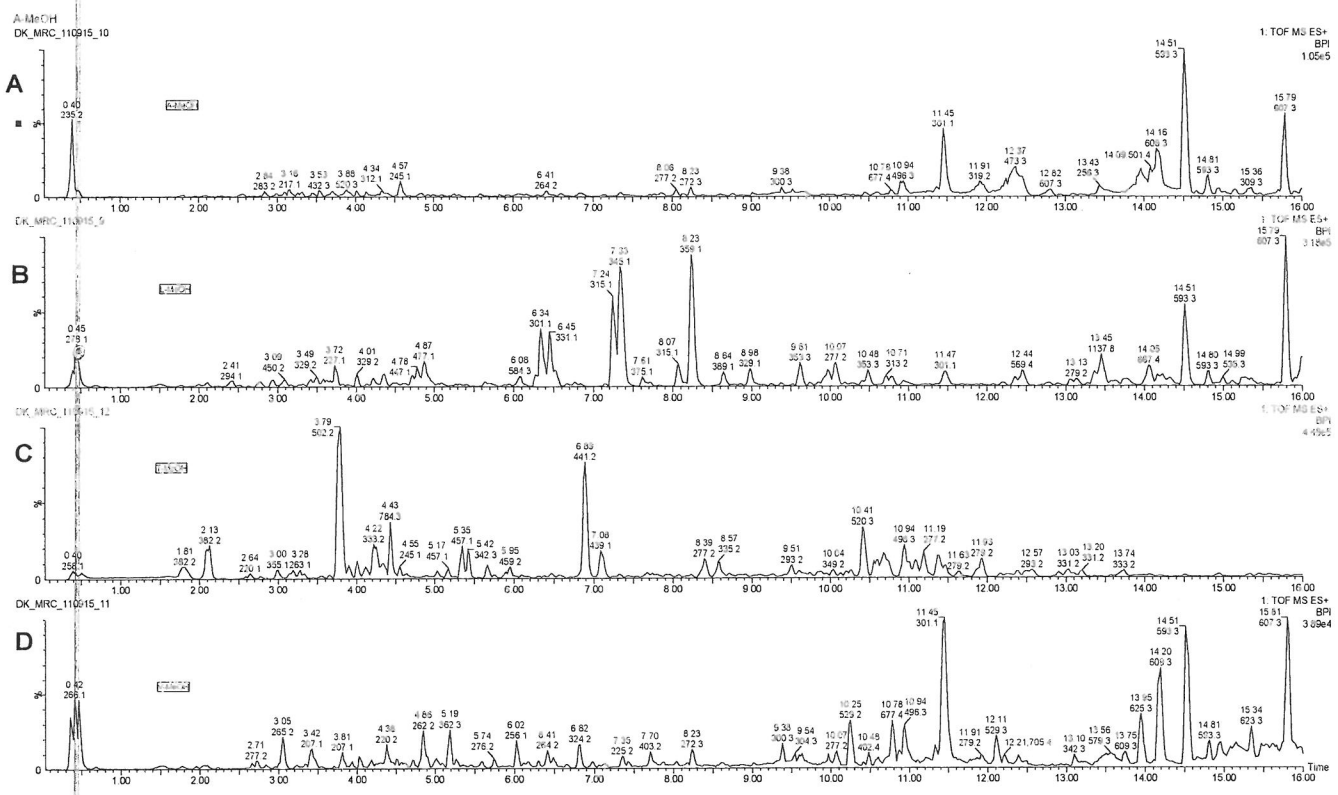


Fig. 6.3: Chromatograms of the MeOH extracts – A= *A. spinosus, B= L. javanica, C= T. minuta,*

Based on the foregoing we attempted to elucidate the structure of some of the most abundant metabolomes using MS / MS.

Based on the foregoing we attempted to elucidate the structures of some of the most abundant metabolomes using MS/MS.

6.3.1 Phenolic structures in the plant extracts

From MS data it was postulated that *L. javanica* and *T. minuta* were phenolic rich while *V. unguiculata* and *A. spinosus* had much lower concentrations of phenolic compounds. This was in fact previously established in chapter 5 (Table 5.1). It is further borne out by the existence of many compounds which are UV active at 280nm (Fig. 6.4) and fragments involving losses of CH_3, H_2O, CO, C_2H_2O, and CO_2 observed in the MS/MS spectra (Liu *et al*, 2008).

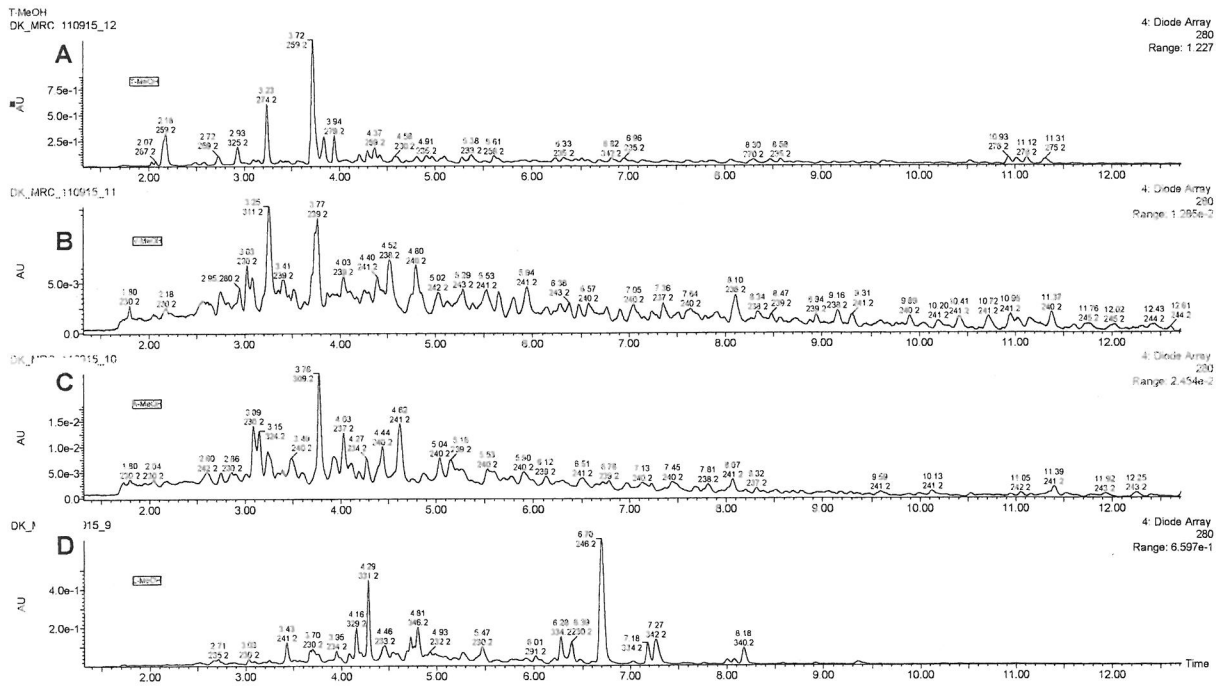


Fig. 6.4: Chromatograms of the MeOH extracts obtained at fix wavelength viz. 280nm. A= *A. spinosus, B= L. javanica, C= T. minuta, D= V. unguiculata.*

The observed phenolics are probably mostly phenolic acids flavonoids and their glycosides. The MS of phenolic aglycones ranges from m/z 220 - 350 (Parejo *et al.* 2004; Lee *et al.* 2005; Plazonic *et al.* 2009).

LA-1 was found in both the methanol, DCM and hexane extracts of *L. javanica*. It had a retention time t_R of 7.3 min. The UV spectrum was typical of flavonoids with maxima at 242, 273 and 342 nm (Fig. 6.5). The m/z was 345.1 (Fig. 6.6) giving a molecular formula [M+1] of $C_{18}H_{17}O_7$. The fragment were [M+1] m/z 345.1 (10%), [M - CH_4] $^{\cdot+}$, 330.1 (100%), [M+1 - CH_3 -H_2O] $^{\cdot+}$,312 (50%), [M+1 - CH_3 -H_2O - CO] $^{\cdot+}$,284.1 (70%). Two fragments at m/z 168 and 136 result from Retro-Diels Alder (RDA) reactions which are characteristic of flavonoid aglycones (Berahia, Gaydou *et al.* 1994). From the foregoing LA-1 was most likely a dihydroxy, trimethoxylated flavone. With the molecular ion at 344.1, this would either be nevadensin (A) or eupatorium (B) (Fig 6.7). With the data at hand it was not possible to be certain which isomer it was. In future either isolation of the compounds from the extract for characterization or comparison with a commercial standard of the proposed compounds should be done for a more conclusive resolution.

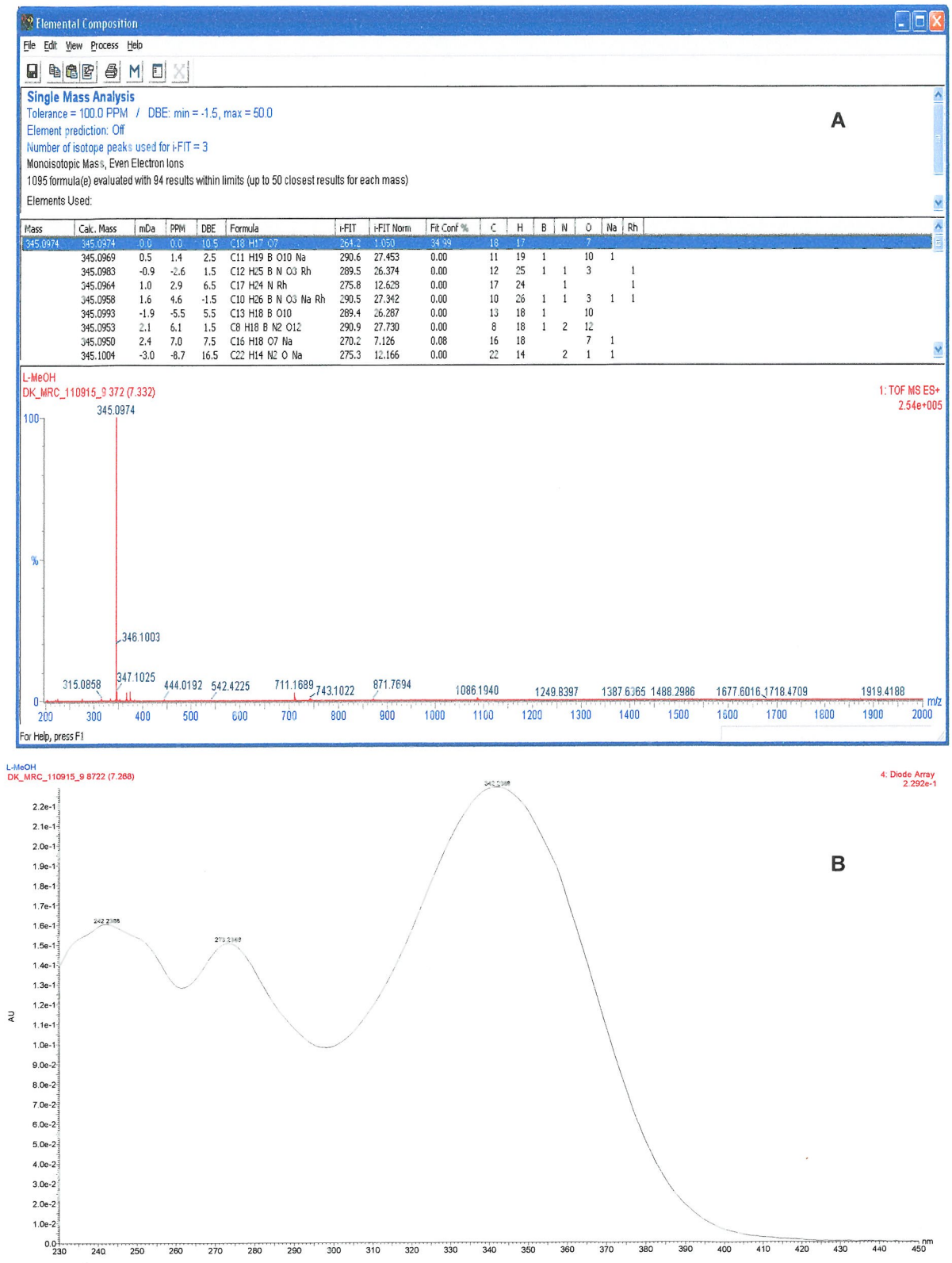


Fig 6.5: Mass ion chromatogram (A) and UV spectrum (B) of LA-1 found in *L. javanica.*

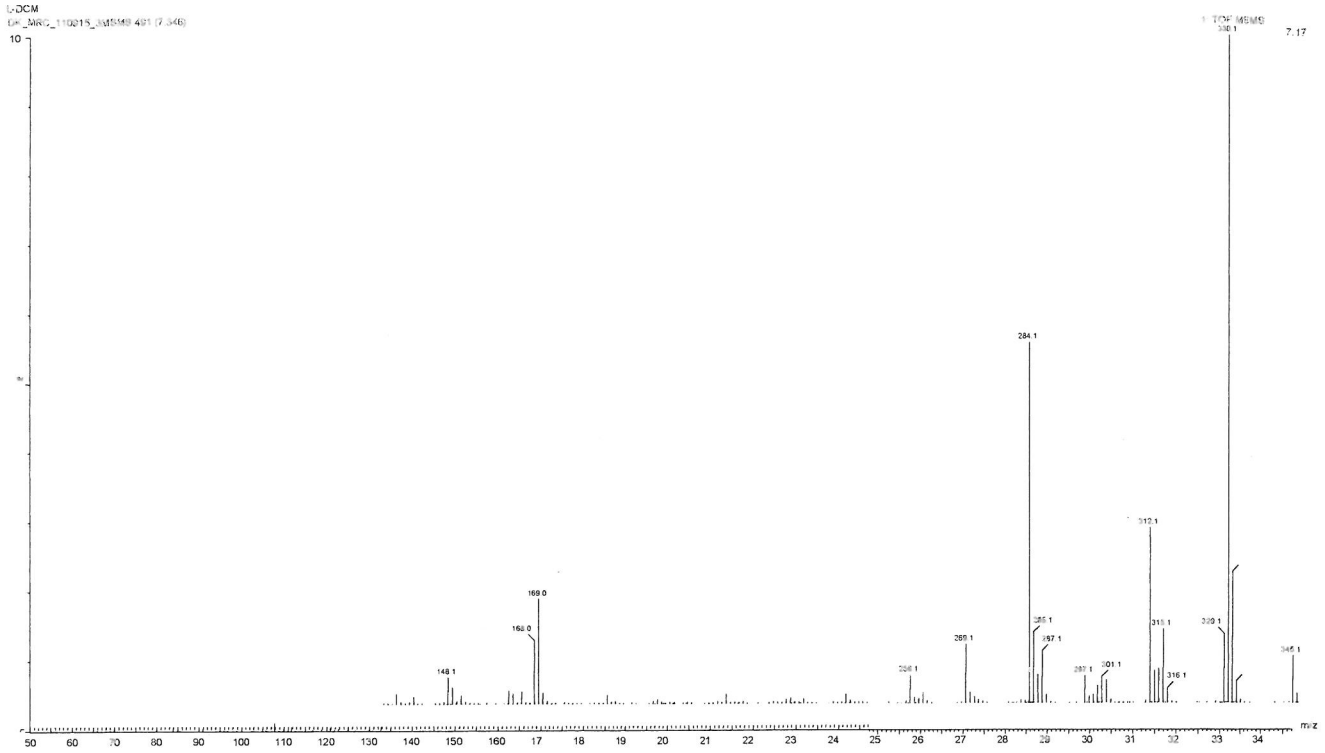


Fig 6.6: MS/MS spectrum of LA 1.

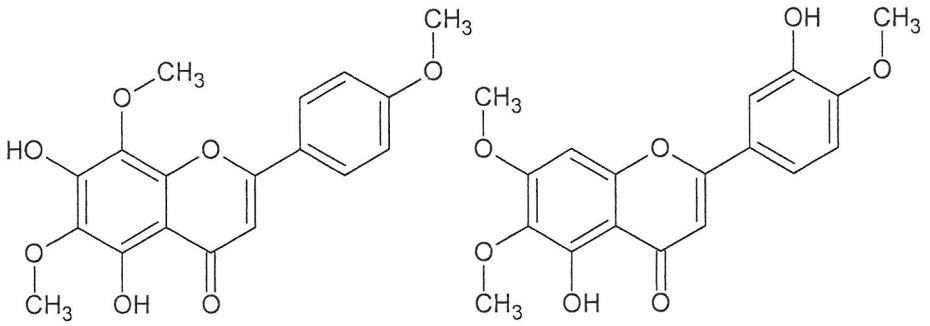


Fig 6.7: Chemical structures of nevadensin (A) and eupatorin (B).

LA-2 was present in the methanol and the DCM extracts of *L. javanica* and it has a molecular ion at m/z 315.1 which corresponded to $C_{17}H_{14}O_6$. It was suggested that that this was 5,7-dihydroxy-4',6-dimethoxyflavone commonly called pectolinarigenin or it's isomer 4',5-dihydroxy-6,7-dimethoxyflavone (cirsimaritin).

LA-3 was present in the methanol, DCM and hexane extracts of *L. javanica.* Fragmentation was typical of flavonoids with the formula $C_{18}H_{16}O_6$, *m/z* 328.1. The daughter ions of the molecular ion *m/z* 329.1 were 316.1 (100%), 301 (20%), 273.1 (15%), 168 (15%) and 140 (3%). This suggested that the compound is probably the trihydroxy, dimethoxylated flavones salvigenin.

LA-4 was present only in the DCM extract with a *m/z* of 359 and fragments at *m/z* 328.1 (60%), 300.1 (100%), 285.1 (10%), 257.1 (10%), 164.1 (10%). This compound was probably retusin (A) or 3,8-dimethoxy-5,7-dihydroxy-3',4'-methylenedioxyflavone (B) (Fig. 6.8).

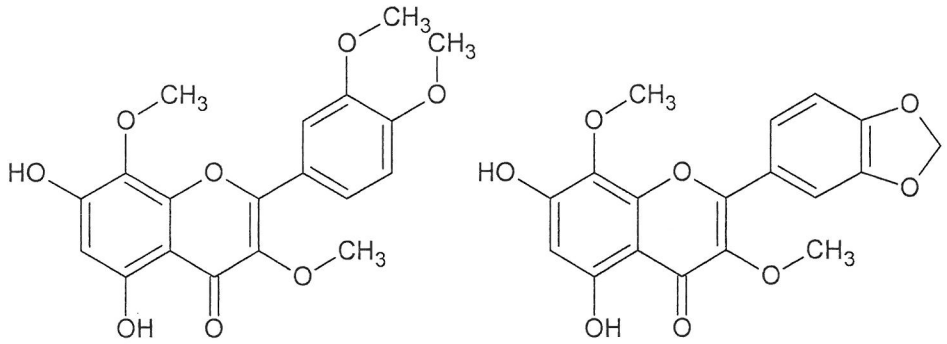


Fig 6.8: Chemical structures of retusin (A) and 3,8-dimethoxy-5,7-dihydroxy-3',4'-methylenedioxyflavone (B).

Lippia species have previously yielded 6-hydroxylated flavones and methoxyflavones but also some flavone sulphates (Pascual *et al.* 2001). Common flavanones (e.g. pinocembrin and naringenin) were identified from *L. graveolens,* and salvigenin, eupatorin, eupafolin, luteolin, hispidulin, diosmetin, cismaritin, cirsiliol, pectolin-arigenin, 6-hydroxyluteolin from *L. citriodora* (Ort.). The types of flavonoids in *L. javanica* are not known. We can predict from this data that these

flavonoids are possibly retusin, salvigenin, eupatorin, cirsimaritin. These mainly occur in the DCM extracts.

With respect to *T. minuta*, TA-1 has a fragmentation pattern diagnostic of a flavonoid. The molecular ion of m/z 253.2 corresponded to a molecular formula of $C_{16}H_{12}O_3$. Upon MS-MS analysis, other important typical fragments obtained were 225.1 (7%), 131.1 (100%), 91.1 (20%) and 77 (7%). The last two are attributed to a tropylium (m/z 91) resulting from a rearrangement of the base peak fragment and the benzene fragment of ring B. The putative major fragments are shown in Fig. 6.9.

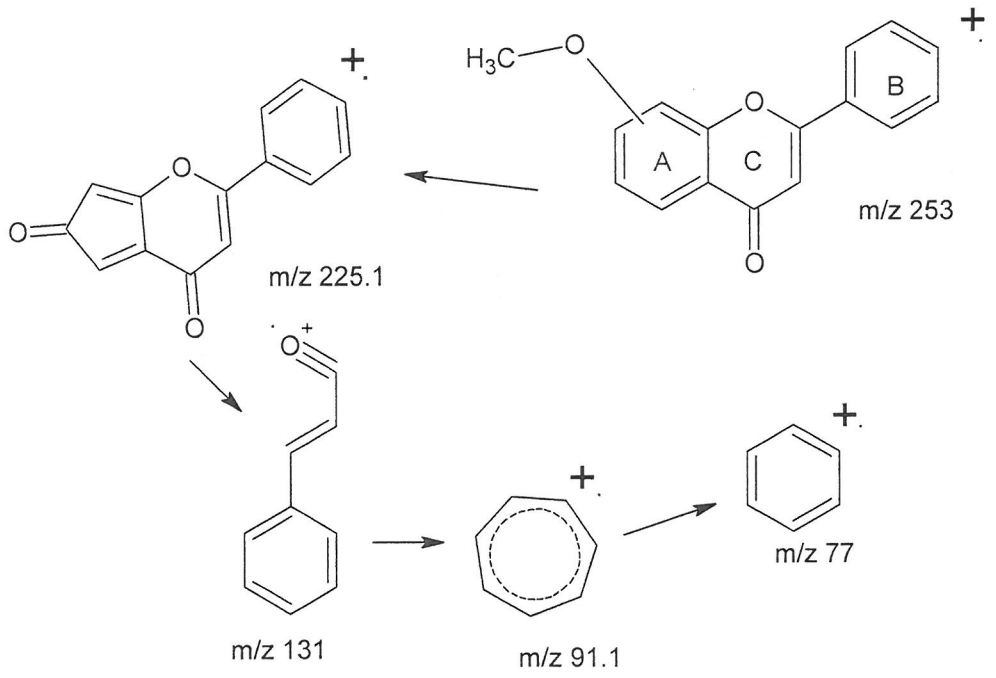


Figure 6.9: Fragmentation pattern typical of flavonoids seen with TA-1.

TA-1 is a methoxyflavone, however, it is not possible to know the exact location of methoxy substituent. In flavones and flavanones, C-5 and or C-7 substitution is the most commonly occurring possibly for biosynthetic reasons (Jaipetch *et al.* 1983;

Cheong *et al.* 1998; Bernini *et a*l. 2011). It can be speculated therefore that TA-1 is either 5 - methoxyflavone or 7-methoxyflavone.

Flavonoids have been known to possess antimicrobial activity (Cushnie and Lamb, 2005, Akroum *et al.* 2009) and show anti-oxidant activity (Pietta, 2000, Braca *et al.* 2002). The phenolic peaks of *Vigna* and *Amaranthus* extracts were very small and as a result no MS / MS was done.

6.3.2 Other constituents found in the plant extracts

The dominant compounds in *V. unguiculata* and *A. spinosus* were generally non-polar eluting above 12 min and of higher molecular weight. These compounds also occurred in *L. javanica* and *T. minuta* and were present in all three extracts. This led us to believe that they are probably not species unique but rather ubiquitous to herbaceous plants. The most interesting compounds were at t_R 14.16 (*m /z* 609.03) (denoted UB-1), t_R 14.50 (*m /z* 593.3) (UB-2) (Figs. 6.10 and 6.11), t_R 15.79 (*m /z* 607.3) (UB-3) and t_R 13.95 *m /z* 625.30 (UB-4). These compounds were resistant to further MS-2 fragmentation and remained largely intact losing what appears to be side chains e.g. CH_3COO (60), H_2O (18), CO (28), CH_4O (32). There were no daughter ions with masses lower than 485 which made structural elucidation difficult. The compounds had poor chromophoric properties at 280nm which implied that they were not polyphenolic in nature (see Appendix 1 and 2). The likelihood being that they are either terpenoids or chlorophylls. HPLC chromatograms show that the extraction method was selective and no overlap of compound between different organic solvents used was observed (Appendix 2).

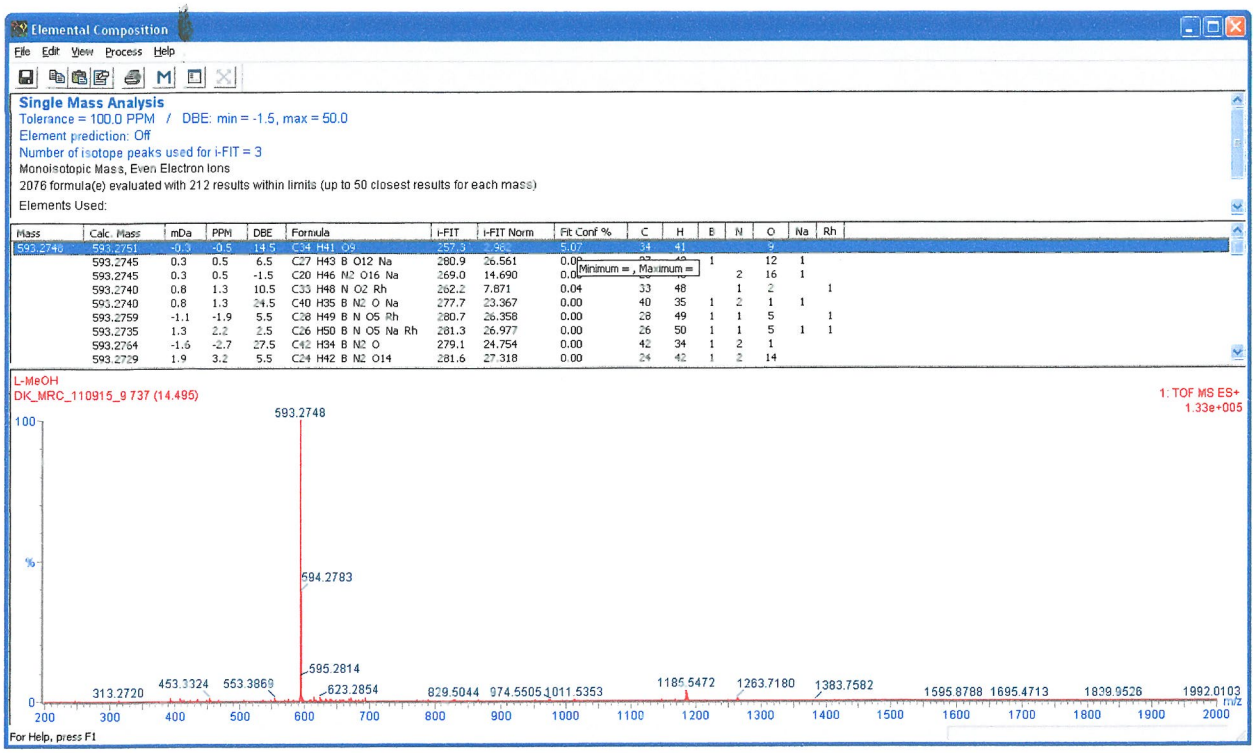


Fig. 6.10: MS Spectrum of UB-2.

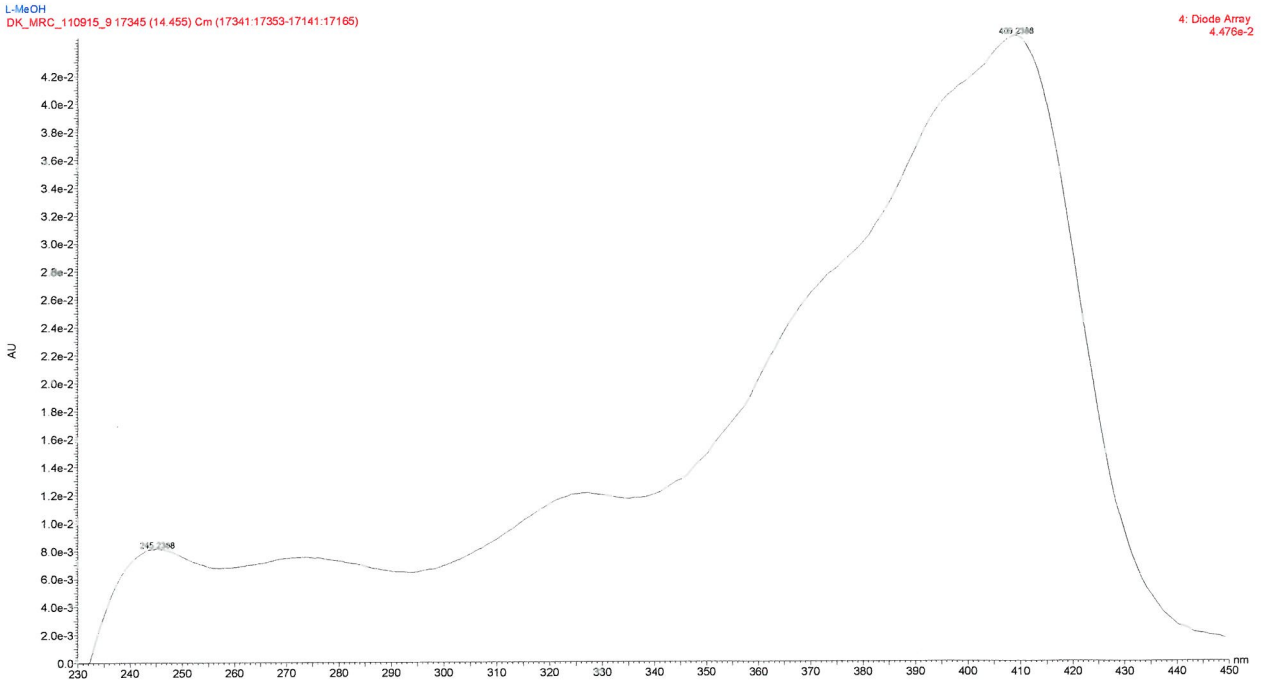


Fig. 6.11: UV Spectrum of UB-2.

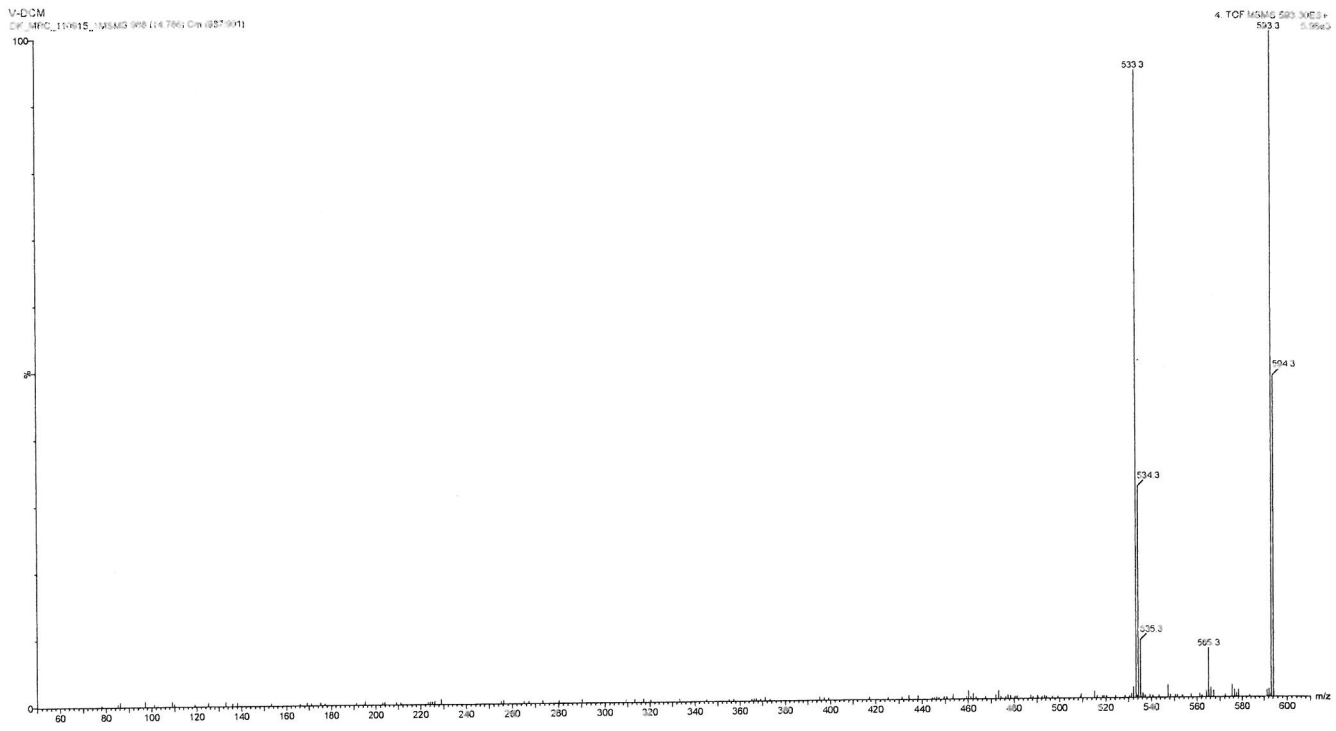


Fig. 6.12: MS / MS spectra of UB-2.

UB-2 eluted at t_R 14.50, showed UV maxima at 409 nm and the molecular ion of m/z 593.3 corresponded to $C_{35}H_{36}N_4O_5$. MS-MS for the compound was $[M+1]^{\cdot+}$ (593.3) (100%), $[M+1 - COOCH_3]^{\cdot+}$ (533.3)(20%) and $[M+1 - COOCH_3]^{\cdot+}$. The peak at m/z 460 (1%) is also typical of the fragmentation (Van Breemen *et al.* 1991). UB3 eluted at t_R 15.79 and the fragmentation was as follows: $[M+1]^{\cdot+}$ (607.3) (100%) and $[M+1 - COOCH_3]^{\cdot+}$ (547.3)(20%). By comparison with the literature it was concluded that UB-2 and UB-3 are pheophorbide *a* and pheophorbide *b* respectively (Fig 6.12). Van Breemen *et al.* 1991 have previously shown that these chlorophyll pigments only show limited fragmentation which compares with what we obtained. Because pheophorbide lack the phytyl chain, the most abundant fragment ions are $[M - CH_2COOH]^{\cdot+}$ or due to the loss of the β-keto ester group $[M-COOCH_3]^{\cdot+}$ (Van Breemen *et al.* 1991).

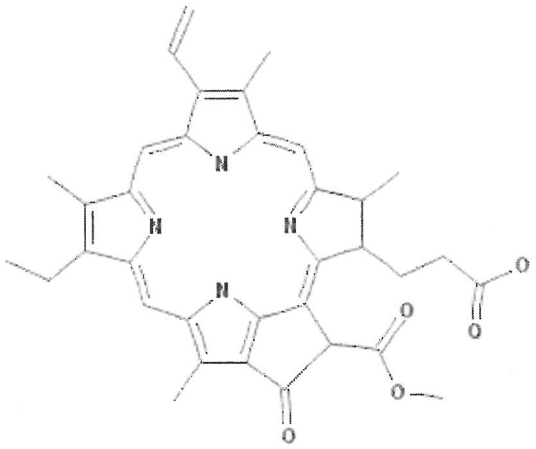


Fig 6.13: Chemical structure of Pheophorbide *a* (source: http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=586455&loc=es_rss#pharmaction).

Pheophorbides have been shown to possess antimicrobial activity particularly against *S. aureus* 6538, *E. faecalis* 29212, *S. tiphymurium* MS7953 and *E. coli* 25922 (Prieto Rodríguez *et al.* 2011). They were also shown to inhibit the Multi-Drug Resistant (MDR) protein efflux pumps in *Staphylococcus aureus* and thus increasing the potency of co-administered antimicrobials (Stermitz *et al.* 2000).

CHAPTER 7

7.1 GENERAL DISCUSSION AND CONCLUSION

In chapter one of this study, a review of mycotoxins, their health implication and agricultural control methods is given. From this review it became apparent that the currently used control methods, which rely largely on the use of synthetic fungicides, are fraught with a number of problems. This situation necessitates the search for alternative control methods that excludes or rely less on chemical fungicides. The results obtained in the current study provide valuable insight with regard to the antifungal and anti-mycotoxigenic activities of extracts of *T. minuta, L. javanica, A. spinosus* and *V. unguiculata.*

Although it is well known that plant extracts contain compounds that have antifungal activity, the effect of extracts of *T. minuta, L. javanica, A. spinosus* and *V. unguiculata* against agricultural fungi have not been investigated before. The results obtained in the present study, as can be seen in chapters 3 and 4, indicated that the *Fusarium* isolates were found to be generally more susceptible to the plant extracts in terms of both the growth and mycotoxin production than the *Aspergillus* isolates. Low amounts (concentrations) of the plant extracts were required to completely inhibit the growth of *Fusarium* isolates whereas the growth of the *Aspergillus* isolates was not inhibited. The MeOH and Hex extracts of *V. unguiculata* and *A. spinosus* were generally more active and stable in inhibiting fungal growth and mycotoxin production, while the DCM extracts were less active. These results suggest that in this study there is correlation between fungal growth and mycotoxin production, i.e. the less the fungal growth, the lesser the toxin produced. These results however, are

in contrast to the findings of Gourama and Bullerman (1995) and Vismer *et al.* (2004), who reported that toxin production, with particular reference to aflatoxin and fumonisins, can be independent of growth.

In the case of *Aspergillus* species tested, the implication of spore formation was not clear. However, a relationship between spore formation and mycotoxin production has been previously reported (Calvo *et al.* 2002). According to these authors, inhibition of spore production has been shown to inhibit the production of aflatoxin. *Aspergillus* mutants deficient in sporulation have been shown to be unable to produce aflatoxin (Calvo *et al.* 2002). Based on this relationship, it can be speculated that the observed spore formation in this study may result in enhanced toxin production. These findings however, are in contrast with those of Gourama and Bullerman (1995), who investigated the relationship between aflatoxin production and mold growth as measured by ergosterol and plate count, and Vismer *et al.* (2004) who studied the production of fumonisins by *Fusarium verticillioides* strains on solid and a defined liquid medium. According to these authors there was no linear relationship between fungal growth, sporulation and toxin production. Spore formation and mycotoxin production in *Aspergillus* species have been reported to be affected by physical factors such as temperature, availability of an air-surface interface and pH (Coty, 1998). Nutritional factors such as carbon and nitrogen sources can also affect mycotoxin production (Keller *et al.* 1997). Additionally, some compounds, e.g. linoleic acid present in the seeds commonly infected by *Aspergillus* species can influence both spore formation and toxin production (Calvo *et al.* 1999). It is possible that some compounds present in the plant species used in this study also influenced sporulation. It is therefore important to further investigate the plant

extracts used in this study to determine the exact effect of these plants species on spore formation.

The results of this study also highlighted the lack of activity of Amphotericin B and Cantus beyond 24 hrs. The implication of these findings is that when susceptibility tests are performed on slow-growing fungi requiring incubation longer than 24 hrs, the levels of resistance may be falsely high because in most cases controls are fungistatic, and fungal regrowth will occur as the activity of the controls fall with prolonged incubation. Therefore, very likely, decay of the active drug is responsible for the apparent recovery of fungi temporarily inhibited by the controls.

From the results obtained in chapters 5 and 6, it can be speculated that the correlation between antioxidant activity and phytochemical content of the plant extracts could be due to the presence of flavonoids. The difference in activity amongst different plant extracts can be attributed to certain phenolic compounds. For example, the results obtained from this study and literature review show that the flavonol rutin is present in both *V. unguiculata* and *A. spinosus* but not in *T. minuta* and *L. javanica*. This compound has been reported to show antioxidant activity and the activity is extremely good when acting in synergy with quercetin (Guardia *et al.* 2001), which is also present in both *V. unguiculata* and *A. spinosus*. While some authors found a strong correlation between phenolic content and the antioxidant activity (Velioglu *et al.* 1998), others found no such relationship (Kaehkoenen *et al.* 1999) showing that the antioxidant activity of an extract cannot be predicted on the basis of its total phenolic content because the antioxidant response of phenolic compounds depending on their chemical structure (Statue-Gracia *et al.* 1997).This is

probably because there are structure – activity relationships governing anti-oxidant activity. Flavones and catechins are in general superior to other polyphenols (for example lignans and coumarins) in this regard (Nijveldt *et al.* 2001). The extent of hydroxylation and glycosylation also plays an important role in determining anti-oxidant potency. The antioxidant activity of flavonoids has been shown to reside in the aromatic OH groups (Rezk *et al.* 2002), with maximum radical scavenging activity displayed by flavonoids with 3-OH groups attached to the 2,3 – double bond neighbouring the carbonyl group in the C-ring (Cotelle, 2001). Resorcinol and phloroglucinol substituents also show substantially higher antioxidant activity compared to phenol (Rezk *et al.* 2002). In this regard, two anti-oxidant pharmacores are now recognized in flavonoids - the catechol structure in ring B and three OH in rings A and C (Rezk *et al.* 2002). It is worth noting that the exact mechanism in which the plant extracts inhibited both fungal growth and mycotoxin production has not been investigated in this study and therefore, is not understood. In general, limited information is available on the antioxidant potential of extracts of plants species used in this study which are important dietary and medicinal plants in rural sub-Saharan Africa. It is also impossible to compare results from other studies because of immense variations in environment, climate, time of harvest among numerous other parameters which affect any wild growing plant.

The use of different assays in this study highlighted the problems or shortcomings and successes associated with these methods. In general, the serial dilution-microtitre method, as was seen in chapter 3, seems to be more trustworthy / valid than the agar diffusion and bioautographic assays. This could be because plant constituents are dissolved, kept in solution and not separated from each other,

allowing all the possible plant constituents in an extract to be in full contact with the fungi. This further allows the detection of possible synergistic effect in a plant extract. According to Masoko and Eloff, 2005, there are some limitations associated with the serial dilution-microtitre method as well. One of the limitations is precipitation of non-aqueous plant constituents in a well containing aqueous medium making it impossible for the plant constituents to fully interact with the fungi, hence fungal growth might not be inhibited, making the scoring of the MIC values difficult. Another limiting factor is that it is not possible to identify active compounds from the plant extract exhibiting activity against fungi. This is mainly because the colour of the plant extract may make it difficult to read the MIC values, resulting in false positive results. Even if this method has some limitations it was an effective method for the analysis of antifungall activity of plant extracts because it was possible to detect the loss or gain of activity.

The maize patty culture method was chosen on the basis that it was found to give high yields of toxin production, exceedingly higher than other media such as liquid medium (Alberts *et al.* 1993, Vismer *et al.* 2004). The fungi used in this study are natural pathogens of maize, thereby, making maize patties an almost natural medium. A significant correlation between biomass and total fumonisins production has been demonstrated (Vismer *et al.* 2004). Even if this method was the best choice, it is time consuming, i.e. maize patties take two days to prepare and another 21 days before the results are obtained provided there is no contamination. Another limitation is that it is difficult to measure the fungal biomass. This is primarily because some patties may show no fungal growth, others will have limited growth

and more importantly, some patties may dry out over the incubation period of 21 days.

Generally, the findings of the present study indicate that plant extracts, particularly *V. unguiculata* and *A. spinosus* may be exploited in treatment of food commodities against fungal infections. These plant species seem to contain chemical constituents that can be developed as potential antifungal agents for agricultural use. It would appear that these plants may be ecologically adapted to withstand fungal infection, i.e. they seem to have developed a huge armament of secondary metabolites (phytoalexins) to resist fungal attack because of their constant exposure to fungi due to co-existence with crop plants. This assertion is supported by Houssou *et al.* (2009), who reported the absence of aflatoxin contamination in *V. unguiculata* collected from four main agroecologial zones in Benin, even though *A. flavus* was most frequently isolated. The broad fungitoxic spectrum of *V. unguiculata* and *A. spinosus* was comparatively lower than that of the synthetic fungicides tested, thereby, presenting the plant extracts as possible fungitoxicants that may be employed successfully in controlling fungi, in particular *Fusarium* species.

The use of plant products in the management of fungi could reduce over-reliance on chemical fungicides, as well as cut down production costs. The plants used in the study are readily available and easy to extract, they can be exploited in the control of fungal infection and mycotoxin production. This is the first report showing the antifungal and antimycotoxigenic activities of extracts of *T. minuta, L. javanica, A. spinosus* and *V. unguiculata*. Further studies, including the isolation and elucidation of the active compounds will be required.

7.2 RECOMMENDATIONS

Although the present study has revealed important insights with regard to the antifungal and antimycotoxigenic activity of extracts of *V. unguiculata, A. spinosus, T. minuta* and *L. javanica*, some aspects on the study still need further clarification. As a result the following are recommended for future research:

- Future research should focus on determining the correlation between total ergosterol levels and fungal deterioration. This is particularly important because ergosterol is the main sterol found in fungal cell membranes, and offers a way of relating fungal biomass to deterioration. Ergosterol assays will determine the "history" of invasion by fungi, as it detects both viable and nonviable fungi.
- Future research should focus on determining the efficiency of the extraction method with respect to recovery, repeatability and linearity of the method.
- Also, research should focus on isolation and characterization of antifungal compounds present in these plant species.

REFERENCES

Aguiar JM, Franco OL, Rigden DJ, Bloch Jnr C, Monteiro ACS, Flores VMQ, Jacinto T, Filho JX, Oliveira AEA, Grossi-de-Sa MF, Fernandes KVS: Molecular modeling and inhibitory activity of cowpea cystatin against bean bruchid pests. Proteins: Structure, Function and Bioinformatics 2006;63:662-670.

Akroum S, Bendjeddou D, Satta D, Lalaoui K: Antibacterial activity and acute toxicity effect of flavonoids extracted from *Mentha longifolia*.

Amin I, Norazaidah Y and Emmy Hainida KI: Antioxidant activity and phenolic content of raw and blanched *Amaranthus* species. Food Chemistry 2006;94:47-52.

Alberts JF, Gelderblom WCA, Vleggaar R, Marasas WFO, Rheeder JP: Production of ^{14}C fumonisin B_1 by Fusarium moniliforme MRC 826 in corn cultures. Applied and Environmental Microbiology 1993;59:2673-2677.

Atanda OO, Akpan I, Oluwafemi F: The potential of some essential oils in the control of A. parasiticus CFR 223 and aflatoxin production. Food Control 2007;18:601-607.

Bakasso S, Lamien-Meda A, Lamien CE, Kiendrebeogo M, Millogo J, Ouedraogo AG, Nacoulma OG: Polyphenol contents and antioxidant activities of five indigofera speciies (Fabaceae) from Burkina Faso. Pakistan Journal of Biological Sciences 2008;11:1429-1435.

Bankole AS, Adebanjo A: Mycotoxins in food in West Africa: current situation and possibilities of controlling it. African Journal of Biotechnology 2003;2:254-263.

Bassole IHN, Ouattara AS, Nebie R, Ouattara CAT, Kabore ZI, Traore SA: Chemical composition and antibacterial activities of the essential oils of *Lippia chevalieri* and *Lippia multiflora* from Burkina Faso. Phytochemistry 2003;62:209-212.

Berahia T, Gaydou EM, Cerrati C, Wallet JC: Mass Spectrometry of Polymethoxylated Flavones. Journal of Agricultural and Food Chemistry 1994:42:1697-1700.

Bardin PG, van SF Eeden, Moolman JA, Foden AP, Joubert JR: rganophosphate and Carbamate Poisoning. Archives of International Medicine 1994;154:1433-1441

Becroft DM, Webster DR: Aflatoxins and Reye's disease. British Medical Journal 1972;4:117.

Beekrum S, Govinden R, Padayachee T, Odhav B: Naturally occurring phenols: a detoxification strategy for fumonisin B1. Food Additives and Contaminants 2003;20:490-493.

Begue WJ, Kline RM: The use of tetrazolium salts in bioautographic procedures. Journal of Chromatography 1972;64:182-184.

Bennett JW, Klich M: Mycotoxins. Clinical Microbiology Reviews 2003;16: 497-516.

Benzie IFF, Strain JJ: The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. Analytical Biochemistry 1996;239:70-79.

Bernini R, Crisante F, Ginnasi MC: A Convenient and Safe O-Methylation of Flavonoids with Dimethyl Carbonate (DMC). Molecules 2011:16:1418-1425.

Bhat RV, Vasanthi C: Food safety in food security and food trade: Mycotoxins and food safety in developing countries. Focus 10, Brief 13 of 17. International Food Policy Institute 2003.

Bii CC, Seboe GM, Mibey RK: Plant essential oils with promising antifungal activity. East African Medicinal Journal 2000;77:319-22.

Block ER and Bennett JE: Stability of Amphotericin B in infusion bottles. Antimicrobial Agents and Chemotherapy 1973:4:648-649.

Bouda H, Tapondjou LA, Fontem DA, Gumedzoe MYD; Effect of essential oils from leaves of *Ageratum conyzoides, Lantana camara* and *Chromolaena odorata* on the mortality of *Sitophilus zeamais* (Coleoptera, Curculionidae). Journal of Stored Products Research 2001;37:103-109.

Bowers HJ, Locke CJ: Effect of botanical extracts on the population density of *Fusarium oxysporum* in soil and control of *Fusarium* Wilt in the Greenhouse. Plant Disease 2000;84:300-305.

Braca A, Sortino C, Politi M, Morelli I, Mendez J: Antioxidant activity of flavonoids from Licania licaniaeflora. Journal of Ethnopharmacology 2002:79:379-381

Brandsberg JW, French ME: In vitro susceptibility of isolates of *Aspergillus fumigatus* and *Sporothrix schenckii* to Amphotericin B. Antimicrobial Agents and Chemotherapy 1972:2:402-404.

Brandt K, Christensen LP, Hansen-Møller J, Hansen SL, Haraldsdottir J, Jespersen L, Purup S, Kharazmi A, Barkholt V, Frøkiær H, Kobæk-Larsen M:

Health promoting compounds in vegetables and fruits: A systematic approach for identifying plant components with impact on human health. Trends in Food Science and Technology 2004:14:383-384

Calvo AM, Hinze LL, Gardner HW, Keller NP: Sporogenic effect of polyunsaturated fatty acids on development of *Aspergillus* spp. Applied and Environmental Microbiology 1999;65:3668-3673.

Calvo AM, Wilson RA, Bok JW, Keller NP: Relationship between secondary metabolism and fungal development. Microbiology and Molecular biology Review 2002;66:447-459.

Cheong H, Ryu SY,Oak MH, Cheon SH, YooGS: Studies of structure activity relationship of flavonoids for the anti-allergic actions. Archives of Pharmacal Research 1998:21:478-480.

Cheung SC, Medoff G, Schlessinger D, Kobayashi GS: Stability of Amphotericin B in Fungal Culture Media. Antimicrobial agents and Chemotherapy 1975:4:426-428.

Connolly JD, Hill RA: Mono- and sesquiterpenoids: In, Dictionary of Terpenoids, Chapman and Hall, London 1991.

Cos P, Ying L, Calomme M, Hu JP, Cimamga K, van Poel B, Pieters L, Vlitinck AJ, Berghe DV: Structure-Activity Relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxides scavengers. Journal of Natural Products 1998;61:71-76.

Cotelle N: Role of flavonoids in oxidative stress. Current topics in Medicinal Chemistry 2001:1: 569-590.

Coty P: Aflatoxin and sclerotial production by *Aspergillus flavus*: influence of pH. Phytopathology 1988;78:1250-1253.

Cowan MM: Plant products as antimicrobial agents. Clinical Microbiology Reviews 1999;12:564-582.

Creppy EE: Update of survey, regulation and toxic effects of mycotoxins in Europe. Toxicology Letters 2002;127:19-28.

Chowdhury AR, Sharma S, Mandal S, Goswami A, Mukhopadhyay S, Majumder H: Luteolin, an emerging anti-cancer flavonoid, poisons eukaryoticDNA topoisomerase I. Journal Biochemistry 2002; 366:653-661.

Cushnie TPT, Lamb AJ: Errata for Antimicrobial activity of flavonoids. International Journal of Antimicrob, Agents 2005: 26:343-356.

Department of Health South Africa, Government Notice No. 26849. Foodstuffs, Cosmetics and Disinfectants Act, 1972 (ACT No. 54 of 1972), Regulations governing tolerance for fungus-produced toxins in foodstuffs, 2004.

Deighton N, Brennan R, Finn C, Davies HV: Antioxidant properties of domesticated and wild Rubus species. Journal of the Science of Food and Agriculture 2000;80:1307-1313.

Dhiraj A V, Reza G and Kalidas S: Enhancing health benefits of berries through phenolic antioxidant enrichment: focus on cranberry. Asia Pacific Journal of Clinical Nutrition 2005; 14:120-130.

D'Mello JPF, MacDonald AMC: Mycotoxins. Animal Feed Science and Technology 1997;69:155-166.

Duan S, Weng XC, Dong XW, Liu YP, Li HP, Jin JR: Antioxidant properties of butylated hydroxytoluene refluxed in ferric chloride solution. Food Chemistry 1998;61:101-105.

Duke JA: Handbook of medicinal herbs. CRC Press, Inc., Boca Raton, Fla. 1985.

Edward SG: Influence of agricultural practices on Fusarium infection of cereals and subsequent contamination of grain by trichothecene mycotoxins. Toxicology Letters 2004;153:29-35.

Elad Y, Yunis H, Katan T: Multiple fungicide resistance to benzimidazoles, dicarboximides and diethofencarb in field isolates of *Botrytis cineria* in Israel. Plant Pathology 2007;41:41-46.

Eloff JN: Which extractant should be used for the screening and isolation of antimicrobial components from plants? Journal of Ethnopharmacology 1998a;60:1-8.

Eloff JN: A sensitive and quick microplate method to determine the minimum inhibitory concentration of plant extracts for bacteria. Planta Medica 1998b;64:711-713.

El-Kady IA, El-Maraghy SSM, Mostafa ME: Natural occurrence of mycotoxins in different spices in Egypt. Folia Microbiologica 2008;40:297-300.

Eskenazi B, Bradman A, Castorina R: Exposure of children to organophosphates and their potential adverse health effects. Environmental Heal th Perspectives 1999;107:409-419.

European Commission, Commission Regulation (EC) No. 1126/2007 of 28 September 2007 amending Regulation (EC) No. 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards Fusarium toxins in maize and maize products. Official Journal of the European Union 2007; No. L 255/14.

Fery R: New opportunities in *Vigna*. Trends in new crops and new uses. ASHS Press, Alexandra, VA 2002; 424-427.

Fisher GW, Holton CS: in "Biology and control of the Smut fungi", chapter 4, pp. 111-143. Ronald Press, New York, 1957.

Food and Agriculture Organization of the United Nations: Food insecurity in the world–eradicating world hunger–key to achieving the millennium development goals 2005. *www.fao.org/news-a.htm. (accessed 30/01/2008).*

Flores GS: Evaluation of anticancer potential of sorghums with different genetic characteristics and levels of phenolic compounds. PhD dissertation, 2009, Texas A&M University.

Frankel EN, Waterhouse AL, Teissedre PL: Principal phenolic phytochemicals in selected California wines and their antioxidant activity inhibiting oxidation of human low-density lipoprotein. Journal of Agriculture and Food Chemistry 1995;43:890-894.

Galvano F, Piva A, Ritieni A, Galvano G: Dietary strategies to counteract the effect of mycotoxins: a review. Journal of Food Protection 2001;64:120-131.

Gao X, Bjork L, Trajkovski V, Uggla M: Evaluation of antioxidant activities of rosehip ethanol extracts in different test systems. Journal of the Science of Food and Agriculture 2000;80:2021-2027.

Gelderblom WCA, Kriek NPJ, Marasas WFO, Thiel PG: Toxicity and carcinogenicity of the *Fusanum monilzforine* metabolite, fumonisin B_1, in rats. Carcinogenesis 1991;12:1247-1251.

Gelderblom WCA, Marasas WFO, Lebepe-Mazur S, Swanefelder S, Vessey CJ, de la M Hall P: Interaction of fumonisin B_1 and aflatoxin B_1 in a short-term carcinogenesis model in rat liver. Toxicology 2002; 171:161-173.

Gelineau-van Waes J, Starr L, Maddox J, Aleman F, Voss KA, Wilberding J, Riley T: Maternal fumonisin exposure and risk for neural tube defects: Mechanisms in *in vivo* mouse model. Birth Defects Res A Clin Mol Terat 2005;73:487-497.

Ghoshal SBN, Krishna P, Lakshmi V: Antiamoebic activity of *Piper longum* fruits against *Entamoeba histolytica* in vitro and in vivo. Journal of Ethnopharmacology 1996;50:167-70.

Gil A, Ghersa CM, Perelman S: Root thiophenes in *Tagetes minuta* L. accessions from Argentina: genetic and environmental contribution to changes in concentration and composition. Biochemical Systematics and Ecology 2002; 30:1-13.

Gómez AM, López CP, De la Ossa EM: Recovery of grape seed oil by liquid and supercritical carbon dioxide extraction: A comparison with conventional solvent extraction. Journal of Chemical Engineering and Biochemical Engineering 1996;61:227-231.

Gong YY, Cardwell KK, Hounsa A, Eggal S, Turner PC, Hall AJ, Wild CP: Dietary aflatoxin exposure and impaired growth in young children from Benin and Togo: a cross sectional study. British Medical Journal 2002;325:20-21.

Gourama H, Bullerman LB: Relationship between aflatoxin production and mold growth as measured by ergosterol and plate count. Lebensm Wiss U Technology 1995;28:185-189.

Grassroot Natural Products. Zinziba oil, *Lippia javanica* (Verbenaceae) 2002. *Website: http:/www. Gnp.co.za/essentialoils/zinziba.htm. Accessed 2007/05/30.*

Grayer RJ, Kokubun T: Plant-fungal interactions: the search for phytoalexins and other antifungal compounds from higher plants. Phytochemistry 2001;56:253-263.

Groopman JD, Zhu JQ, Donahue PR, Pikul A, Zhang LS, Chen JS, Wogan GN: Molecular dosimetry of urinary aflatoxin-DNA adducts in people living in Guangxi autonomous region, People's Republic of China. Cancer Research 1992;52:45-52.

Guardia T, Rotelli AE, Juarez AO, Pelzer LE: Anti-inflamatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat. Il Farmaco 2001;56:683-687.

Hagerman AE, Robbins CT, Weerasuriya Y, Wilson TC, McArthur C: Tannin Chemistry in relation to digestion. Journal of Range Management 1992; 57-62.

Hammer KA, Carson CF, Riley TV: Antimicrobial activity of essential oils other plant extracts. Journal Applied Microbiology 2001;86:985-990.

Harborne JB: The flavonoids: Advances in Research since 1986, Chapman and Hall, London 1994.

Harborne JB, Williams CA: Advances in flavonoid Research since 1992. Phytochemistry 2000; 481-504.

Hatem NI, Hassah HM, Abd Al-Rahman EM, El-Deeb SA, El-Sayed ARL: Prevalence of aflatoxins in blood and urine of egyptian infants with protein-energy malnutrition. Food Nutrition Bulletin 2005;26:49-56.

Heinonen M, Lehtonen PJ, Hopla A: Antioxidant activity of berry and fruit wines and liquor. Journal of Agriculture and Food Chemistry 1998;43:890-894.

Hesseltine CW: Conditions leading to mycotoxin contamination of foods and feeds: In Mycotoxins and other fungal related food problems. Advances in Chemistry, American Chemical Society, Washingrton, DC, 1976: 62-123.

Horžić D, Komes D, Belščak A, Ganić K K, Iveković D, Karlović D: The composition of polyphenols and methylxanthines in teas and herbal infusions. Food Chemistry 2009;115:441-448.

Houssou PA, Ahohuendo BC, Fandohan P, Hounhouigan DJ, Jakobsen M: Natural infection of cowpea (*Vigna unguiculata* L. Walp.) by toxigenic fungi and mycotoxin contamination in Benin, West Africa. Journal of Stored Products Research 2009;45:40-44.

Hoyer AP, Jorgensen T, Grandjean P, Hartvig HB: Repeated measurements of organochlorine exposure and breast cancer risk (Denmark). Cancer Causes and Control 2000;11:117-84.

Hua SST, Grosjean OK, Baker JL: inhibition of aflatoxin biosynthesis by phenolic compounds. Letters in Applied Microbiology 1999;29:289-291.

Hussein HS, Brasel JM: Toxicity, metabolism, and impact of mycotoxins on humans and animals. Toxicology 2001;167:101-134.

Huwig A, Freimund S, Kappeli O, Ditler H: Mycotoxin detoxication of animal feed Martini ND, Katerere DRP, Eloff JN: Biological activity of five antibacterial flavonoids from *Combretum erythrophyllum* (Combretaceae). Journal of Ethnopharmacology 2004;93:207-212.

Ickes GR, Fong HHS, Schiff Jr. PL, Perdue RE, Farnsworth NR: Antitumor activity and preliminary phytochemical examination of *Tagetes minuta* (compositae). Journal of Pharmaceutical Sciences 2006;62:1000-1011.

Jaipetch T, Reutrakul V, Tuntiwachwuttikul P, Santisuk T: Flavonoids in the black rhizomes of Boesenbergia panduta. Phytochemistry 1983:22:625-626.

Jayashree T, Subramanian C: Antiaflatoxigenic activity of eugenol is due to inhibition of lipid peroxidation. Letters in Applied Microbiology 2002;189-193.

Javanmardi JC, Stushnoff EL, Vivano JM: antioxidant activity and total phenolic content of Iranian *Ocimum accessions*. Food Chemistry 2003;83:547-550.

Jones BN, Gilligan JP: *o*-Phthaldialdehyde precolumn derivatization and reversed-phase high-performance liquid chromatography of polypeptide hydrolysates and physiological fluids. Chromatography. 1983;266:471-82.

Joseph GS, Jayaprakasha GK, Selvi AT, Jena BS, Sakariah KK: Antiaflatoxigenic and antioxidant activities of *Garcinia* extracts. International Journal of Food Microbiology 2005;101:153-160.

Jouany JP: Methods for preventing, decontaminating and minimizing the toxicity of mycotoxins in feeds. Animal Feed Science and Techology 2007;137:342-362.

Kaaya GP: The potential of anti-tick plants as components of integrated tick control strategy. Annals of New York Academy of Science 2002;916:576-582.

Kaehkoenen M, Hopia A, Vuorela H, Rauha J: Antioxidant Activity of Plant Extracts Containing Phenolic Compounds. Journal of agricultural and food chemistry 1999:47: 3954-3962.

Katerere DR: Phytochemical and Pharmacological Studies of species of African *Combretaceae*. PhD Thesis 2001, University of Strathclyde, UK

Katerere DRP, Eloff JN: Management of diabetes in African traditional medicine. In: Traditional medicines for modern times: Antidiabetic plants. Taylor and Francis Group, CRC Press 2006;57-158.

Katerere DR, Shephard GS, Faber M: Infant malnutrition and chronic aflatoxicosis in Southern Africa: Is there a link?. International Journal of Nutrition and Public Health 2008;1:127-135.

Keetch DP, Webster JW, Ngqaka A, Akanbi R, Mahlanga P: Bt maize for small scale farmers: a case study. African Journal of Biotechnology 2005;4:1505-1509.

Keller NP, Nesbitt C, sarr b, Phillips TD, burrow GB: pH regulation of sterigmatocystin and aflatoxin biosynthesis in *Aspergillus* spp. Phytopathology 1997;87:643-648.

Kellerman TS, Marasas WFO, Thiel PG, Gelderblom WCA, Cawood ME, Coetzer JA: W. leukoencephalomalacia in two horses induced by oral dosing of fumonisin B_1. Onderstepoort Journal of Veterinary Research 1990;57:269-275.

Kritzinger Q, Lall N, Aveling TAS: Antimicrobial activity of cowpea (*Vigna unguiculata*) leaf extracts. South African Journal of Botany 2005;71:45-48.

Kuhajek JM, Jeffers SN, Slatterey M, Wedge DE: A rapid microbioassay for discovery of novel fungicides for Phytophthora spp. American Phytopathology Society 2003;93: 46-53.

Kirby AJ, Schmidt RJ: The antioxidant activity of Chinese herbs for eczema and of placebo herbs - I. Journal of Ethnopharmacology 1997103-108.

Lakew EJ: The persistent food crisis in Ethiopia: causes, government responses and household strategies; the case of Enebse Sar Midir district. Masters thesis, Norwegian University of Science and Technology, 2006.

Langyintuo AS, Lowenberg-DeBoer J, Faye M, Lambert D, Ibro G, Moussa B, Kergna A, Kushwaha S, Musa S, Ntoukam G: Cowpea supply and demand in west and central Africa. Field Crops Research 2003;82:215-231.

Lattanzio V, Arpaia S, Cardinati A, Di Veupse D, Linsahata V: Role of endogenous flavonoids in resistance mechanism of *Vigna* to Aphids. Journal of Agriculture and Food Chemistry 2000; 5316-5320.

Lee JS, Kim DH, Liu KH, Tae K, Choong HL: Identification of flavonoids using liquid chromatography with electrospray ionization and ion trap tandem mass spectrometry with an MS/MS library. Rapid communications in mass spectrometry 2005;19:3539-3548.

Leroux P, Lanen C, Fritz R: Similarities in the antifungal activities of fenpiclonil, iprodione and tolclofos-methy against *Botrytis cinerea* and *Fusarium nivale*. Pesticide Science 1992;36:325-326.

Leroux P, Chapeland F, Desbrosses, Gredt M: Patterns of cross-resistance to fungicides in *Botrytinia fuckeliana* (*Botryti cinerea*) isolates from French vineyards. Crop Protection 1999;18:687-697.

Lewis L, Onsongo M, Njapau H, Schurz-Rogers H, Luber G, Kieszak S, Nyamongo J, Backer L, Mohamud Dahiye A, Misore A, DeCock K, Rubin C: Aflatoxin Contamination of Commercial Maize Products during an Outbreak of Acute

Aflatoxicosis in Eastern and Central Kenya. Environmental Health Perspective 2005;113:1762-1767.

Lipkin A, Anisimova V, Nikonorova A, Babokov A, Krause E, Bienert M, Grishin E, Egorov T: An antimicrobial peptide AR-AMP from *Amaranthus* (*Amaranthus retroflexus*) seeds. Phytochemistry 2005;66:2426-2431.

Liu H, Qiun N, Ding H, Yao R: Polyphenols contents and antioxidant capacity of 68 Chinese herbals suitable for medical or food uses. Food Research International 2008;41:363-370.

Lopez-Malo A, Alzamora SM, Palou E: *Aspergillus flavus* growth in the presence of chemical preservatives and naturally occuring antimicrobial compounds. International Journal of Food Microbiology 2005;99:119-128.

Mabogo DEN: The Ethnobotany of the Vhavenda. MSc thesis. University of Pretoria 1990.

Magan N, Hope R, Colleate A, Baxter ES: Relationship between growth and mycotoxin production by Fusarium species, biocides and environment. European Journal of Plant Pathology 2002;108:685-690.

Mahanil S, Attajarusit J, Stout MJ, Thipyapong P: Overexpression of tomato polyphenol oxidase increases resistance to common cutworm. Plant Science 2008;174:456-466.

Manandhar NP: A survey of medicinal plants of jajarkot district, Nepal. Journal of Ethnopharmacology 1995;48:1-6.

Manenzhe NJ, Potgieter N, van Ree T: Composition and antimicrobial activities of volatile components of *Lippia javanica*. Phytochemistry 2004;65:2333-2336.

Manson MM, Ball HW, Barrett MC, Clark HL, Judah DJ, Williamson G, Neal GE: Mechanism of action of dietary chemoprotective agents in rat liver: induction of phase I and II drug metabolizing enzymes and aflatoxin B1 metabolism. Carcinogenesis, 1997:1729-1738

Marasas WFO, Nelson PE: Mycotoxicology: In Introduction to the Mycology, Plant Pathology, Chemistry, Toxicology and Pathology of naturally occurring Mycotoxicoses in animals and man. The Pennsylvania State University Press, University Park, London 1986.

Marasas WFO, Kellerman TS, Gelderblom WCA, Coetzer JAW, Thiel PG, van der Lugt JJ: Leukoencephomalacia in horse induced by Fumonisin B1 isolated from *Fusarium moniliforme*. Onderstepoort Journal of Veterinary Research 1988; 55:197-203.

Marasas WFO: Fumonisins: their implications for human and animal health. Natural Toxins 1995;3:193-198.

Martini ND, Katerere DRP, Eloff JN: Biological activity of five antibacterial flavonoids from *Combretum erythrophyllum* (Combretaceae). Journal of Ethnopharmacology 2004; 93;207-212.

Masoko P, Eloff JN: Bioautography indicates the multiplicity of antifungal compounds from twenty-four southern African *Combretum* species (Combretaceae). African Journal of Biotechnology 2006;5:1625-1647.

Mathieu G, Meissa D: Traditional leafy vegetables in Senegal: diversity and medicinal uses. African Journal of Traditional, Complementary and Alternative Medicines 2007; 4:469-475.

Mazzola M, Fujimoto DK, Thomashow LS, Cook RJ: Variation in sensitivity of *Gaeumannomyces graminis* to antibiotics produced by fluorescent *Pseudomonas* spp. and effect on biological control of take-all of wheat. Applied and Environmental Microbiology 1995;2554-2559.

Mayer AM: Polyphenol oxidases in plants and fungi: Going places? A review. Phytochemistry 2006;67:2318-2331.

McGarvey DJ, Croteau R: Terpenoid metabolism. The Plant Cell 1995;1015-1026.

McKean C, Tang L, Tang M, Billam, Wang Z, Theodorakis CW, Kendall RJ, Wang JS: Comparative acute and combinative toxicity of aflatoxin B_1 and fumonisin B_1 in animals and human cells. Food and Chemical Toxicology 2006;44:868-876.

Miliauskas G, Venskutonis PR, van Beek TA: Screening of radical scavenging activity of some medicinal and aromatic plant extracts Food Chemistry 2004; 85:231-237

Missmer SA, Suarez L, Felkner M, Wang E, Merrill AH, Rothman KJ, Hendricks KA: Exposure to fumonisin and the occurance of neural tube defects along the Texas-Mexico border. Environmental Health Perspectives 2006;114:237-241.

Mohana DC, Raveesha KA, Lokanath Rai KM: Herbal remedies for the management of seed-borne fungal pathogens by an edible plant *Decalepis hamiltonii* (Wight & Arn). Archives of Phytopathology and Plant Protection 2008; 41:38-49.

Moss H: Under-explored Food Plants in Botswana. Unpublished report for Veld Products Research, Gaborone 1988.

Muchuweti M, Nyamukonda L, Chagonda LS, Ndhlala AR, Mupure C, Benhura M: Total phenolic content and antioxidant activity in selectedmedicinal plants of Zimbabwe. International Journal of Food Science and Technology 2006;41:33-38

Mucsi I, Gyulai Z, Beladi I: Combined effects of flavonoids and acyclovir against herpesviruses in cell cultures. Acta Microbiology 1992;39:137-47.

Mwanda OW, Otieno CF, Omonge E, Acute aflatoxicosis: case report, East African Medical Journal 2005; 82:320-324.

Ndube N, van der Westhuizen L, Shephard GS: Determination of fumonisins in maize by HPLC with ultraviolet detection of o-phthaldialdehyde derivatives. Mycotoxin Research 2009:25:225-228.

Ngouajio M, McGiffen Jr ME, Hutchinson CM: Effect of cover crop and management system on weed populations in lettuce. Crop Protection 2003;22: 57-64.

Nijveldt RJ, van Nood E, van Hoorn DE, Boelens P, van Norren K, van Leeuwen PA: Flavonoids: a review of probable mechanisms of action and potential applications. The American journal of clinical nutrition 2001: 74: 418-425.

Oigiangbe NO, Onigbinde AO: The association between some physico-chemical characteristics and susceptibility of cowpea (*Vigna unguiculata* (L) Walp) to *Callosobruchus maculates* (F.). Journal of Stored Products Research 1996;32:7-11.

Omolo MO, Okinyo D, Ndiege IO, Lwande W, Hassanali A: Repellency of essential oils of some Kenyan plants against *Anopheles gambiae.* Phytochemistry 2004;65:2797-2802.

Pascual ME, Slowing K, Carretero E, Mata SD, Villar A: Lippia: traditional uses, chemistry and pharmacology: a review. Journal of Ethnopharmacology 2001;76:201-214.

Pasquet R: Wild cowpea *(Vigna unguiculata)* evolution. In: B. Pickersgill and J.M. Lock (editors). Advances in Legume Systematics 8: Legumes of Economic Importance 1996;95-100. Royal Botanic Gardens, ICe

Parejo I, Jauregui O, Viladomat F, Bastida J, Codina C: Characterization of acylated flavonoid-*O*-glycosides and methylated flavonoinds from *Tagetes maxima* by liquid chromatography coupled to electrospray ionization tandem mass spectrometry. Rapid Communication in Mass Spectrometry 2004;18:2801-2810.

Paterson RRM: Some fungicides and growth inhibitor/biocontrol-enhancer 2-deoxy-D-glucose increase patulin from *Penicillium expansum* strains in vitro. Crop Protection 2007;26:543-548.

Pauli A: Anticandidal low molecular compounds from higher plants with special reference to compounds from essential oils. Medicinal Research Reviews 2006;26:223-268.

Pelletier SW (ed). Alkoloids: Biological and Chemical Perspectives 1996. Elservier Science, Ltd, Oxford.

Phillips TD: Dietary Clay in the chemoprevention of aflatoxin-induced disease. Toxicological Sciences 1999;52:118-126.

Pietta PG: Flavonoids as antioxidants. Journal of Natural Products 2000:63:1035-1042.

Plazonic A, Bucar F, Males Z, Mornar A, Nigovic B, Kunjundzic N: Identification and quantification of flavonoids and phenolic acids in burr parsley (Caucalis platycarpos L.), using high-performance liquid chromatography with diode array detection and electrospray ionization mass spectrometry. Molecules 2009:14:2466-2490.

Polychronaki N, Wild CP, Mykkanen H, Amra H, Abdel-Wahhab M, Sylla A, Dialo M, El-Nezami H, Turner PC: Urinary biomarkers of aflatoxin exposure in young children from Egypt and Guinea. Food and Chemical Toxicology 2008;46:519-526.

Probst C, Njapau H, Cotty P J: Outbreak of an acute aflatoxicosis in Kenya in 2004: Identification of the causal agent. Applied and Environmental Microbiology 2007;73:2762-2764.

Pussemier L, Larondelle Y, van Peteghem C, Huyghebaert A: Chemical safety of conventionally and organically produced foodstuffs: a tentative comparison under Belgian conditions. Food Control 2006;17:14-21.

Rajapakse R, Van Emden HF: Potential of four vegetable oils and ten botanical powders for reducing infestation of cowpeas by *Callosobruchus maculatus*, *C. chinesis* and *C. rhodesianus.* Journal of Stored Products Research, 1997: 59-68.

Rauha JP, Remes S, Heinonen M, Hopia A, kahkonen M, Kujala T, Pihlaja K, Vuorela H, Vuorela P: Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. International Journal of Food Microbiology 2000;56:3-12.

Rasooli I, Owlia P: Chemoprevention by thyme oils of *Aspergillus parasiticus* growth and aflatoxin production. Phytochemistry 2005;66:2851-2856.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C: Antioxidant activity applying an improved ABTS radical cation decolorazation assay. Free Radical Biology and Medicine 1999;26:1231-1237.

Rex JH, Pfaller MA, Barry AL, Nelson PW, Webb CD: Antifungal susceptibility testing of isolates from a randomized, multicenter trial of Fluconazole versus Amphotericin B as treatment of nonneutropenic patients with Candidemia. Antimicrobial agents and Chemotherapy 1993;40-44.

Rezk BM, Haenen GR, van der Vijgh WJ, Bast A: The antioxidant activity of phloretin, the disclosure of a new antioxidant pharmacophore in flavonoids. Biochemical and biophysical research communications 2002:295: 9-13.

Rheeder JP, Marasas WFO, Vismer HF: Production of Fumonisin Analogs by *Fusarium* Species. Applied and Environmental Microbiology 2002;68:2101-2105

Rice-Evans C, Miller N, Paganga G: Antioxidant properties of phenolic compounds. Trends in Plant Science, 1997:152-159.

Riley RT, Hinton DM, Chamberlain WJ, Bacon CW, Wang E, Merrill AH, Jr, Voss KA: Dietary Fumonisin B_1 Induces Disruption of Sphingolipid Metabolism in Sprague-Dawley Rats: A New Mechanism of Nephrotoxicity. Journal of Nutrition 1994;594-603.

Rios JL, Recio MC; Medicinal plants and antimicrobial activity. Journal of Ethnopharmacology 2005;100:80-84.

Ross PF, Nelson PE, Richard JL, Osweiler GD, Rice LG, Plattner RD, Wilson TM: Production of fumonisins by *Fusarium moniliforme* and *Fusarium proliferatum* isolates associated with equine leukoencephalomalacia and pulmonary edema syndrome in swine. Applied and Environmental Microbiology 1990;56:3225-3226.

Russell L, Cox DF, Larsen G, Bodwell K, Nelson CE: Incidence of molds and mycotoxins in commercial animal feed mills in seven midwestern states 1988-1989. Journal of Animal Science 1991;69:5-12.

Ruuhola T, Juha-Pekka S, Haviola S, Yang S, Rantala MJ: Immunological Memory of Mountain Birches: Effects of Phenolics on Performance of the Autumnal Moth Depend on Herbivory History of Trees. Journal of Chemical Ecology 2007;33:160-1176.

Salah N, Miller NJ, Paganga G, Tijburg L, Bolwell GP, Rice-Evans C: polyphenolic flavonoids as scavengers of aqueous phase radicals and as chain-breaking antioxidants. Archives of Biochemistry and Biophysics 1995;322:339-346.

Samapundo S, de Meulenaer B, Osei-Nimoh D, Lamboni Y, Debevere J, Devlieghere F: Can phenolic compounds be used for the protection of corn from fungal invasion and mycotoxin contamination during storage? Food Microbiology 2007;24:465-473.

Sánchez E, Heredia N, García S: Inhibition of growth and mycotoxin production of *Aspergillus flavus* and *Aspergillus parasiticus* by extracts of *Agave* species. International Journal of Food Microbiology 2005;98:271-279.

Sarr M, Ngom S, Kane M, Wele A, Diop D, Sarr B, Gueye L, Andriantsitohaina R, Diallo A: *In vitro* vasorelaxation mechanisms of bioactive compounds extracted from *Hibiscus sabdariffa* on rat thoracic aorta. Nutrition and Metabolism 2009:6,45.

Scalbert A: Antimicrobial properties of tannins. Phytochemistry 1991;30;3875–3883.

Scorzini L, Benaducci T, Almeida AMF, Silva DHS, da Silva Bolzani V, Gianinni MJSM: The use of standard methodology for determination of antifungal activity of natural products against medicinal yeasts *Candida* sp. and *Cryptococcus* sp. Brazilian Journal of Microbiology 2007;38:1517-8382.

Schmidt H: Phenol oxidase (E.I.14.18.1), a marker enzyme for defense cells. Progress in Histochemistry and Cytochemistry 1988; 17. Gustav Fischer, New York.

Selvi AT, Joseph GS, Jayaprakasha GK: Inhibition of growth and aflatoxin production in *Aspergillus flavus* by *Garcinia indica* extract and its antioxidant activity. Food Microbiology 2003;20:455-460.

Shadomy S, Bummer DL, Ingroff AV: Light sensitivity of prepared solutions of Amphotericin B. American Review Respir. Dis. 1973:107:303-304

Shephard GS, Stdenham EW, Thiel PG, Gelderblom WCA: quantitative determination of fumonisins B1 and B2 by high-performance liquid chromatography with fluorescence detection. Journal of Liquid Chromatography 1990;13:-2077-2087.

Shephard GS, Thiel PG, Stockenstrom S, Sydenham EW: Worldwide survey of fumonisin contamination of corn and corn-based products. Journal of AOAC International 1996;79:671-687.

Shephard GS: Chromatographic determination of the fumonisin mycotoxins. Journal of chromatography A 1998;815:31-39.

Shephard GS: Aflatoxin and food safety, recent African perspectives, in H.K Abbas (Ed). Aflatoxin and Food safety, Florida: taylor and Francis 2005.

Sinha KK, Sinha AK: Impact of stored grain pests on seed deterioration and aflatoxin contamination. Journal of Stored Products Research 1992;28:211-219.

Singh V, Singh B, Kaul VK: Domestication of wild Marigold (*Tagetes minuta* L.) as a potential economic crop in western Himalaya and northern Indian plains. Economic Botany 2000;57:535-544.

Skouras PJ, Margaritopoulos JT, .Seraphides NA , Ioannides IM, Kakani EG, Mathiopoulos KD, Tsitsipis JA: Organophosphate resistance in olive fruit fly, *Bactrocera oleae,* populations in Greece and Cyprus. Pest management Science 2006;63:42-48.

Soliman KM, Badeaa RI: effect of oil extracted from some medicinal plants on different mycotoxigenic fungi. Food and Chemical Toxicology 2002;40:1669-1675.

Souza EL, Stamford TLM, Lima EO, Trajano VN: Effectiveness of *Origanum vulgare* L. essential oil to inhibit the growth of food spoiling yeast. Food Control 2007;18:409-413.

Srinivasan D, Nathan S, Suresh T, Perumalsamy PL: Antimicrobial activity of certain Indian medicinal plants used in folkloric medicine. Journal of Ethnopharmacology 2001;74:217-220.

Statue-Gracia M, Heionen M, Frankel E: Antioxidant activity of anthocyanin in LDL and lecithin liposome systems. Journal of Agricultural and Food Chemistry 1997:5: 3362-3367.

Stermitz FR, Tawara-Matsuda J, Scriven LN, Tegos G, Lewis K: Methoxyhydnocarpin-D and Pheophorbide A: Berberis Species components that potentiate Berberine growth inhibition of resistant *Staphylococcus aureus*. Journal of Natural Products 2000:63:1146-1149

Stern JL, Hagerman AE, Steinberg PD, Mason PK:.Phlorotannin-protein interactions. Journal of Chemical Ecology 1996;22:1887-1899..

Stintzing FC, Kammerer D, Schieber A, Hilou A, Nacoulma OG, Carle R: Betacyanins and phenolic compounds from *Amaranths spinosus* L. and *Boerhavia erecta* L. Naturforsch 2004;59:1-8.

Sundaresan P, Raja NU: Gunasekaran PInduction and accumulation of phytoalexins in cow pea roots infected with a mycorrhezalfungus *Glomus fasciculatum* and their resistance to *Fusarium* wilt disease. Journal of Bioscience 1993;18:291-301.

Surveswaran S, Yi-Zhong Cai, Corke H and Sun M: Systematic evaluation of natural phenolic antioxidants from 133 Indian medicinal plants. Food Chemistry 2007;102:938-953.

Sydenham EW, Shephard GS, Thiel PG, Stockenstrom S, Snijman PW, Van Schalkwyk DJ: Liquid chromatographic determination of fumonisins B_1, B_2 and B_3 in corn: AOAC-IUPAC collaborative study. Journal of AOAC International 1996;79:688-696 .

Taylor RSL, Edel F, Manandhar NP, Towers GHN: Antimicrobial activities of southern Nepalese medicinal plants. Journal of Ethnopharmacol.ogy 1996;50:97-102.

Tepe B, Daferera D, Sokmen M, Polissiou M: Antimicrobial and antioxidant activities of the essential oil and various extracts of *Salvia tomentosa* Miller (Lamiaceae). Food Chemistry 2005;90:333-340.

Ueno Y, Iijima K, Wang SD, Sugiura Y, Sekijima M, Tanaka T, Chen C, Yu S.Z: Fumonisins as a possible contributory risk factor for primary liver cancer: A 3-year

study of corn harvested in Haimen, China, by HPLC and ELISA. Food and Chemical Toxicology 1997;35:1143-1150.

van Breemen RB, Canjura FL, Schwartz SJ: Identification of chlorophyll derivatives by mass spectrometry. Journal of Agricultural and Food Chemistry 1991:39:1452-1456.

van Wyk B, van Oudtshoorn B, Gericke N: Medicinal plants of Southern Africa. Briza, Pretoria 1997.

Vasudevan P, Kashyap S, Sharma S: *Tagetes*: a multipurpose. Plant Biosource Technology 1997;62:29-35.

Velioglu YS, Mazza G, Gao L, Oomah BD: Antioxidant Activity and Total Phenolics in Selected Fruits, Vegetables, and Grain Products. Journal of Agricultural and Food Chemistry 1998;46:4113-4117.

Viljoen AM, Subramoney S, van Vuuren SF, Baser KHC, Demirci B: The composition, geographical variation and antimicrobial activity of *Lippia javanica* (Verbenaveae) leaf essential oils. Journal of Ethnopharmacology 2005;96:271-277.

Vismer HF, Snijman PW, Marasas WFO, van Schalkwyl RJ: Production of Fumonisins by *Fusarium verticilliodes* strains on solid and in a defined liquid medium – effects of L-methionine and inoculum. Mycopathologia 2004;158:99-106.

Wang J, Zhou Y, Wang Q: Analysis of mycotoxin fumonisins in corn products by high-performance liquid chromatography coupled with evaporative light scattering detection. Food Chemistry 2008;107:970-976.

Wareing PW, Westby A, Gibbs JA, Allotey LT, Halm M: Consumer preferences and fungal and mycotoxin contamination of dried cassava products from Ghana. International Journal of Food Science Technology 2001:36:1-10.

Watkinson E, Makgetla N: South Africa's food security crisis. National Labour and Economic Development Institute 2002;1-17.

WHO/UNICEF: Global strategy for infant and young child feeding, Geneva, Switzerland: World Health Organisation 2003.

Wild CP, Hudson GJ, Sabbioni G, Chapot B, Hall AJ, Wogan GN, Whittle H, Montesano R, Groopman JD: Dietary intake of aflatoxins and the level of albumin-bound aflatoxin in peripheral blood in The Gambia, West Africa. Cancer Epidemiology Biomarkers and Prevention 1992;1:229-234.

Williams JH, Phillips TD, Jolly PE, Stiles JK, jolly CM, Aggarwal D: Human aflatoxicoses in developing countries: a review of toxicology, exposure, potential health consequences and interventions. American Journal of Clinical Nutrition 2004;80:1106-1122.

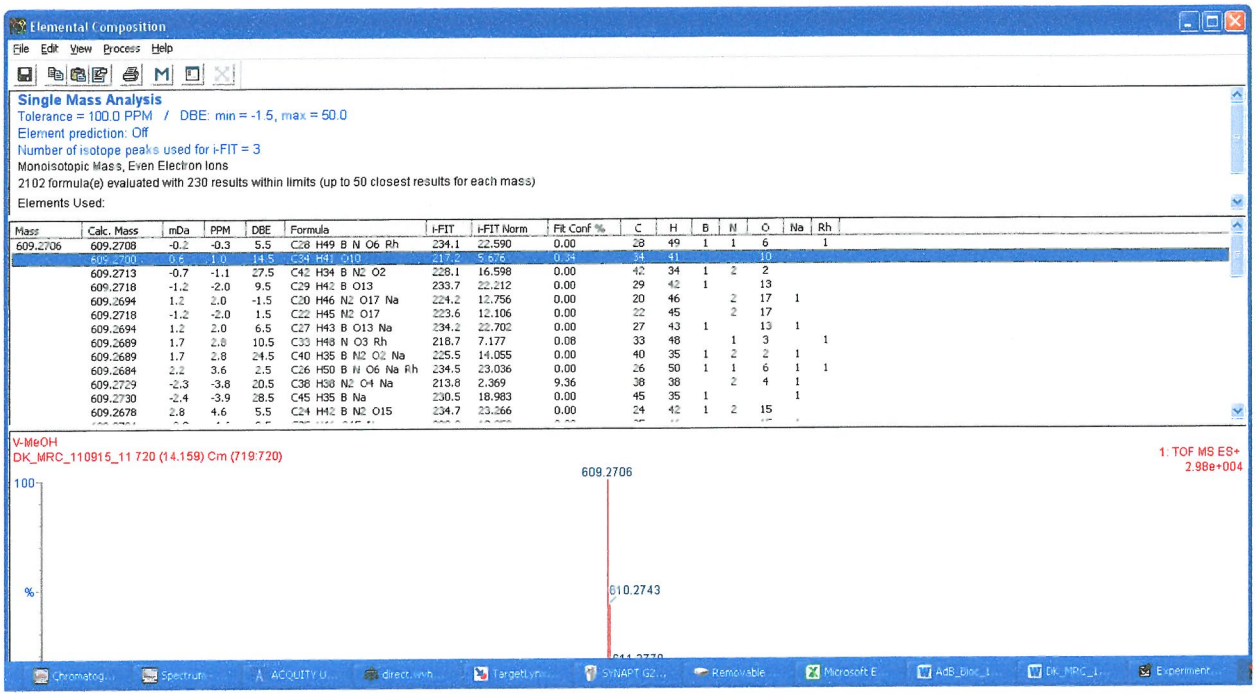
Wilson CL, Solar JM, El-Ghaouth A, Wisniewski ME: Rapid Evaluation of Plant Extracts and Essential Oils for Antifungal activity against *Botrytis cinerea*. Plant Disease 1997;81:204-210.

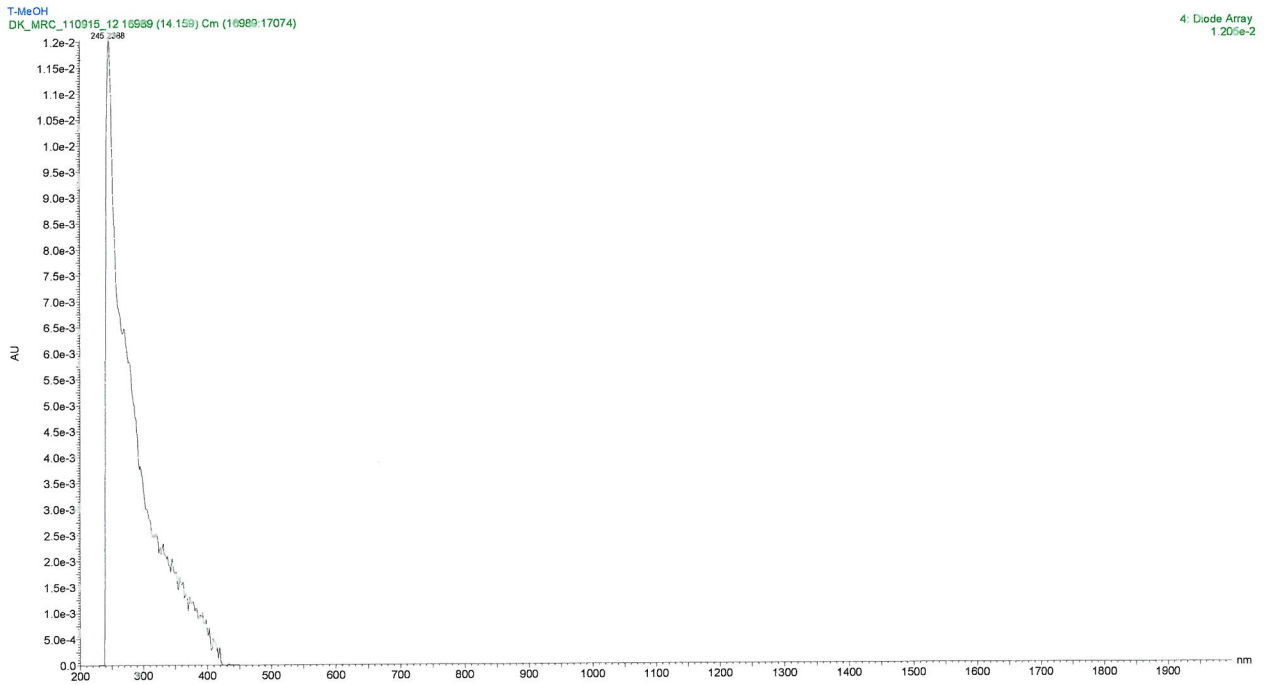
Winkelhausen E, Pospiech R, Laufenberg G: Antifungal activity of phenolic compounds extracted from dried olive pomace. Bulletin of the Chemists and Technologists of Macedonia 2005; 24: 41-46.

Worthington V: Effect of agricultural methods on nutritional quality: a comparison of organic with conventional crops. Alternative Therapies in Health and Medicine 1998;4:58-69.

APPENDIX 1

TOP – BOTTOM: POOR CHROMOPHORIC PROPERTIES OF UB-4





APPENDIX 2

SUMMARY OF THE MAJOR COMPOUND PEAKS FOUND AND THEIR MOLECULAR MASSES

Molecular ion	Retention time	AM	LM	TM	VM	AD	LD	TD	VD	AH	LH	TH	VH
235.5	0.4	xx											
382	2.13			x									
505.5	3.79			xxxx									
784.3	4.43			x									
245.1	4.57											xxxx	
209.1	5.9											x	
256.1	6.02				x								
301.1	6.32		xx				x						
331.1	6.46		xx				x						
295.2	6.84											x	
441.2	6.89			xxx									
324.2	6.82				x								
235.5	7.08							x					
315.1	7.26		xxx				xx						
415.2	7.26											xx	
345.1	7.35		xxxx				xxxx				xxxx		
327.1	7.72							x					
315.1	8.07										xxx		
313.2	8.09											xx	
359.1	8.25												
			xxxx				xxxx				xxxx		
329.1	8.96										xxxx		
253.2	9.12							xxx					
367.2	9.65											x	
529.2	10.25				x				x				
520.3	10.41			xx									
279.2	11.93			x		x				x		x	x
467.3	11.91										x		
419.3	11.97						x						
529.3	12.11				x								
569.4	12.35										xx		
569.4	12.44										xxxx		
459.4	12.43							xxxx					
591.4	12.71										xx		
589.4	13.03										xx		
573.4	13.77										x		
625.3	13.95				x	x			xx				xxx
609.3	14.16	x			xx	x		x	xx	xx			xxx

553.4	14.41									xx		
593.3	14.51	xxxx	xxx	xxxx	xxxx		xxxx	xxxx	xx			xx
593.3	14.81				x			x	x			
523.4	15.34				x					x	xx	
623.3	15.34			x		x						
607.3	15.82	xxx	xxxx	xxxx		xx			xxxx			xxx

AM = *Amaranthus* MeOH extract, LM = *Lippia* MeOH extract, TM = *Tagetes* MeOH extract, VM = *Vigna* MeOH extract, AD = *Amaranthus* DCM extract, LD = *Lippia* DCM extract, TD = *Tagetes* DCM extract, VD = *Vigna* DCM extract, AH = *Amaranthus* Hex extract, LH = *Lippia* Hex extract, TH = *Tagetes* Hex extract, VH = *Vigna* Hex extract.

X = arbitrary scale used to indicate the concentration of compounds based on the area under the curve.

APPENDIX 3

PAPERS PUBLICATIONS FROM THIS THESIS

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ORIGINAL ARTICLE

Antifungal activity of four weedy plant extracts against selected mycotoxigenic fungi

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Keywords
Antimicrobial(s), fungi (*Aspergillus, Fusarium*), mycotoxins, pesticides, weedy plants.

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Abstract

Aims: To investigate the antifungal activity of aqueous and organic extracts of four weedy plant species *viz. Tagetes minuta, Lippia javanica, Amaranthus spinosus* and *Vigna unguiculata* against isolates of four agriculturally important fungi, i.e. *Fusarium verticillioides, F. proliferatum, Aspergillus flavus* and *A. parasiticus.*

Methods and Results: Dried powdered aerial parts of the plants were extracted sequentially with hexane, dichloromethane, methanol and water and tested for activity using a serial microdilution assay. Results were read every day over 120 h. All extracts except for the water extracts showed growth inhibitory activity against most isolates of the *Fusarium* spp. The most active were the methanol and hexane extracts of *V. unguiculata* and *A. spinosus* with minimum inhibitory concentration (MIC) values of <0·5 mg ml^{-1} after 48 h against *Fusarium* spp. No inhibition of the *Aspergillus* spp. tested was observed, but conidium formation was stimulated on plates treated with plant extracts when visually compared to the growth controls.

Conclusions: The results obtained from this study indicated that chemical constituents from these plant species may be developed as potential agrochemical fungicides.

Significance and Impact of the Research: Food and feed are subject to infection by a variety of micro-organisms that can induce spoilage and/or produce metabolites that are toxic to humans and animals. Extracts of *V. unguiculata* and *A. spinosus* were most active and maybe developed into environmentally friendly fungicides, which are affordable to rural farmers in developing countries.

Introduction

In rural Africa as in most of the developing world, subsistence farming is common, with home-grown crops being the major source of food for many households, irrespective of quality considerations (Shephard 2003). Subsistence farming is a strategy by poor rural households to reduce expenditure on food (Watkinson and Makgetla 2002). Food production and storage, therefore, play an important role in stabilizing seasonal food production (FAO 2005). The risk of natural contamination, especially by mycotoxigenic fungi, is an important safety concern. The fungal contaminants most frequently found in food and feed are *Fusarium* and *Aspergillus* species among others, both in the field and in storage (Bankole 2006). Species that are of major concern are *Fusarium verticillioides, F. proliferatum, Aspergillus flavus* and *A. parasiticus,* and these occur mainly in staple food commodities, such as grains and grain-based products (Lewis *et al.* 2005), peanuts and cassava (Wareing *et al.* 2001).

Apart from the production losses that these fungi cause directly, they also produce mycotoxins, which pose a health hazard to both humans and animals and impact on the commercial trade of agricultural produce. The most important toxins produced by some strains of *Fusarium* and *Aspergillus* species are fumonisins and aflatoxins,

respectively, of which fumonisin B_1 (FB_1) and aflatoxin B_1 (AFB_1) are considered to be most toxic (Shephard *et al.* 1996). Fumonisins cause leukoencephalomalacia (LEM) in horses and pulmonary oedema in pigs (PPE) (Marasas 1995) and have been associated with a higher risk of oesophageal and liver cancer in humans (Gelderblom *et al.* 1988; Ueno *et al.* 1997) as well as neural tube defects in infants (NTDs) (Missmer *et al.* 2006). Aflatoxins are potent carcinogens and can act in synergy with hepatitis B virus (HBV) to cause hepatocellular carcinoma (Bhat and Vasanthi 2003). Peri-natal exposure to aflatoxins has been shown to lead to stunted growth (low height for age) and may contribute to infant mortality because of protein energy malnutrition (Gong *et al.* 2002).

Several strategies have been proposed to minimize the loss of food and other agricultural commodities as a result of fungal spoilage and also to reduce exposure to mycotoxins (Huwig *et al.* 2001; Katerere *et al.* 2008). These strategies include among others, the use of synthetic chemicals, microwave or sonic drying and physical separation (Bankole and Adebanjo 2003). Although some of these methods are effective they do not fulfil all the requirements, especially those concerning safety and safeguarding of the nutritional characteristics of treated food and feed (Piva *et al.* 1995).

Fungicides are at present the most important containment tools but they are expensive and inaccessible to rural farmers in many African countries. Most of these chemicals are residual and not biodegradable posing danger to the environment and to the users. More importantly, increasing instances of pesticide resistance have been reported (Wilson *et al.* 1997). There is an urgent need to find affordable interventions, which can be safely used, e.g. fungicides without the prohibitive cost and disadvantages of the currently used synthetic compounds. Extracts from plants may be a viable alternative for such utilization.

Although the interest in research of antifungal compounds from plants is increasing (Sinha and Bhatnagar 1998; Martini *et al.* 2004), most studies tend to focus mainly on activity against human pathogens, and few lead compounds have been developed for use in food and crop protection (Cowan 1999; Agarwal *et al.* 2000; Quiroga *et al.* 2001; Shai *et al.* 2008;). Results obtained from studies on some plant species that have been examined for antifungal activity against plant pathogens are encouraging. For example, extracts of *Allium sativum* have been reported to have potent fungicidal activity, effectively protecting peaches against brown rot caused by *Monilinia fructicola* (Wilson *et al.* 1997). Extracts of *Mentha spicata* L. were reported to completely inhibit the mycelial growth of *Fusarium oxysporum* (Singh *et al.* 1994), whereas essential oils of flowerheads of *Chrysanthemum coronarium* have been shown to inhibit hyphal growth of phytopathogens (Alvarez-Castellanos *et al.* 2001).

In the present study, weedy dietary and medicinal plants were specifically targeted for screening. *Tagetes minuta* (Family: Asteraceae), [common names: wild marigold (English), kambanje (Shona), Kakiebos (Afrikaans), isangwana (Zulu)] is used in rural villages as an insect repellent in granaries (Grassroots Natural Products 2008) and drunk as a medicinal tea for the treatment of various diseases (Rios and Recio 2005). *Lippia javanica* (Family: Verbenaceae) [common names: lemon bush (English), lemoenbossie (Afrikaans), zumbani (Shona), musukudu (Sotho)] has been used by the Vhavenda people of South Africa as an anthelmintic, while the Xhosa people use it for the disinfection of anthrax-infected meat (Viljoen *et al.* 2005). In Zimbabwe, it is used as a mosquito repellent and to treat nightmares and fevered convulsions in young children (Lukwa 1994). Essential oils of *L. javanica* were reported to have toxic and/or repellent effects against insects when used as fumigants in granaries (Omolo *et al.* 2005). *Vigna unguiculata* (Family: Fabaceae) [common names: cowpea (English), munyemba (Shona), boontjies (Afrikaans), isihlumaya (Zulu)] and *Amaranthus spinosus* (Family: Amaranthaceae) [common names: Spiny amaranth (English), thepe (Sotho), mowa (Shona), imbuya (Zulu)] are both cooked fresh or dry and eaten as relish with maize staple porridge (pap or sadza). These plant species play an important role as famine foods in rural Southern Africa, and they may be cooked with a tomato or peanut sauce and served alone or with meat as relish. Both species have been cited for the treatment of diabetes mellitus (Katerere and Eloff 2006) and to control weed infestation when used as cover crop (Ngouajio *et al.* 2003).

This study investigated the activity of aqueous and organic extracts of four weedy plant species against isolates of *F. verticillioides, F. proliferatum, A. flavus* and *A. parasiticus* using the microtitre assay (Eloff 1998). These fungi are important plant pathogens, which produce mycotoxins of public health importance.

Material and methods

Chemicals

All solvents used were of analytical grade. Methanol, hexane and dichloromethane were purchased from Merck (Darmstadt, Germany). Amphotericin B, an antifungal agent used to treat human pathogens and Cantus®, an agricultural fungicide whose active ingredient is boscalid was a kind donation from a farm manager. These were used as positive controls. *P*-iodonitrotetrazolium violet (INT) was obtained from Sigma-Aldrich, Germany.

Plant collection and preparation

Tagetes minuta and *L. javanica* were collected from Onderstepoort, Pretoria, South Africa. *Amaranthus spinosus* and *Vigna unguiculata* were collected from Moruleng village of Rustenburg, North West Province, South Africa. The plants were collected between March and May in 2006, and their identity was confirmed by the University of the Western Cape herbarium where voucher specimens are deposited. The aerial parts were dried at room temperature in the shade and then ground into powder using a Romer Labs Series II Grinding/Subsampling mill, USA.

Preparation of the plant extracts

For each plant species, the dried powdered aerial parts (80 g) were extracted sequentially with 600 ml of each of the following solvents: hexane (HEX), dichloromethane (DCM), methanol (MeOH) and water (H_2O) in a centrifuge tube by blending in a Polytron (Kinematica PT 3100 Lucerne, Switzerland) for 5 min at 2000 *g*. The extracts were centrifuged at 4000 rpm (3000 *g*) for 10 min in a Sorvall Instruments RC-3B Refrigerated Centrifuge (Newtown, CT) and filtered through Whatman no. 1 filter paper. To improve yields, each extraction was repeated twice. The organic extracts were dried on a rotary evaporator (Buchi, Germany) at temperatures of between 55 and 60°C, whereas the aqueous extracts were freeze dried. All the extracts were stored desiccated at 4°C prior to use.

Fungal isolates

Isolates of four fungal species, *Fusarium verticillioides* (MRC 826, 8267 and 8559); *F. proliferatum* (MRC 2301, 6908 and 7140), *Aspergillus flavus* (MRC 3951, 3953 and 3954) and *A. parasiticus* (MRC 200, 352 and 2528) kept in the PROMEC Unit Culture Collection of the South African Medical Research Council (MRC) were used. The *Fusarium* isolates used produced high levels of fumonisin B_1 (Table 1). *Fusarium verticillioides* strains were mainly isolated from maize, and *F. proliferatum* strains were obtained from diverse commodities including maize, rice and asparagus. AFB_1 production of the *A. flavus* and *A. parasiticus* isolates in maize cultures varied between extremely high and very low. The *Fusarium* isolates were grown on Carnation Leaf Agar (CLA) slants, and *Aspergillus* isolates were grown on Potato Dextrose Agar (PDA) for 7–14 days at 25°C and stored in a cold room at 4°C prior to use (Leslie and Summerell 2006). Fungal suspensions were prepared by dislodging the conidia in a 20 ml aqueous solution of 0·05% Tween 20 per slant. Conidium suspensions were standardized to a 0·5 McFarland concentration and were added (12·5 ml) to 37·5 ml Potato Dextrose Broth (PDB).

Table 1 Fungal isolates used with the respective FB_1 and AFB_1 production profiles on maize*

Fungal isolate	Origin of isolate	FB_1 level (g kg^{-1})
Fusarium verticillioides		
MRC 826	Maize	9·05
MRC 8267	Maize	12·09
MRC 8559	Maize	5·6
Fusarium proliferatum		
MRC 2301	Maize	6·5
MRC 6908	Rice	2·8
MRC 7140	Asparagus	2·01
		AFB_1 level (mg kg^{-1})
Aspergillus flavus		
MRC 3951	Maize	18·7
MRC 3953	Maize	0·041
MRC 3954	Maize	3·5
Aspergillus parasiticus		
MRC 200	Maize	4·86
MRC 352	Peanut	73·0
MRC 2528	Unknown	11·8

*Data obtained from MRC/PROMEC Unit culture collection database.

Microtitre assay

A serial dilution microplate technique (Eloff 1998) was used to determine the minimum inhibitory concentration (MIC) for plant extracts against the above-mentioned standardized conidial fungal suspensions. Each plant extract (100 mg) was solubilized/suspended in acetone (1 ml) and diluted in water (9 ml) to obtain a final concentration of 10 mg ml^{-1}. Water (100 μl) was added to all 96 wells of a microplate using a multichannel pipette. The solubilized plant extracts (100 μl) were added in each of the first wells and serially diluted so as to reduce the concentration of each plant extract by 50%. The conidium suspension, prepared in PDB (100 μl), was added to each well. The final concentrations were 2·5, 1·25, 0·64, 0·32, 0·16, 0·08, 0·04 and 0·02 mg ml^{-1}. A solvent control of acetone: water (1:9) was used, and amphotericin B and Cantus™ (boscalid) were included as positive controls. Growth controls for the respective fungal conidium suspensions without the plant extracts were also included. Forty microliters (40 μl) of *p*-iodonitrotetrazolium violet (INT), an indicator of fungal growth, was dissolved in water (0·2 mg ml^{-1}) and added to each well and the covered microplates incubated at 35°C and 100% relative humidity. The plates were examined visually every 24 h

Table 2 Extraction yields (%) from weedy plants using different solvents

Solvent	*Tagetes minuta*	*Lippia javanica*	*Vigna unguiculata*	*Amaranthus spinosus*
Hex	13·1	13·2	11·9	15·9
DCM	16·1	12·0	11·3	6·0
MeOH	31·8	30·7	30·1	19·8
H_2O	35·5	36·0	32·1	40·6
Total yield	96·5	91·9	85·4	82·3

Each plant was extracted three times with each solvent.
Hex, hexane; DCM, dichloromethane; MeOH, methanol; H_2O, water.

up to a total time of 120 h, and the presence of colour formation (pink) indicating viability. The MIC values, i.e. the lowest concentration of the different plant extracts that inhibited fungal growth for each time interval, were recorded.

Results

The highest extraction yields were obtained with water and methanol while hexane and dichloromethane gave similar but lower values (Table 2). However, with *A. spinosus* the recovery with DCM was lower when compared to the other plants while the MeOH extract was also less when compared to H_2O. The lowest total yields were obtained from *V. unguiculata* and *A. spinosus*.

The plant extracts showed no growth inhibitory effects against isolates of the *Aspergillus* spp although conidium formation was stimulated to form visually more conidia compared to the fungal growth control. In general, the DCM extract was more effective in inducing conidia formation followed by HEX and MeOH while no effect was observed with the H_2O extracts. Amphotericin B and Cantus®, the positive controls used, did not have an effect on fungal sporulation.

When considering the *Fusarium* species, the water extracts exhibited no activity at the highest concentration (2·5 mg ml^{-1}) used. The MeOH, HEX and DCM extracts of *V. unguiculata* and *A. spinosus* exhibited the broadest spectrum of antifungal activity after 48 h (Table 3 and 4). Generally, extracts of *T. minuta* and *L. javanica* exhibited weaker activity although differences existed.

To determine the stability of the plant extracts regarding their growth inhibitory effects (fungicidal and/or fungistatic), assays were carried out up to 120 h (Table 3 and 4). As no viability tests were conducted, the fungicidal effects were not evaluated during the present experimental conditions. A decrease in activity could imply that the fungi either became resistant to the extracts or that the active compound(s) became unstable during the incubation period. If the low MIC values obtained after 48 h are maintained over 120 h, the extracts presumably exhibited a fungicidal effect. If the MIC increased during the incubation period then an apparent fungistatic effect is implied. When considering the control antifungal agents, the inhibitory effect was already lost after 48 h, indicating a typical fungistatic effect (Hawser and Islam 1999). The MeOH extract of *T. minuta* exhibited growth inhibitory

Table 3 MIC values of plant extracts against *Fusarium proliferatum*

		MIC (mg ml^{-1}) 48 h			72 h			96 h			120 h		
Plants	Extracts	*6908	*2301	*7140	6908	2301	7140	6908	2301	7140	6908	2301	7140
Tagetes minuta	MeOH	2·50	0·08	2·50	2·50	0·64	2·50	2·50	0·64	2·50	2·50	0·64	2·50
	Hex	2·50	0·02	0·02	2·50	0·32	0·16	2·50	0·64	0·32	2·50	0·64	0·32
	DCM	0·02	0·16	0·02	0·02	0·64	0·64	0·16	2·50	2·50	2·50	2·50	2·50
Lippia javanica	MeOH	0·32	2·50	2·50	0·64	2·50	2·50	0·64	2·50	2·50	1·25	2·50	2·50
	Hex	0·64	0·02	0·02	1·25	0·32	0·64	2·50	2·50	1·25	2·50	2·50	2·50
	DCM	0·32	0·02	0·08	0·32	2·50	1·25	1·25	2·50	2·50	1·25	2·50	2·50
Vigna unguiculata	MeOH	0·02	0·08	0·32	0·08	0·08	0·32	0·16	0·32	0·32	0·32	0·32	0·32
	Hex	0·32	0·08	0·02	0·32	0·08	0·16	0·32	0·64	0·16	0·64	0·64	0·32
	DCM	0·04	0·32	0·32	0·16	1·25	1·25	0·16	2·50	2·50	0·32	2·50	2·50
Amaranthus spinosus	MeOH	0·16	0·04	0·02	0·16	0·04	0·02	0·16	0·04	0·04	0·32	0·16	0·16
	Hex	0·04	0·04	0·02	0·04	0·08	0·08	0·04	0·16	0·08	0·08	0·16	0·16
	DCM	0·04	1·25	0·32	0·08	2·50	2·50	0·08	2·50	2·50	0·08	2·50	2·50
Amphotericin B†			0·04			2·50			2·50			2·50	
Cantus†			0·04			2·50			2·50			2·50	

MIC, minimum inhibitory concentration; DCM, dichloromethane; Hex, hexane; MeOH, methanol.
*MRC isolates. Values are means of triplicate determinations.
†MIC of controls was the same for all strains as shown.
MIC of aqueous extracts showed activity >2·50 mg ml^{-1}.

Table 4 MIC values of plant extracts against *Fusarium verticillioides*

		MIC (mg ml^{-1})											
		48 h			72 h	96 h					120 h		
Plants	Extracts	*826	*8559	*8267	826	8559	8267	826	8559	8267	826	8559	8267
Tagetes minuta	MeOH	2·50	0·02	0·02	2·50	0·64	2·50	2·50	2·50	2·50	2·50	2·50	2·50
	Hex	0·04	0·32	0·32	0·16	0·32	0·32	0·64	0·64	0·64	0·64	0·64	0·64
	DCM	0·08	0·32	0·32	0·08	0·32	0·64	0·64	2·50	2·50	1·25	2·50	2·50
Lippia javanica	MeOH	0·32	0·64	0·32	0·32	0·64	0·64	0·64	2·50	2·50	1·25	2·50	2·50
	Hex	0·08	0·02	1·25	0·32	0·02	2·50	0·64	2·50	2·50	1·25	2·50	2·50
	DCM	0·08	0·16	0·32	0·08	0·64	0·64	0·64	2·50	2·50	1·25	2·50	2·50
Vigna unguiculata	MeOH	0·02	0·02	0·32	0·08	0·02	0·32	0·08	0·64	0·32	0·08	0·64	0·32
	Hex	0·04	0·02	0·16	0·08	0·02	0·16	0·16	0·64	0·64	0·16	0·64	0·64
	DCM	0·02	0·16	0·08	0·04	0·16	0·16	0·08	0·32	2·50	0·32	0·32	2·50
Amaranthus spinosus	MeOH	0·16	0·02	0·16	0·16	0·02	0·32	0·32	0·04	0·32	0·32	0·32	0·64
	Hex	0·04	0·02	0·16	0·16	0·04	0·32	0·32	0·16	0·64	0·64	0·32	0·64
	DCM	0·64	0·16	0·04	0·64	0·16	2·50	1·25	1·25	2·50	1·25	1·25	2·50
Amphotericin B†			0·02			2·50			2·50			2·50	
Cantus†			0·04			2·50			2·50			2·50	

MIC, minimum inhibitory concentration; DCM, dichloromethane; Hex, hexane; MeOH, methanol.
*MRC isolates. Values are means of triplicate determinations.
†MIC of controls was the same for all strains as shown.
MIC of aqueous extracts showed activity >2·50 mg ml^{-1}.

activity against *F. proliferatum* isolate MRC 2301, but showed no effect against two isolates MRC 6908 and MRC 7140. The MeOH extract, however, lost its activity after 72–96 h implying a fungistatic effect, presumably, to acquired resistance or the instability of the active plant extract. The HEX extract lost their activity to some extent over the 120 hr incubation period, although not to the same extent as the MeOH extract. The DCM extract initially showed activity against all the isolates of *F. proliferatum* and *F. verticilliodes*, which was lost after 72–96 h, except against *F. verticillioides* isolate MRC 826.

A similar response was observed regarding the time-dependent inhibitory effect with the other plant extracts although the selectivity of the extracts to the *Fusarium* isolates differs. In general, it would appear that the different extracts from *V. unguiculata* and *A. spinosus* exhibited the highest inhibitory and stability effects over the 120-hr incubation period against all *Fusarium* strains. Once again the DCM extract seemed to lose its activity more rapidly than the MeOH and HEX extracts. It would appear that the DCM extracts are not stable over the experimental time period.

Discussion

Various publications have documented factors that affect conidia formation in *Aspergillus* spp (Cotty 1988; Guzman-de-Pen a and Ruiz-Herrera 1997; Feng and Leonard 1998). Conidia formation is known to occur when fungi are under stress because of fungicides, nitrogen fertilizers and environmental factors such as temperature, pH, water availability or co-inoculation (Leandro *et al.* 2003; Paterson 2007; Xu *et al.* 2007). It is likely that the conidiium stimulation observed in this study was in reaction to treatment with the plant extracts. The implications of conidia formation by *A. flavus* and *A. parasiticus* when exposed to the different extracts are not clear at this stage, but we can speculate that this may influence mycotoxin production, either positively or negatively.

When considering the *Fusarium* spp, the activity of some extracts of the different plants after 48 h was similar to that of Amphotericin B and Cantus®, the positive controls used. There is no validated criteria for the MIC end points for *in vitro* testing of plant extracts; however, Souza *et al.* (2007) working on essential oils proposed classification for plant materials based on MIC results after 48 h as follows: strong inhibitors (MIC < 0·5 mg ml^{-1}); moderate inhibitors (MIC between 0·6 and 1·5 mg ml^{-1}) and weak inhibitors (MIC > 1·6 mg ml^{-1}). Based on the above classifications, the MeOH extract of *T. minuta* exhibited weak (*F. proliferatum* MRC 6908, MRC 7140 and *F. verticillioides* MRC 826) to strong activity (*F. proliferatum* MRC 2301 and *F. verticillioides* MRC 8559, MRC 8267). The HEX and DCM extracts were strong inhibitors (MIC < 0·5 mg ml^{-1}) for all isolates of the two *Fusarium* spp except for the HEX extracts against *F. proliferatum* MRC 6908 (MIC > 1·6 mg ml^{-1}).

Extracts of *L. javanica* were generally strong inhibitors (MIC < 0·5 mg ml^{-1}) of both these *Fusarium* species, except for the MeOH extracts against *F. proliferatum* isolates MRC 2301, MRC 7140 (MIC > 1·6 mg ml^{-1}) and for the HEX extract against *F. verticillioides* isolate MRC 8267.

All the extracts of *V. unguiculata* and *A. spinosus* were strong inhibitors (MIC < 0·5 mg ml^{-1}) against all isolates of the two *Fusarium* spp except for the DCM extract that exhibited a moderate response against *F. proliferatum* MRC 2301 and *F. verticillioides* MRC 826.

The fungal isolates showed varying degrees of sensitivity and tolerance to the plant extracts. The results showed that there was no uniform response within or between fungal isolates of the same species in terms of susceptibility to antifungal activity in the extracts. Variation in sensitivity of bacterial and fungal isolates has been previously reported (Mazzola *et al.*1995; Sandrock and van Etten 1998). These differences in sensitivity among the microorganisms to antimicrobial substances in plant extracts may be explained by the differences in cell wall composition and/or inheritance genes on plasmids that can be easily transferred among bacterial or fungal isolates (Sandrock and van Etten 1998). In this study, it is possible that a resistance mechanism is operating in isolates that exhibited reduced sensitivity to the plant extracts. According to Mazzola *et al.* (1995), if resistance to a xenobiotic is conferred by way of detoxification mechanism, co-existence of sensitive and resistant strains at a particular microsite may have the potential to decrease the efficacy of that substance. More recent literature suggests the role of ATP-binding cassette (ABC) drug efflux transporters in conferring both virulence and xenobiotic resistance in fungi e.g., *Aspergillus nidulans* (de Waard *et al.* 2006) and Candida species (Kolaczkowski *et al.* 2009). Thakur *et al.* 2008 have shown that the overexpression of membrane efflux pumps, mainly ABC transporters leads to the expulsion of anti-fungal compounds.

The polarity of the extraction solvents used sequentially decreases in the order of MeOH > DCM > Hex. Generally, it appears that the inhibitory effect of the plant extracts varies depending on the specific plant and solvent used as well as the fungal isolate with no specific trend related to the polarity of the solvent. When considering the different solvent systems used a diversity of molecules with distinct polarities are extracted from plants. For example, MeOH tends to extract a diversity of compounds such as polyphenols, glycosides and to some extent flavonoids (Rauha *et al.* 2000) that can be assumed to contribute to the inhibitory effect of the extract. DCM and hexane tend to extract mainly nonpolar constituents such as fats, fatty acid and terpenoids (Gómez *et al.* 1996). It would appear that both the polar and nonpolar constituents contributed to the antifungal activity of the plant extracts, although some fungal strains, especially *F. proliferatum* were less susceptible to the MeOH extracts of *T. minute* and *L. javanica*.

The sensitivity of a specific fungal isolate to a specific extract is also worth considering. This would imply that the diverse fungal genetics and the selectivity of specific plant constituents are important when developing plant fungicides. Care should therefore be exercised as this could result in the selection of a specific resistant genotype that can either be less or be more mycotoxigenic. For example, the MeOH extract of *T. minuta* will inhibit the growth of *F. verticillioides* MRC 8559 and MRC 8267 but not MRC 826 which is still a high producer of FB_1. This might therefore inadvertently lead to selective evolution of resistant strains. The effect of the plant extracts on actual mycotoxin production in culture is presently under investigation.

The results obtained from this study indicate that dietary plant species, particularly *V. unguiculata* and *A. spinosus* contain chemical constituents that can be developed as potential antifungal agents for agricultural use. These plants may be ecologically adapted to withstand fungal infection, i.e. they seem to have developed a huge armament of secondary metabolites (phytoalexins) to resist fungal attack because of their constant exposure to fungi because of co-existence with crop plants (Eloff *et al.* 2007).

There has been a recent resurgence of interest in the use of botanicals in agricultural pest management because of the deleterious environmental and health effects of conventional agrochemicals (Isman 2008). This study illustrates the potential that certain plant species may have to be used as botanicals in the burgeoning organic food production sector in industrialized countries and more importantly in resource-poor developing countries where traditional plant use is widespread but lacks scientific rationale (Isman 2006). Furthermore, the plants investigated in this study may provide potential leads for novel bioactives.

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References

Agarwal, S.K., Singh, S.S., Verma, S. and Kumar, S. (2000) Antifungal activity of anthraquinone derivatives from *Rheum emodi*. *J Ethnopharmacol* 72, 43–46.

Alvarez-Castellanos, P.P., Bishop, C.D. and Villalobos, M.J.P. (2001) Antifungal activity of the essential oil of flower-heads of garland chrysanthemum (*Chrysanthemum coronarium*) against agricultural pathogens. *Phytochemistry* **57**, 99–102.

Bankole, S.A. (2006) Mycotoxins in food systems in Sub-Saharan Africa: a review. *Mycotoxin Research* **22**, 163–169.

Bankole, S.A. and Adebanjo, A. (2003) Mycotoxins in food in West Africa: current situation and possibilities of controlling it. *Afr J Biotechnol* **2**, 254–263.

Bhat, R.V. and Vasanthi, C. (2003) Food safety in food security and food trade: mycotoxins and food safety in developing countries. *Focus* **10**, 1–2. Brief 13 of 17. International Food Policy Institute.

Cotty, P. (1988) Aflatoxin and sclerotial production by *Aspergillus flavus*: influence of pH. *Phytopathology* **78**, 1250–1253.

Cowan, M.M. (1999) Plant products as antimicrobial agents. *Clinical Microbiology Review* **12**, 564–582.

Eloff, J.N. (1998) A sensitive and quick microplate method to determine the minimum inhibitory concentration of plant extracts for bacteria. *Planta Med* **64**, 711–713.

Eloff, J.N., Mdee, L.K. and Masoko, P. (2007) Invasive and weedy species can be used as a source of antifungal compounds to control plant fungal pathogens. *S Afr J Bot* **73**, 287 [Published abstract].

Feng, G.H. and Leonard, T.J. (1998) Culture conditions control expression of the genes for aflatoxin and sterigmatocystin biosynthesis in *Aspergillus parasiticus* and *A. nidulans*. *Appl Environ Microbiol* **64**, 2275–2277.

Food and Agriculture Organization of the United Nations (2005) Food insecurity in the world–eradicating world hunger–key to achieving the millennium development goals 2005. http://www.fao.org/news-a.htm. (accessed 30/01/2008).

Gelderblom, W.C.A., Jaskiewickz, K., Marasas, W.F.O., Thiel, P.G., Horak, R.M., Vleggaar, R. and Kriek, N.P.J. (1988) Fumonisins- novel mycotoxins with cancer promoting activity produced by *Fusarium moniliforme*. *Appl Environ Microbiol* **54**, 1806–1811.

Gómez, A.M., López, C.P. and de la Ossa, E.M. (1996) Recovery of grape seed oil by liquid and supercritical carbon dioxide extraction: a comparison with conventional solvent extraction. *Journal of Chemical Engineering and Biochemical Engineering* **61**, 227–231.

Gong, Y.Y., Cardwell, K., Hounsa, A., Eggal, S., Turner, P.C., Hall, A.J. and Wild, C.P. (2002) Dietary aflatoxin exposure and impaired growth in young children from Benin and Togo: cross-sectional study. *Br Med J* **325**, 20–21.

Grassroots Natural Products (2008). Zinziba oil, *Lippia javanica* (Verbenaceae) 2002. Website: http://www.Gnp.co.za/essentialoils/zinziba.htm (accessed 30/01/2008).

Guzman-de-Peña, D. and Ruiz-Herrera, J. (1997) Relationship between aflatoxin biosynthesis and sporulation in *Aspergillus parasiticus*. *Fungal Genet Biol* **21**, 198–205.

Hawser, S. and Islam, K. (1999) Comparisons of the effects of fungicidal and fungistatic antifungal agents on the morphogenetic transformation of *Candida albicans*. *J Antimicrob Chemother* **43**, 411–413.

Huwig, A., Freimund, S., Kappeli, O. and Dutler, H. (2001) Mycotoxin detoxication of animal feed by different adsorbents. *Toxicol Lett* **122**, 179–188.

Isman, M.B. (2006) Botanical Insecticides, Deterrents, And Repellents In Modern Agriculture And An Increasingly Regulated World. *Annu Rev Entomol* **51**, 45–66.

Isman, M.B. (2008) Botanical insecticides: for richer, for poorer. *Pest Manag Sci* **64**, 8–11.

Katerere, D.R.P. and Eloff, J.N. (2006). Management of diabetes in African traditional medicine. In *Traditional Medicines for Modern Times: Antidiabetic Plants* ed. Soumyanath, A., Boca Raton, FL/London/New York: Taylor and Francis Group, CRC Press.

Katerere, D.R., Shephard, G.S. and Faber, M. (2008) Infant malnutrition and chronic aflatoxicosis in Southern Africa: is there a link? *Int J Food Safety, Nutrition and Public Health* **1**, 2.

Kolaczkowski, M., Kolaczkowska, A. and Stermitz, F.R. (2009) Modulation of the Antifungal Activity of New Medicinal Plant Extracts Active on *Candida glabrata* by the Major Transporters and Regulators of the Pleiotropic Drug-Resistance Network in*Saccharomyces cerevisiae*. *Microb Drug Resist* **15**, 11–17.

Leandro, L.F.S., Gleason, M.L., Nutter, F.W. Jr, Wegulo, S.N. and Dixon, P.M. (2003) Strawberry plant extracts stimulate secondary conidiation by *Colletotrichum acutatum* on symptomless leaves. *Phytopathology (Ecology and Population Biology)* **93**, 1285–1291.

Leslie, J.F. and Summerell, B.A. (2006) *The Fusarium Laboratory Manual*. Iowa, USA: Blackwell Publishers.

Lewis, M., Onsongo, H., Njapau, H., Schurz-Rogers, G., Luber, S., Kieszak, J., Nyamongo, L., Backer, A. *et al.* (2005) Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in Eastern and Central Kenya. *Environ Health Perspect* **113**, 1762–1767.

Lukwa, N. (1994) Do traditional mosquito repellent plants work as mosquito larvicides? *Cent Afr J Med* **40**, 306–309.

Marasas, W.F.O. (1995) Fumonisins: their implications for human and animal health. *Nat Toxins* **3**, 193–198.

Martini, N.D., Katerere, D.R.P. and Eloff, J.N. (2004) Biological activity of five antibacterial flavonoids from *Combretum erythrophyllum* (Combretaceae). *J Ethnopharmacol* **93**, 207–212.

Mazzola, M., Fujimoto, D.K., Thomashow, L.S. and Cook, R.J. (1995) Variation in sensitivity of *Gaeumannomyces graminis* to antibiotics produced by fluorescent *Pseudomonas* spp. and effect on biological control of take-all of wheat. *Appl Environ Microbiol* **61**, 2554–2559.

Missmer, S.A., Suarez, L., Felkner, M., Wang, E., Merrill, A.H., Rothman, K.J. and Hendricks, K.A. (2006) Exposure to

fumonisin and the occurence of neural tube defects along the Texas-Mexico border. *Environ Health Perspect* **114**, 237–241.

Ngouajio, M., McGiffen, M.E. and Hutchinson, C.M. (2003) Effect of cover crop and management system on weed populations in lettuce. *Crop Prot* **23**, 57–64.

Omolo, M.O., Okinyo, D., Ndiege, I.O., Lwande, W. and Hassanali, A. (2005) Fumigant toxicity of the essential oils of some African plants against *Anapheles gambiae* sensu stricto. *Phytomedicine* **12**, 241–246.

Paterson, R.R.M. (2007) Some fungicides and growth inhibitor/biocontrol-enhancer 2-deoxy-D-glucose increase patulin from *Penicillium expansum* strains in vitro. *Crop Prot* **26**, 543–548.

Piva, G.F.P., Galvano, F.R.D., Pietri, A.A.P. and Piva, A.R.D. (1995) Detoxification methods of aflatoxins. A review. *Nutrition Research* **15**, 767–776.

Quiroga, E.N., Sampietro, A.R. and Vattuone, M.A. (2001) Screening antifungal activities of selected medicinal plants. *J Ethnopharmacol* **74**, 89–96.

Rauha, J.P., Remes, S., Heinonen, M., Hopia, A., Kähkönen, M., Kujala, T., Pihlaja, K., Vuorela, H. *et al.* (2000) Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *Int J Food Microbiol* **56**, 3–12.

Rios, J.L. and Recio, M.C. (2005) Medicinal plants and antimicrobial activity. *J Ethnopharmacol* **100**, 80–84.

Sandrock, R.W. and van Etten, H.D. (1998) Fungal sensitivity to and enzymatic degradation of the phytoanticipin α-tomatine. *Biochem Cell Biol* **2**, 37–43.

Shai, L.J., McGaw, L.J., Aderogba, M.A., Mdee, L.K. and Eloff, J.N. (2008) Four pentacyclic triterpenoids with antifungal and antibacterial activity from *Curtisia dentata* (Burm.f) C.A. Sm. leaves. *J Ethnopharmacol* **119**, 2238–2244.

Shephard, G.S. (2003) Aflatoxin and food safety: Recent African perspectives. *Toxicology Review* **22**, 267–286.

Shephard, G.S., Thiel, P.G., Stockenstrom, S. and Sydenham, E.W. (1996) Worldwide survey of fumonisin contamination of corn and corn-based products. *J AOAC Int* **79**, 671–687.

Singh, J., Dubeyd, A.K. and Tripathi, N.N. (1994) Antifungal activity of *Mentha spicata*. *Pharm Biol* **32**, 314–319.

Sinha, K.K. and Bhatnagar, D. (1998). *Mycotoxins in Agriculture and Food Safety*. New York/Basel/Hong Kong: Marcel Dekker, Inc.

Souza, E.L., Stamford, T.L.M., Lima, E.O. and Trajano, V.N. (2007) Effectiveness of *Origanum vulgare* L. essential oil to inhibit the growth of food spoiling yeast. *Food Control* **18**, 409–413.

Thakur, J.K., Arthanari, H., Yang, F., Pan, S-J., Fan, X., Breger, J., Frueh, D.P., Gulshan, K. *et al.* (2008) A nuclear receptor-like pathway regulating multidrug resistance in fungi. *Nature* **452**, 604–611.

Ueno, Y., Iijima, K., Wang, S.D., Sugiura, Y., Sekijima, M., Tanaka, T., Chen, C. and Yu, S.Z. (1997) Fumonisins as a possible contributory risk factor for primary liver cancer: a 3 year study of corn harvested in Haimen, China by HPLC and ELISA. *Food Chem Toxicol* **35**, 1143–1150.

Viljoen, A.M., Subramoney, S., Van Vuuren, S.F., Baser, K.H.C. and Demirci, B. (2005) The composition, geographical variation and antimicrobial activity of *Lippia javanica* (Verbenaceae) leaf essential oils. *J Ethnopharmacol* **96**, 271–277.

de Waard, M.A., Andrade, A.C., Hayashi, K., Schoonbeek, H., Stergiopoulos, I. and Zwiers, L.-H. (2006) Impact of fungal drug transporters on fungicide sensitivity, multidrug resistance and virulence. *Pest Manag Sci* **62**, 95–207.

Wareing, P.W., Westby, A., Gibbs, J.A., Allotey, L.T. and Halm, M. (2001) Consumer preferences and fungal and mycotoxin contamination of dried cassava products from Ghana. *International Journal of Food Science Technology* **36**, 1–10.

Watkinson, E. and Makgetla, N. (2002) *South Africa's Food Security Crisis*. Pretoria, South Africa: National Labour and Economic Development Institute (NALEDI). http://www.naledi.org.za/docs/food_security.pdf (accessed 7 June 2010)

Wilson, C.L., Solar, J.M. and El Ghaouth, A. (1997) Rapid evaluation of plant extracts and essential oils for antifungal activity against *Botrytis cinerea*. *Plant Dis* **81**, 204–210.

Xu, X., Nicholson, P. and Ritieni, A. (2007) Effects of fungal interactions among Fusarium head blight pathogens on disease development and mycotoxins accumulation. *Int J Food Microbiol* **119**, 67–71.

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A preliminary survey of mycological and fumonisin and aflatoxin contamination of African traditional herbal medicines sold in South Africa

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Traditional medicine is an important aspect of healthcare delivery in South Africa and is used by at least 70% of the country's population. The trade in medicinal plants is a multi-million rand business which is a major driver for rural economies. However, the conditions in which these plant products are transported and stored make them prone to fungal contamination which results in economic losses to the traders and pose potential health hazards to consumers. Of major concern is the possible presence of toxigenic fungi and mycotoxins. This study assessed fungal and mycotoxin contamination of African herbal products sold in Cape Town and Tshwane (formerly Pretoria) in South Africa. Of the 16 samples analyzed, 15 were contaminated with at least one of these three fungal genera: *Aspergillus*, *Fusarium*, and *Penicillium*. Fumonisin B_1 was present in 13 of the samples in quantities ranging from 14 to 139 µg/kg (detection limit 5 µg/kg). None of the samples was contaminated with aflatoxigenic fungi or aflatoxin (detection limit 0.5 µg/kg). This is the first study to report on mycological and mycotoxin contamination of commercial traditional African medicines in South Africa. There is a need to expand the study to other urban centers to gain enough insight into this problem and then to intervene with measures that can protect the public from potential harm.

Key words: aflatoxin; African herbal medicines; fumonisin; fungal contamination; muthi; mycotoxin

Introduction

African traditional herbal medicine, commonly called muthi (Zulu), emayeza (Xhosa) or mushonga (Shona) in Southern Africa is an important part of healthcare delivery in the subcontinent. It is actively promoted by the South African government and it is used by at least 70% of the country's population.[1]

The trade in traditional herbal medicines in South Africa has been described as a multi-million rand "hidden economy."[2] This is, in part, because the demand for indigenous plant-based medicines has created business that has been estimated in the past to be worth more than R270 million a year with traded amounts topping 20,000 tonnes.[3,4] More recently, turnover has been estimated to exceed R520 million annually.[5] The trade is essentially subsistence based and employs about 130,000 people, mainly rural women, within the informal economy.[5] Market networks for medicinal plants extend across east and southern Africa, with plants being harvested primarily from communal land and/or protected areas.[6] Because of the high demand for traditional herbal medicines in urban areas, the plant material is often transported soon after it is harvested, leaving little time for it to dry before arriving at the market where it may be processed, that is, ground, dried, and/or packaged. Plant products are at risk of contamination from insects, rodent droppings, and other objects/contaminants, for example, human and animal feces in the vicinity of the plant material at the time of harvesting, transporting, and point of sale storage.

A great cause for concern, however, is the possibility of microbial contamination. Though bacterial contamination has been reported and appears to be common place in some products traded in South Africa,[7] fungal contamination is of greater concern because of the potential of mycotoxin production by some fungi and the possibility of acute and chronic mycotoxicosis.

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Aflatoxins are produced by *Aspergillus flavus, A. parasiticus,* and *A. nomius* and exposure may lead to hepatic necrosis, childhood cirrhosis, immune suppression, and hepatocellular carcinomas.[8] Aflatoxin B_1 (AFB_1) is listed by the International Agency for Research on Cancer (IARC) as a human carcinogen.[9] Fumonisins however are produced mainly by *Fusarium verticillioides* (formerly known as *F. moniliforme*) and *F. proliferatum* and cause equine leukoencephalomalacia and porcine pulmonary edema; and FB_1, the most abundant analog, has been declared a possible human carcinogen (group 2B carcinogen) by the IARC based on the toxic and carcinogenic effects in experimental animals.[10] Exposure to fumonisin has also been associated with esophageal cancer and neural tube defects (NTDs) in neonates.[11]

Fungal and mycotoxin contamination of herbal medicines has been reported elsewhere especially in Nigeria and the Indian sub-continent.[12–14] This is the first study of its kind on African herbal medicines bought directly at the point of sale. The long-term goal will be to inform and educate harvesters, traders, healers, and consumers of African traditional herbal medicines of best practices in managing the supply chain of such products to improve and preserve quality and protect consumers.

Materials and methods

Reagents and standards

Pure standards of fumonisin B_1, B_2, and B_3 isolated using the method of Cawood, *et al.*[15] were obtained from PROMEC Unit, Tygerberg, South Africa. A stock solution was made up to 250 µg/mL in a mixture of acetonitrile/H_2O (1:1) for each of the standards. This was diluted to individual working solutions of 49, 20, and 22 µg/mL. AFB_1 was purchased from Sigma (St Louis, Missouri, USA). A stock solution of 250 µg/mL was prepared and diluted to give a working solution of 550 ng/mL. All solvents and chemicals were analytical grade from Merck (Darmstadt, Germany). Water was purified in a Milli-Q system (Millipore, Bedford, Massachusetts, USA).

Acquisition of herbal medicines

Samples (16) were purchased anonymously in different herbal (muthi) markets in Cape Town, and at Marabastad, Tshwane (formerly Pretoria) in South Africa during 2006 (Table 1). The samples were purchased anonymously and placed in sample sacks, taken to the laboratory, photographed, and stored at 4 °C. They were then taken to Kirstenbosch Botanical Gardens of the South African Biodiversity Institute (SANBI) (Cape Town) and as many of the samples as possible were identified by a team of taxonomists to genus and species level.

Mycological assays

Muthi samples were dried at 45 °C for 3 days in an oven. Some samples did, however, remain moist and could not be dried completely. Small parts of the visible fungal growth from these samples were cut out and plated directly onto malt extract agar (MEA) plates containing 150 mg novobiocin/L of agar and incubated at 25 °C in the dark until good growth was obtained. The samples that could be dried were finely ground using a small electric laboratory mill (Falling AB Stockholm, Sweden) and stored at 4 °C. The mill was thoroughly cleaned between samples to prevent cross-contamination. Fungal counts were done on these samples by using a plate dilution technique. In all, 1 g of the material was suspended in 9 mL sterile distilled water and serially diluted 10 times. In all, 1 mL of each dilution was placed in a 90 cm Petri dish and 15 mL of cooled MEA (including novobiocin) added, mixed, and incubated at 25 °C for 3–5 days. Counts (colony forming units [cfu]) of each of the fungal species identified were recorded at the appropriate dilution. Selected isolates from both direct plating and plate dilution methods were single spored and identifications confirmed using cultural and microscopic morphological characteristics. Cultural characteristics included colony diameters and pigmentation on Potato Dextrose Agar (PDA) after incubation in the dark for 3 days at 25 °C and 30 °C, colony morphology on PDA, and carnation leaf agar after incubation for 10–14 days under mixed lights with a 12-h photo period.

Extraction of aflatoxin B_1

AFB_1 was extracted from dried plant material using a modification of published methods.[16,17] Briefly, 1 g of milled plant sample was mixed with 1 g NaCl and extracted with 25 mL of 80% aqueous methanol. This was blended with a Polytron PT 31000 homogenizer (Kinematica, Switzerland) at 7000–9000 rpm for 5 min then centrifuged at 4000 *g* for 10 min and filtered through Macherey-Nagel (Durn, Germany) MN 617 filter paper. The filtrate (10 mL) was diluted with 40 mL of distilled water and subsequently filtered through Schleicher and Schuell filter paper (Dassel, Germany) and then via a 0.45 µm acetate syringe filter. In all, 10 mL of the filtrate was loaded onto Aflatest column (Vicam, Massachusetts, USA) and passed through at a rate of

Table 1 Mycological and chemical analyses of muthi samples collected from Cape Town and Pretoria

Plant species (common name)		*Fungi isolated: sample a = dilution plating (cfu/g × 10^4); sample b = direct plating*						*Fumonisin B_1 (μg/kg)*
		A. n	Fus *spp.*	Mucor *spp.*	Pen *spp.*	Rhiz spp.	*Other fungi*	
Dioscorea sp. (Uthuvana) (heartwood)	a	4	>100	1	–	–	1 (mixed growth)	40
	b	+	–	+	+	+	*Sten* sp.	
Myrsiphyllum asparagoides Willd (Isicakatha) (roots)	a	>100	–	–	–	–	–	87
	b	+	–	–	+	–	–	
Unidentified legume (Iqwili) (rootstock)	a	1	2	–	–	–	6 (mixed growth)	ND
	b	+	+	–	–	–	–	
Pelargonium sp. (Umsila wengwe) (rootstock)	a	20	–	20	40	–	–	14
	b	–	–	–	–	–	*Alt* sp. *Phoma* sp.	117
Ganoderma sp. (Sibindi) (bracket fern)	a	+	–	–	–	+	–	30
	b	+	–	–	–	+	–	
Scilla sp. (Mudhora) (corm)	a	30	>100	20	–	–	50	25
	b	–	+	–	–	+	–	
Haemanthus coccineus L. (Matunga) (corm)	a	–	20	–	–	–	–	139
	b	–	–	+	–	+	(Bacteria)	
Urginea sp. (Mredeni) (corm)	b	–	+	+	+	–	–	21
Kedrostis nana lam. (Bogo) (bulb)	a	–	>100	–	>100	–	–	
	b	–	+	–	–	–	*Phoma* sp.	
Kniphofia sp. (Red Carrot) (rootstock)	b	+	–	–	+	–	*Alt*sp.;*Trich* sp.	30
Mentha longifolia HERBA (Chrismint) (aerial parts)	a	–	–	–	–	–	>100	ND
	b	+	–	–	–	–	*Clad*sp.;*Asp* sp.	
Cinnamonum camphora (Roselina) (bark)	b	+	–	–	–	+	*Trich* sp.	126
Bidens pilosa (Seloka) (aerial parts)	b	–	+	–	–	–	*Phoma*sp.;*Alt* sp.	67
Amaranthus thunbergii (Thepe) (leaves)	b	–	+	–	–	–	*Phoma*sp.;*Alt* sp.	26
Hypoxis hemerocallidea fisch. (Thlonya) (corm)	a	–	–	–	>100	–	–	ND
	b	–	+	–	–	–	–	
Unidentified (Wagapa) (stem)	b	–	–	–	–	–	–	ND

cfu, colony forming units; +, species present in the sample; –, species absent in sample; ND, not detected (detection limit = 5 μg/kg); *A. n*, *Aspergillus niger*; *Alt*, *Alternaria* spp; *Clad* sp., *Cladosporium* spp.; *Fus*, *Fusarium* spp.; *Muc*, *Mucor* spp.; *Pen*, *Penicillium* spp.; *Rhiz*, *Rhizopus* spp.; *Sten*, *Stenocarpella* spp.; *Trich*, *Trichosporum* spp.

1–2 drops/s. AFB_1 was thereafter eluted under gravity by passing 3 mL of High Performance Liquid Chromatography (HPLC) grade methanol through the column and the eluate collected in scintillation vials and dried under nitrogen at 60 °C.

Recoveries were performed on three different plant products after spiking samples at 5.5 μg/kg and 11 μg/kg with AFB_1 to take into account matrix effects.

Extraction of fumonisins (FB_1, FB_2, and FB_3)

The extraction method[18] was modified for use on plant matrix. Milled plant sample of 5 g was mixed with 25 mL of methanol and then blended with a Polytron PT 31000 homogenizer at 8000–10000 rpm for 5 min. The mixture was then centrifuged at 1000 *g* for 10 min at 4 °C and the supernatant filtered through Macherey-Nagel (Durn, Germany) MN 617 filter paper. An aliquot of 6 mL of the filtrate was then diluted with 6 mL of distilled water and then adjusted to pH 6–6.5. A 10 mL aliquot of this was loaded on conditioned strong anionic exchange (SAX) columns (Bond Elut, Varian, Massachusetts, USA) and the procedure used by Shephard, *et al.*[19] followed.

Three different samples were spiked at 52 μg/kg (for FB_1), 48 μg/kg (for FB_2), and 54 μg/kg (for FB_3) and recoveries performed.

HPLC analysis

Chromatograms were recorded using a Hewlett Packard Agilent 1100 Series Fluorescent detector (Darmstadt, Germany) set at ex = 365, em = 440 for AFB_1 and ex = 335, em = 440 for the three fumonisins. HP ChemStation for LC software was used for data processing.

AFB_1 was analyzed using post-column iodine derivatization as followed by Shephard, *et al.*[20] The dried extracts were reconstituted in 200 μL of methanol. In all, 5 μL and 10 μL aliquots of the standard and extracts were injected, respectively. AFB_1 was separated on an Ultracarb 3 μm ODS (100 × 4.6 mm) (Phenomenex, Torrance, California, USA) with 0.01 M KH_2PO_4:CH_3CN:CH_3OH (690:200:75) mobile phase pumped at a flow rate of 1.0 mL/min.

FB_1, FB_2, and FB_3 were analyzed after derivatization with *o* – phthaldialdehyde (OPA) before

injecting on the column according to the method.[19] Separation was performed on a Phenomenex (Torrance, California, USA) Synergi 4 µm MAX-RP column (75 × 4.6 mm) using a mobile phase of methanol:0.1 M sodium phosphate buffer (73:27, pH 3.35) pumped at a flow rate of 1.0 mL/min. Quantification of the analytes was by peak area comparison with standards.

Results and discussion

In all, 15 of the 16 samples were found to be contaminated by various species of fungi (Table 1). *A. niger* was the most common contaminant being isolated from 50% of the samples, followed by *Fusarium* (6/16) and *Penicillium* (5/16). Approximately 60% of the samples were co-infected with *Alternaria* and *Rhizopus* spp, whereas one sample showed bacterial contamination.

Recoveries of AFB_1 from three different spiked plant samples were 71% (RSD = 22%), 66% (3.5%), and 77% (3.5%) at 5.5 µg/kg and 57% (20%), 71% (23%), and 88% (17%) at 11 µg/kg which were considered satisfactory. None of the samples was found to be naturally contaminated with AFB_1 (detection limit <0.5 µg/kg). The recoveries of fumonisins from three different spiked samples were 76% (RSD = 2%), 80% (6.9%), and 74% (7.5%) for FB_1 (52 µg/kg), FB_2 (48 µg/kg), and FB_3 (54 µg/kg), respectively. In all, 13 of the 16 samples were positive for FB_1 (detection limit 5 µg/kg). FB_2 and FB_3 were not detected (Table 1).

Thiel, *et al.*[21] have recently developed a method for the analysis of aflatoxin in medicinal herbs. However, because of chemical constituents of plant products which vary with locality, season, and species, it is almost impossible to have one method which works for all plant products. This is evident from the variability of recoveries among the spiked samples. This is the reason why recoveries were performed on three different species to validate the method in this study which was the best compromise available. Arranz, *et al.*[22] have noted that the complexity of chemical constituents of herbs tends to impact on aflatoxin binding on immunoaffinity columns by affecting pH, salt concentration, and competition for binding sites. The methods used by Ip and Che[16] and Sewram, *et al.*[17] were initially attempted without modification and this resulted in recoveries below 30%. They were then adapted and modified by reducing the amounts extracted and not using Tween-20 as a diluent, among other changes. This resulted in recoveries which were generally above 70% for both fumonisins and aflatoxin.

One of the most common ways that plant products may be contaminated is through their mode of transportation to the market. Harvesters commonly convey plants to the markets in plastic or hessian bags previously used to store maize or maize meal.[23] Fungi such as *A. flavus* and *A. parasiticus* (known aflatoxin producers) and *F. verticillioides* (a major fumonisin producer) are known to grow on maize. If medicinal plants are placed in infected bags, then cross contamination may occur. After the plants are placed in the bags, they are usually taken to the markets in the luggage compartments of buses, cars, and commuter taxis, and the insufficient air circulation leads to increased humidity which favors fungal growth. The relative humidity at the market site and the manner in which the plants are stored can further augment conditions favorable for the growth of fungal contaminants and also cross contamination.

Underground plant parts are most likely to be contaminated than aerial parts. This may be because they are fleshy and moist and therefore may not dry properly from the time of harvesting to the shop-floor. Roots and corms also appear to be the most popular parts in African traditional medicine because they are not seasonal (unlike leaves and other aerial parts). This popularity along with their susceptibility to contamination poses a potential public health problem.

There have been several recent publications on mycotoxin contamination of botanical products, particularly of Chinese medicines.[17,24] These studies have shown the presence of aflatoxins and ochratoxin A, a nephrotoxin produced by *A. ochraceus* and *Penicillium verrucosum* in numerous botanical products including ginseng, ginger, licorice, devil's claw, and turmeric. African traditional herbal medicines are different from botanicals in form and presentation. They are not commercially packaged and they are not sold in conventional retail outlets, health food stores, or pharmacies. Instead, they are sold in make-shift stalls or on pavements in almost all towns in Africa.

Ip and Che[16] previously analyzed dietary and medicinal plants used in the Eastern Cape province of South Africa. They also reported no AFB_1 contamination; however, they reported levels of FB_1 contamination ranging from 8 to 1553 µg/kg. The highest concentration in the present study was only 139 µg/kg, well below the highest reported in the Eastern Cape study. However, the plant samples were collected from botanical gardens by the investigators themselves[16] and not purchased from the

markets as with those in this study. No mycology was reported in that instance, but the results appear to suggest that the plants were colonized by mycotoxicogenic fungi and the possibility of external contamination can be ruled out.

The present study however provides important information about the mycological and toxin contamination of the product at the point of sale. It is worth noting that most users of medicinal plants buy them from markets and traditional healers and that in most cases they appear unconcerned about the visible signs of contamination. See pictures in Figure 1. There is also a belief in some instances that the more effective herbs are those that look unhygienic. B-E. Van Wyk (personal communication) argues that these products may become more efficacious due to some fermentative breakdown and by the formation of bio-active by-products or alcohol.

The presence of fungal and mycotoxin contamination can be potentially harmful especially because the consumers are already suffering from ill-health. Fumonisins have been associated with esophageal cancer in parts of South Africa and China and in the increased incidence of NTDs.[11,16] Aflatoxin exposure leads to hepatocellular carcinoma and stunted growth.[8] Exposure to fungal spores is a leading cause of allergic reactions in young children and respiratory infections particularly in immune compromised persons.[25]

The absence of AFB_1 which is a more pernicious mycotoxin particularly in a country with a high burden of infectious disease (hepatitis B and HIV/AIDS being of greatest concern) such as South Africa is a good outcome. However, this may be due to the geographical location of the markets; both Cape Town and Tshwane (Pretoria) are outside the hot and humid sub-tropical climes which are ideal for the growth of aflatoxigenic *Aspergillus* species. There is a need to focus on the hot and humid areas of South Africa and investigate aflatoxin occurrence there in a bigger and more focused study. It will also be worth tracking plant products brought in from Mozambique, Swaziland and the tropical climes of Central Africa as these are known hotspots of aflatoxin.[26] As Williams[6] has noted, herbal medicines are imported from as far as the Democratic Republic of Congo and environs to Johannesburg and Durban markets.

Conclusion

For economic, social, and cultural reasons the use of African herbal medicines continues to increase. Although there is huge interest into research to validate the efficacy of medicinal plants, what is of immediate importance is the need to assure the quality and safety of commercially traded herbs. This study though limited by the data set has

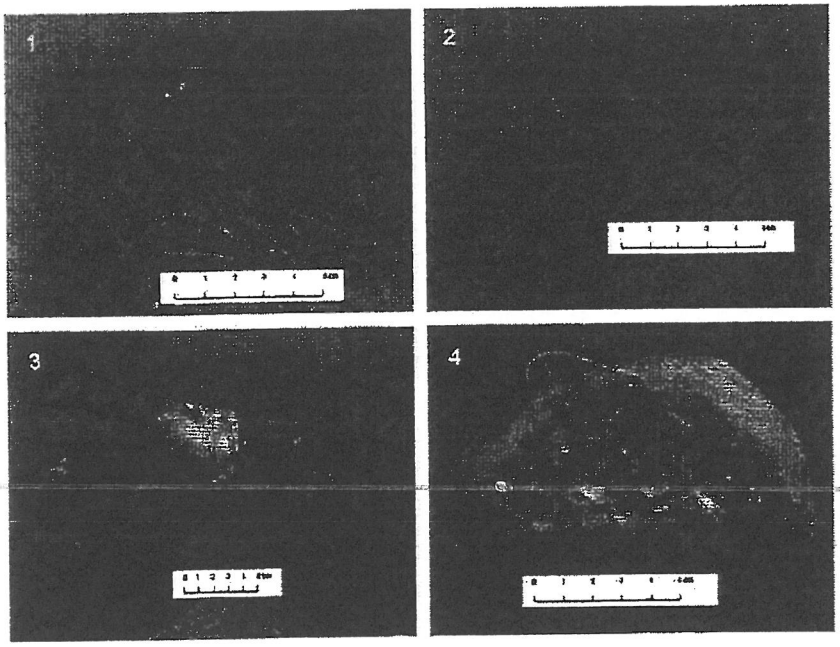

Figure 1 Four examples of medicinal plants purchased from muthi markets for this study.

shown that there is potential harm due to possible contamination of muthi products with fungi and mycotoxins. There is a need to explore these results further by expanding the area survey.

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References

1 Springfield, EP, Eagles, PKF, Scott, G. Quality assessment of South African herbal medicines by means of HPLC fingerprinting. *J Ethnopharmacol* 2005; **101**: 75–83.
2 Cunningham, AB. Herbal medicine trade: a hidden economy. *Indicator S Afr* 1989; **6**: 51–54.
3 Mander, M. Marketing of indigenous medicinal plants in South Africa – a case study in KwaZulu Natal. Rome: FAO; 1998.
4 Dold, AP, Cocks, ML. The trade in medicinal plants in the Eastern Cape Province, South Africa. *S Afr J Sci* 2002; **98**: 589–597.
5 Inggs, M. Plant strippers threaten traditional healing. Business Report 2007; 9.
6 Williams, VL. Market networks for medicinal bark in southern Africa. Paper presented at the indigenous plant use forum Conference (IPUF), 3–6 July 2006. Gaborone: University of Botswana. Conference Abstract, 1–69.
7 Govender, S. du Plessis - Stoman, D, Downing, TG, van de Venter, M. Traditional herbal medicines: microbial contamination, consumer safety and the need for standards. *S Afr J Sci* 2006; **102**: 253–255.
8 Williams, JH, Phillips, TD, Jolly, PE, Stiles, JK, Jolly, CM, Aggarwal, D. Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *Am J Clin Nutr* 2004; **80**: 1106–1122.
9 International Agency for Research on Cancer (IARC). Monograph on the evaluation of carcinogenic risk to humans. Lyon, France. *IARC* 1987; (Suppl. 1): 82–87.
10 Shephard, GS, Thiel, PG, Stockenström, S, Sydenham, EW. Worldwide survey of fumonisin contamination of corn and corn-based products. *J AOAC Int* 1996; **79**: 671–687.
11 Hendricks, K. Fumonisins and neural tube defects in South Texas. *Epidemiology* 1999; **10**: 198–200.
12 Esimone, CO, Oleghe, PO, Ibezim, EC, Okeh, CO, Iroha, IR. Susceptibility – resistance profile of microorganisms isolated from herbal medicine products sold in Nigeria. *Afr J Biotechnol* 2007; **24**: 2766–2775.
13 Kneifel, W, Czech, E, Kopp, B. Microbial contamination of medicinal plants – a review. *Planta Med* 2002; **68**: 5–15.
14 Aziz, NH, Youssef, YA, El-Fouly, MZ, Moussa, LA. Contamination of some common medicinal plant samples and spices by fungi and their mycotoxins. *Bot Bull Acad Sinica* 1998; **39**: 279–285.
15 Cawood, ME, Gelderblom, WCA, Vleggaar, R, Behrend, Y, Thiel, PG, Marasas, WFO. Isolation of the fumonisin mycotoxins - a quantitative approach. *J Agric Food Chem* 1991; **39**: 1958–1962.
16 Ip, S-P, Che, C-T. Determination of aflatoxins in Chinese medicinal herbs by high – performance liquid chromatography using immunoaffinity column cleanup improvement of recovery. *J Chromatogr A* 2006; **1135**: 241–244.
17 Sewram, V, Shephard, GS, van der Merwe, L, Jacobs, TV. Mycotoxin contamination of dietary and medicinal wild plants in the Eastern Cape Province of South Africa. *J Agric Food Chem* 2006; **54**: 5688–5693.
18 Trucksess, M, Weaver, C, Oles, C, DOvidio, K, Rader, J. Determination of aflatoxins and ochratoxin a in ginseng and other botanical roots by immunoaffinity column cleanup and liquid chromatography with fluorescence detection. *J AOAC Int* 2006; **89**: 624–630.
19 Shephard, GS, Thiel, PG, Sydenham, EW. Determination of fumonisin B1 in plasma and urine by high – performance liquid chromatography. *J Chromatogr* 1992; **574**: 229–304.
20 Shephard, GS, van der Westhuizen, L, Gatyeni, PM, Katerere, DR, Marasas, WFO. Do fumonisin mycotoxins occur in wheat. *J Agric Food Chem* 2005; **53**: 9293–9296.
21 Thiel, PG, Stockenström, S, Gathercole, PS. Aflatoxin analysis by reverse phase HPLC using post-column derivatization for enhancement of fluorescence. *J Liq Chromatogr* 1986; **9**: 103–112.
22 Arranz, I, Sizoo, E, van Egmond, H, Kroeger, K, Legarda, TM, Burdaspal, P, *et al.* Determination of aflatoxin B1 in medical herbs: interlaboratory study. *J AOAC Int* 2006; **89**: 595–605.
23 Williams, VL. Hawkers of health: an investigation of the Faraday Street traditional medicine market in Johannesburg, Gauteng. Final Report to the Gauteng Directorate of Nature Conservation, DACEL, 2003. Plant Ecology and Conservation Series vol. 15. University of the Witwaterand, South Africa, 1–23.
24 DOvidio, K, Trucksess, M, Weaver, C, Horn, E, McIntosh, M, Bean, G. Aflatoxins in ginseng roots. *Food Addit Contam* 2006; **23**: 174–180.
25 Bush, RK, Portnoy, JM, Saxon, A, Terr, AI, Wood, RA. The medical effects of mold exposure. *J Allergy Clin Immunol* 2006; **117**: 326–333.
26 Van Rensburg, SJ, Cook-Mozaffari, P, Van Schalkwyh, DJ, Van Der Watt, JJ, Vincent, TJ, Purchase, IF. Hepatocellular carcinoma and dietary aflatoxin in Mozambique and Transkei. *Br J Cancer* 1985; **51**: 713–726.

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Full Length Research Paper

Antioxidant activity of some African medicinal and dietary leafy African vegetables

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Seven medicinal and dietary plant species from Southern Africa were analysed for their antioxidant and total phenolic content. These were *Lippia javanica, Tagetes minuta, Bidens pilosa, Vigna unguiculata, Amaranthus spinosus, Telfairia occidentalis* and *Corchorus olitarius*. Aqueous methanol extracts were tested for free radical scavenging and anti-oxidant activity using three standard assays including 2,2'azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing antioxidant potential (FRAP). The Folin-Ciocalteu assay was used to determine the total phenolic content with gallic acid as a standard. The antioxidant activity of the plants ranged from 0.76 to 5.77 mmol TEAC/100 g (ABTS), 16.29 to 1711.22 mmol TEAC/100 g (DPPH) and 0.58 to 6.12 mmol TEAC/100 g (FRAP). *B. pilosa* and *C. olitarius* had the best activity in all assays, while *V. unguiculata* and *A. spinosus* were the least active. The total phenolic content ranged from 19.79 to 333.56 mg gallic acid equivalent (GAE)/100 g. In general, there was a good correlation between antioxidant activity and total phenolic content. These results imply that these plant species may possess health promoting effects and might be potential sources of potent natural antioxidants.

Key words: Dietary plants, metabolic stress, antioxidant activity, total phenolic content.

INTRODUCTION

Free radicals are generally unstable reactive molecules that are produced in animals and humans under physiological and pathological conditions (Fang et al., 2002). There is increased scientific evidence that oxidative stress which results in the generation of free radicals contributes to many common ailments including cancer, cardiovascular disease, cataract formation, as well as accelerating the ageing process (Bagchi et al., 2000; Fang et al., 2002; Bugg et al., 2006; Dasgupta et al., 2007). Epidemiological studies have shown a strong and consistent protective effect of dietary antioxidants against the risk of such illnesses (Block et al., 1992; Varma et al., 1995; Steinmetz and Potter, 1996; Hunter and Fletcher, 2002). This protective effect is often attributed to different antioxidant components, such as vitamin C, vitamin E, carotenoids, polyphenolic compounds and other phytochemicals (Amin et al., 2006). A high intake of food rich in natural antioxidants has been shown to increase the antioxidant capacity of the plasma and reduce the risk of some, but not all, cancers, heart diseases and stroke (Kris-Etherton et al., 2002). Recent research has also shown that through overlapping or complementary effects, the complex mixture of phytochemical compounds in fruits and

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Abbreviations: **ABTS**, 2,2'Azinobis-3-ethylbenzthiazoline-6-sulphonic acid; **DPPH**, 1,1-diphenyl-2-picrylhydrazyl; **FRAP**, ferric reducing antioxidant potential; **GAE**, gallic acid equivalent.

vegetables provides a protective effect on health than single phytochemicals (Eberhardt et al., 2000).

The present study investigated the total phenolic content and related total antioxidant potential of extracts of *Amaranthus spinosus* (Amaranthaceae), *Vigna unguiculata* (Fabaceae), *Lippia javanica* (Verbenaceae), *Tagetes minuta* (Asteraceae), *Bidens pilosa* (Asteraceae), *Telfairia occidentalis* (Cucurbitaceae) and *Corchorus olitarius* (Tiliaceae). The plant species were selected because of their dietary use in southern Africa. In addition, all of them are important in the traditional medical armamentarium. *L. javanica*, for example, has been reported to have antimicrobial activity and is used in the treatment of colds, coughs and bronchial problems (Muchuweti et al., 2006). The species has also been reported to be effective against fever caused by malaria, influenza and measles (Viljoen et al., 2005). For example, *T. minuta* has been reported to have antibacterial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis* (Vasudevan et al., 1997). It is used in the treatment of colds, diarrhoea and suspected liver ailments (Rios and Recio, 2005). *A. spinosus* is used in the treatment of menorrhagia, gonorrhoea, eczema and colic (Azhar et al., 2004) and is also cited in the treatment of diabetes (Katerere and Eloff, 2005). The root infusion of *V. unguiculata* is used to treat dysmenorrhoea and epilepsy, while a soup made from the seeds is reported for the treatment of bilharziasis (Gelfand et al., 1993). *B. pilosa* has been reported to have antibacterial, antifungal and anticancer activities (Khan et al. 2001), and *T. occidentalis* is used to treat anaemia (Aiyeloja and Bello, 2006; Mensah et al., 2008).

Furthermore, the leaves of *C. olitarius* have been reported to possess diuretic, antipyretic, analgesic and antimicrobial activity, to contain antitumor compounds (Furumoto et al., 2002) and antioxidant carotenoids and flavonoids (Azuma et al., 1999; Khan et al., 2001; Zeid, 2002). *A. spinosus, V. unguiculata, B. pilosa, T. occidentalis* and *C. olitarius* are eaten as a relish with maize staple (pap or sadza). These plant species play an important role as famish foods in rural Southern Africa and they may be cooked (fresh or dry) with tomato or peanut sauce and served alone or with meat. Aerial parts of *T. minuta* and *L. javanica* are consumed as herbal teas and commercially available in some African countries, for example, in Ghana and Kenya.

However, there are few studies on the healthful properties of these dietary plant species. In this study, we screened them for both antioxidant activity and total phenolic content, considering the increasing importance of both parameters in human health and nutrition.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade. 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), 2,2'azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), gallic acid and Trolox were obtained from Sigma Aldrich Co. (St. Louis, MO). Anhydrous sodium carbonate (Na_2CO_3), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), Folin–Ciocalteu phenol reagent, hydrochloric acid (HCl), glacial acetic acid, potassium persulphate, methanol and sodium acetate trihydrate were obtained from Merck (Darmstadt, Germany).

Plant materials

T. minuta, L. javanica and *B. pilosa* were collected from Onderstepoort, Pretoria, South Africa, while *A. spinosus, V. unguiculata, T. occidentalis* and *C. olitarius* were collected from Moruleng village of Rustenburg, North West Province, South Africa. Voucher specimens were collected and deposited at the UWC herbarium in Cape Town. The plants were collected between March and April 2007, oven-dried at 50°C and separately ground into a fine powder using a Romer Labs Series II Grinding/Sub-sampling mill (Romer Labs, Tulln, Austria). They were then shipped to the University of Naples, Federico II in Naples, Italy, where the analysis was done.

Extract preparation

Three grams (3 g) of each plant material were extracted with 30 ml of 70% aqueous methanol by sonication for 30 min and centrifuged (Jouan CR3i of BICASA Spa, Italy) for 5 min at 4000 rpm. The extracts were filtered using Whatman no. 1 filter paper and the aliquots were analyzed for their antioxidant capacity and total phenol content using the methods subsequently described. Each sample was prepared and analyzed for each assay in triplicate.

ABTS radical scavenging assay

The free radical scavenging activity of the plant extracts was determined using 2,2'azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical cation decolorization assay and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) as a standard (Re et al., 1999). ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulphate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12 to 16 h before use. The free radical was stable in this form for more than two days when stored in the dark at room temperature. For this study $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm. Reagent blank reading was also taken (A_0). After the addition of 1.0 ml of diluted $ABTS^{\bullet+}$ solution to 100 µL of each plant extract, the absorbance reading (A_e) was taken exactly 2.5 min after initial mixing. The plant extracts were first adequately diluted (where necessary) to fit within the linearity range. The percentage inhibition of absorbance at 734 nm was calculated using the formula: % inhibition = $(1 - A_e / (A_0) \times 100$. The antioxidant capacity based on the ABTS free radical scavenging ability of the extract was expressed as mmol Trolox equivalence antioxidant capacity (TEAC) per 100 g of plant material.

Ferric reducing antioxidant potential assay (FRAP)

The FRAP assay was carried out according to the method of Benzie and Strain (1996) with few modifications. The FRAP reagent was prepared from sodium acetate buffer (300 mmol/L, pH 3.6), 10 mmol/L 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) solution in 40 mmol/L HCl and 20 mmol/L $FeCl_3$ solution in proportions of 10:1:1 (v/v),

Table 1. Total antioxidant activity and phenolic content of the plant extracts.

Plant species	TEAC (mmol/100 g)			Phenolic Content
	ABTS	DPPH	FRAP	(mg GAE/100 g)
Vigna unguiculata	0.76	95.93	0.58	109.14
Amaranthus spinosus	1.02	16.29	0.75	79.79
Tagetes minuta	2.3	1399.42	4.22	216.84
Lippia javanica	1.5	1462.54	2.38	221.31
Bidens pilosa	5.77	1210.05	6.12	333.56
Telfairia occidentalis	3.37	293.29	2.68	222.94
Corchorus olitarius	4.42	1711.22	5.04	316.34

ABTS, 2,2'Azinobis-3-ethylbenzthiazoline-6-sulphonic acid; DPPH, 1,1-diphenyl-2-picrylhydrazyl; FRAP, ferric reducing antioxidant potential; GAE, gallic acid equivalent

respectively. The FRAP reagent was prepared fresh daily. Antioxidant potential was determined by adding 651 µL sodium acetate buffer solution, 279 µL of FRAP reagent and 70 µL of each plant extract. The absorbance reading was taken at 593 nm exactly 4 min after initial mixing. The plant extracts were first adequately diluted to fit within the linear dynamic range. Solvent blanks were also prepared and the absorbance reading taken. The antioxidant capacity based on the ability to reduce ferric ions of the extract was expressed as mmol Trolox equivalents (TEAC) per 100 g of plant material.

DPPH radical scavenging assay

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity of plant extracts was determined using the method by Yen and Chen (1995). The DPPH radical ($DPPH^{\cdot}$) solution was prepared in MeOH to make a 1 mM $DPPH^{\cdot}$ solution. DPPH solution was further diluted with MeOH at the ratio of 1:25. The scavenging activity of the plant extracts was determined by adding 600 µl of diluted DPPH solution and 300 µl of each plant extract. The absorbance reading of 0.900 ± 0.020 at 517 nm was taken exactly 4 min after initial mixing. The plant extracts were first adequately diluted to fit within the linearity range. The absorbance of the DPPH radical without antioxidant (the control) was also measured. All determinations were carried out in triplicate. The percentage inhibition of the DPPH radical by the samples was calculated using the same formula as in ABTS assay. The antioxidant capacity based on the DPPH free radical scavenging ability of the extract was expressed as mmol Trolox equivalents (TEAC) per 100 g of plant material.

Determination of total phenolic content (TPC)

Total phenolic concentration in plant extracts was determined spectrophotometrically by the Folin–Ciocalteu assay (Singleton and Rossi, 1965) using gallic acid as a standard. An aliquot of 125 µL of each plant extract was mixed with 125 µL of Folin–Ciocalteu phenol reagent and allowed to react for 6 min. Afterward, 1.25 ml of saturated Na_2CO_3 solution (7.5%) was added and allowed to stand for 90 min before the absorbance of the reaction mixture was measured at 760 nm. The total phenolic content of the plant extracts was expressed as mg gallic acid equivalents (GAE) per 100 g of plant material.

Statistical analysis

All determinations of the antioxidant activity by the assays were conducted in triplicate. The reported values of each sample and the correlation coefficients (R^2) were calculated as the mean of three measurements using Microsoft Excel 2000.

RESULTS AND DISCUSSION

Antioxidant activity

The ABTS, FRAP and DPPH assays were used to measure the antioxidant activity of the plant extracts. Because different antioxidant compounds may act *in vivo* through different mechanisms, no single method can fully evaluate the total antioxidant capacity of foods. For this purpose, in this study three different assays were applied to obtain robust data on antioxidant activity of the selected plants (Table 1). The ABTS and DPPH assays measure the ability of antioxidants to quench a radical cation, while the FRAP assay evaluate the reducing potential of the samples.

The TEAC values of ABTS assay ranged from 0.76 - 5.77 mmol Trolox/100 g, FRAP from 0.58 - 6.12 mmol Trolox/100 g and for DPPH from 16.29 - 1711.22 mmol Trolox/100 g. *B. pilosa* was found to have the highest antioxidant activity in both ABTS (5.77 mmol/100 g) and FRAP (6.12 mmol/100 g) assays, while *C. olitarius* had the highest antioxidant activity in DPPH (1711.22 mmol/100 g) assay. *V. unguiculata* had the lowest antioxidant activity in both ABTS (0.79 mmol/100 g) and FRAP (0.58 mmol/100 g) assays. Moreover, the antioxidant activity obtained from ABTS and FRAP were better correlated (R^2 = 0.8296) compared to those obtained from ABTS-DPPH (R^2= 0.2585) and DPPH-FRAP (R^2 = 0.5943). This implies that these plants contain compounds that are capable of scavenging free cation radicals ($ABTS^{*+}$) as well as reducing oxidants (ferric ions). Activity against DPPH radical was not potent on the other hand. According to Surveswaran et al. (2007), it is not surprising to find differences in antioxidant activity among the assays, as each has a different mechanism of action and different reaction conditions. ABTS*+ is soluble in both aqueous and organic solvents and so can determine both hydrophilic

and lipophilic antioxidant capacities (Arnao, 2000; Thaipong et al. 2006).

V. unguiculata and *A. spinosus* showed the lowest antioxidant activity in all the assays, but this activity was similar to that previously reported for other commonly consumed African green leafy vegetables (Akindahunsi and Salawu, 2005; Oboh, 2006). Antioxidant activity of *V. unguiculata* seeds has been previously reported and it was demonstrated that the DPPH radical and ABTS cation radical scavenging activities were well correlated with the ferric reducing antioxidant capacity (Oboh and Akindahunsi, 2004; Siddhuraju and Becker, 2007). Oboh (2005) reported the antioxidant activity of the aqueous and ethanolic extracts of *T. occidentalis* leaf showing the hepatoprotective properties after garlic-induced oxidative stress in rats; in particular the aqueous extract is more effective than the ethanolic extract and this could be attributed to the higher antioxidant activity of the aqueous extract. To date, the reported antioxidant activity of *L. javanica* and *T. minuta* are mainly from essential oils (Muyima et al., 2004). A comparative study of the antioxidant properties of hydrophilic and lipophilic extract constituents of the *C. olitorius* leaves by Oboh et al.,(2009) reported that hydrophilic extract showed a higher DPPH radical-scavenging ability, reducing power and trolox equivalent antioxidant capacity (TEAC), while lipophilic extract showed a higher OH scavenging ability. Zeashan et al. (2009) also reported that ethanolic extracts of *A. spinosus* possess significant hepatoprotective activity which might be due to antioxidant capacity, and they attributed this to its high phenolic content.

In general, limited information is available on the antioxidant potential of extracts of these plants which are important dietary and medicinal plants in rural sub-Saharan Africa. Our results show that the free radical scavenging ability of the plants analyzed was generally lower than that of fruits and vegetables like spinach and red pepper which were previously studied by Chu et al. (2002). It is also complicated to compare results from other studies because of immense variations in environment, climate and time of harvest, among numerous other parameters which affect any wild growing plant (Howard et al., 2003; Zhou and Yu, 2004).

Total phenolic content

Phenolics constitute one of the major groups of compounds which act as primary antioxidants (Muchuweti et al., 2006). They inhibit autoxidation of unsaturated lipids, thus preventing the formation of oxidized low-density lipoprotein (LDL), which is considered to induce cardiovascular diseases. The assay used for total phenols determination detects phenolic acids, flavonoids, tannins, anthocyanins, lignans and coumarins. The content of phenolic compounds is expressed as milligrams gallic acid equivalence (mg GAE) per 100 g plant sample. The amounts of total phenolics of the plant extracts are shown in Table 1. The total phenolic content of the plants decreased in the order: *B. pilosa* (333.56 mg GAE/100 g) > *C. olitarius* (316.34 mg GAE/100 g) > *T. occidentalis* (222.94 mg GAE/100 g) > *L. javanica* (221.31 mg GAE/100g) > *T. minuta* (216.84 mg GAE/100g) > *V. unguiculata* (109.14 mg GAE/100 g) > *A. spinosus* (79.79 mg GAE/100 g).

While some authors found a strong correlation between phenolic content and the antioxidant activity (Velioglu et al., 1998), others found no such relationship (Kaehkoenen et al., 1999) showing that the antioxidant activity of an extract cannot be predicted on the basis of its total phenolic content because the activity of phenolic compounds depends on their chemical structure (Statue-Gracia et al., 1997). This is probably because there are structure-activity relationships governing anti-oxidant activity. Flavones and catechins are, in general, superior to other polyphenols (for example, lignans and coumarins) in this regard (Nijveldt et al., 2001). In addition, the extent of hydroxylation and glycosylation also plays an important role in determining anti-oxidant potency. The antioxidant activity of flavonoids has been shown to reside in the aromatic hydroxyl groups (Rezk et al., 2002) with maximum radical scavenging activity displayed by flavonoids with 3-OH groups attached to the 2,3–double bond neighboring the carbonyl group in the C-ring (Cotelle, 2001). Resorcinol and phloroglucinol substituents also show substantially higher antioxidant activity compared to phenol (Rezk et al., 2002). In this regard, two anti-oxidant pharmacores are now recognized in flavonoids - the catechol structure in ring B and in rings A and C (Rezk et al., 2002).

In this study, the findings show that phenolic content was better correlated with both FRAP (R^2= 0.8923) and ABTS (R^2= 0.8266) assays than with that of DPPH (R^2= 0.622) (Table 2). These results suggest that in the plants analyzed in the current study, 89% of the ferric reducing power and 82.6% of the ability to scavenge the $ABTS^{*+}$ radical cations, is probably due to phenolic compounds. Also, it can be concluded that antioxidant activity of plant extracts is not limited to phenolics. Activity may also be due to the presence of other secondary metabolites such as volatile oils, carotenoids, and vitamins, among others, that in this case contributed to 11% (for FRAP assay) and 17.4% (for ABTS assay), respectively to the antioxidant capacity. The non-polyphenolic compounds appear to be more important in contributing to activity in the DPPH assay. The antioxidant activity of phenolics is mainly due to the ease with which they can be involved in redox reactions, which implies that they can act as reducing agents, hydrogen donors and singlet oxygen quenchers.

The extract with the lowest total polyphenolic content was that of *A. spinosus*, which also showed the lowest antioxidant activity in both assays. Our results confirm

Table 2. Correlation between the different assays used in this study.

Correlation	R^2
ABTS - DPPH	0.8296
ABTS - FRAP	0.2585
DPPH - FRAP	0.5943
ABTS - TPC	0.8266
DPPH - TPC	0.622
FRAP - TPC	0.8923

previous observations. Muchuweti et al. (2006) reported that the measured antioxidant activity of plant extracts may be due to the synergistic effect of polyphenolics with one another and/or with other components present in an extract. However, there is a need to identify and to characterize active components within each plant extract since it is reported in the literature that different classes of phenolics have varying antioxidative strengths and that synergy of polyphenolics (condensed tannins, gallotannins and flavonoids) with one another or with other components present in an extract may contribute to the overall observed antioxidant activity (Shahidi et al., 1994).

Conclusion

African leafy vegetables have long been known and reported to have health protecting properties and uses. They have a long history of being consumed by humans as both food and medicine. However, due to westernization and urbanization, the consumption of these vegetables appears to be declining and yet they may be an important resource in promoting good nutrition in sub-Saharan Africa (Odav et al., 2007; Smith and Eyzaguirre, 2007). The current study shows the potential of some common African vegetables as functional foods whose uses should be further investigated and encouraged. The antioxidant activity and total phenolic content of the extracts analyzed were appreciable. Results highlighted a good correlation between antioxidant activity and total phenolic content. This may indicate that phenolic compounds play a role in the antioxidant activity of plant materials. Meanwhile, further work is necessary to elucidate the identity the compounds responsible for the antioxidant activity of these plant species.

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REFERENCES

Aiyeloja A, Bello O (2006). Ethnobotanical potentials of common herbs in Nigeria: A case study of Enugu state. Edu. Res. Review 1: 16-22.
Akindahunsi A, Salawu S (2005). Antioxidant indices of some green leafy vegetables. *Trop. Sci.* 45: 33-35.
Amin I, Norazaidah Y, Hainida K (2006). Antioxidant activity and phenolic content of raw and blanched Amaranthus species Food Chemistry, 94:47-52.
Arnao MB (2000). Some Methodological Problems in the Determination of Antioxidant Activity Using Chromogen Radicals: A Practical Case. Trends Food Sci. Tech. 11, 419-421.
Azhar ul H, Malik A, Khan A, Shah M, Muhammad P (2004). Spinoside, new coumaroyl flavone glycoside from Amaranthus spinosus. Archiv.pharmaceut. Res. 27(12): 1216-1219.
Azuma K, Nakayama M, Koshioka M, et al (1999). Phenolic Antioxidants from the Leaves of Corchorus olitorius L. J Agric Food Chem. 47(10): 3963-3966.
Bagchi D, Bagchi M, Stohs SJ, Das DK, Ray SD, Kuszynski CA, Joshi SS, Pruess HG (2000). Free radicals and grape seed proanthocyanidin extract: importance in human health and disease prevention. Toxicology, 148(2-3): 187-197.
Benzie IFF, Strain JJ (1996). The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. Analytical biochemistry, 239: 70-76
Block G, Patterson B, Subar A (1992). Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. Nutr Cancer.18(1):1-29.
Bugg GJ, Crocker I, Baker PN, Johnston TA, Taggart MJ (2006). The effect of superoxide free radicals on human myometrial contractility in vitro. Eur J Obstet Gynecol Reprod Biol.125(1): 140-141.
Chu YF, Sun J, Wu X, Liu RH (2002). Antioxidant and antiproliferative activities of common vegetables. J. Agric. Food Chem. 50(23): 6910-6916.
Cotelle N (2001). Role of flavonoids in oxidative stress. Curr. Top. Med. Chem. 1(6): 569-590.
Dasgupta N, De B (2007). Antioxidant activity of some leafy vegetables of India: A comparative study. Food Chemistry, 101: 471-474.
Eberhardt MV, Lee CY, Liu RH (2000). Antioxidant activity of fresh apples. Nature,405(6789): 903-904.
Fang YZ, Yang S, Wu G (2002). Free radicals, antioxidants, and nutrition. Nutrition, 18(10): 872-879.
Furumoto T, Wang R, Okazaki K, AFMF H, Ali M, Kondo A, Fukui H, (2002). Antitumor promoters in leaves of jute (Corchorus capsularis and Corchorus olitorius). Food Sci Technol Res 8 239-243.
Gelfand M, Mavi S, Drummond R, Ndemera B (1993) *The traditional medical practitioner in Zimbabwe* 2nd ed. Gweru: Mambo Press.
Howard LR, Clark JR, Brownmiller C (2003). Antioxidant capacity and

phenolic content in blueberries as affected by genotype and growing season. J. Sci. Food Agric. 83: 1238-1247
Hunter K, Fletcher J (2002). The antioxidant activity and composition of fresh, frozen, jarred and canned vegetables. Innovative Food Science & Emerging Technologies, 3: 399-406.
Kaehkoenen M, Hopia A, Vuorela H, Rauha J (1999). Antioxidant Activity of Plant Extracts Containing Phenolic Compounds. J. Agric. Food Chem. 47: 3954-3962.
Katerere D, Eloff J (2005). Management of diabetes in African traditional medicine. In: Soumyanath A, ed. Tradi. Med. modern times: Antidiabetic plants: Taylor and Francis Group, CRC Press. pp. 157-158.
Khan MR, Kihara M, Omoloso AD (2001). Anti-microbial activity of Bidens pilosa, Bischofia javanica, Elmerillia papuana and Sigesbekia orientalis. Fitoterapia, 72(6): 662-665.
Kris-Etherton PM, Hecker KD, Bonanome A, Coval SM, Binkoski AE, Hilpert KF, Griel AE, Etherton TD (2002). Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. Am. J. Med. 113(9): 71-88.
Mensah J, Okoli R, Ohaju-Obodo J, Eifediyi K (2008). Phytochemical, nutritional and medical properties of some leafy vegetables consumed by Edo people of Nigeria. . Afr. J. Biotechnol. 7: 2304-2309.
Muchuweti M, Nyamukonda L, Chagonda L, Ndhlala A, Mupure C, Benhura M (2006). Total phenolic content and antioxidant activity in selected medicinal plants of Zimbabwe. Int. J. Food Sci. Technol. 41: 33-38.
Muyima N, Nziweni S, Mabinya L (2004). Antimicrobial and antioxidative activities of Tagetes minuta, Lippia javanica and Foeniculum vulgare essential oils from the Eastern Cape Province of South Africa. J. Essential Oil-Bearing Plants, 7: 68-78.
Nijveldt RJ, van Nood E, van Hoorn DE, Boelens PG, van Norren K, van Leeuwen PA (2001). Flavonoids: a review of probable mechanisms of action and potential applications. Am. J. Clin. Nutr. 74(4): 418-425.
Odav B, Beekrum U, Baijnath H (2007). Preliminary assessment of nutritional value of traditional leafy vegetables in KwaZulu-Natal. J. Food Compos. Anal. 20:430-435.
Oboh G (2006). Antioxidant properties of some commonly consumed and underutilized tropical legumes. Eur. Food Res. Technol. 224 61-65.
Oboh G, Akindahunsi A (2004). Change in the ascorbic acid, total phenol and antioxidant activity of sun-dried commonly consumed green leafy vegetables in Nigeria. Nutrition and Health, 18: 29-36.
Oboh G (2005). Effect of blanching on the antioxidant properties of some tropical green leafy vegetables. Food Sci. Technol. 38: 513-517.
Oboh G, Raddatz H, Henle T (2009). Characterization of the antioxidant properties of hydrophilic and lipophilic extracts of Jute (Corchorus olitorius) leaf. Int. J. Food Sci. Nutr. 60: 124-134.
Rezk BM, Haenen GR, van der Vijgh WJ, Bast A (2002). The antioxidant activity of phloretin: the disclosure of a new antioxidant pharmacophore in flavonoids. Biochem. Biophy. Res. Commun. 295(1): 9-13.
Rios JL, Recio MC (2005). Medicinal plants and antimicrobial activity. J Ethnopharmacol. 100 (1-2): 80-84.
Steinmetz K, Potter J (1996). Vegetables, fruits and cancer prevention: a review. J. Am. Diet Assoc. 96: 1027-1039.
Statue-Gracia M, Heionen M, Frankel E (1997). Antioxidant activity of anthocyanin in LDL and lecithin liposome systems. J. Agric. Food Chem. 5: 3362-3367.
Shahidi F, Wanasundara UN, Amarowicz R (1994). Natural antioxidants from low-pungency mustard flour. Food Res. Int. 27(5): 489-493.
Siddhuraju P, Becker K (2007). The antioxidant and free radical scavenging activities of processed cowpea (Vigna unguiculata (L.) Food Chemistry, 101: 10-19.
Singleton VL, Rossi JA (1965). Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. Am. J. Enol. Vitic. 16(3): 144-158.
Smith F, Eyzaguirre P (2007). African leafy vegetables: Their role in the World Health Organisation's global fruit and vegetables initiative. Afr. J. Food, Agric, Nutr. Develop.7(3).
Surveswaran S, Yi-Zhong C, Corke H, Sun M (2007). Systematic evaluation of natural phenolic antioxidants from 133 Indian medicinal plants. Food Chemistry, 102: 938-953.
Thaipong K, Boonprakob U, Crosby K, Cisneros-Zevallos L, Byrne D (2006). Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. J. Food Compos. Anal. 19: 669-675.
Varma S, Devamanoharan P, Morris S (1995). Prevention of cataracts by nutritional and metabolic antioxidants. . Crit. Rev. Food Sci. Nutr. 35: 111-129.
Vasudevan P, Kashyap S, Sharma S (1997). Tagetes: A multipurpose plant. Bioresour Technol. 62 (1-2): 29-35.
Velioglu Y, Mazza G, Gao L, Oomah B (1998). Antioxidant activity and total phenolics in selected fruits, vegetable, and grain products, . J. Agric.Food Chem. 46: 4113-4117.
Viljoen AM, Subramoney S, van Vuuren SF, Baser KH, Demirci B (2005). The composition, geographical variation and antimicrobial activity of Lippia javanica (Verbenaceae) leaf essential oils. J Ethnopharmacol. 96 (1-2): 271-277.
Yen G-C, Chen H-Y (1995). Antioxidant Activity of Various Tea Extracts in Relation to Their Antimutagenicity. J Agric Food Chem. 43(1): 27-32.
Zeashan H, Amresh G, Singh S, Rao CV (2009). Hepatoprotective and antioxidant activity of Amaranthus spinosus against CCl4 induced toxicity. J Ethnopharmacol. 125(2): 364-366.
Zeid A (2002). Stress metabolites from Corchorus olitorius L. leaves in response to certain stress agents. Food Chemistry, 76: 187-195.
Zhou K, Yu L (2004). Antioxidant Properties of Bran Extracts from Trego Wheat Grown at Different Locations. J. Agric .Food Chem. 52(5): 1112-1117.