FUMONISIN PRODUCTION BY AND BIOLOGICAL CONTROL OF *FUSARIUM* SPECIES ASSOCIATED WITH COWPEA SEED

BY

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DECLARATION

I declare that the thesis/dissertation, which I hereby submit for the degree of Master of science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signed ……..
Date……..

DEDICATION

This thesis is dedicated to Vincent Wooko Etambakonga, for his positive support when I was away for my studies.

Cette thèse est dedié à Vincent Wooko Etambakonga, pour tout son soutien pendant la durée de mes études.

“ Leye endodo wami Vincent Wooko, alako la olombwelo waye loloseno lami wambotana dihole lashina nd’ otemami. One ele tshembetelo yayo lo nkumbo kaso”.

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Department: **Plant Science**

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ABSTRACT

Cowpea, *Vigna unguiculata* (L.) Walp. is an important edible legume crop gaining recognition in the world generally and in Africa particular. The seed is a very good source of vegetable protein for millions of people living in sub-Saharan Africa. Cowpea seed are susceptible to global post-harvest losses caused by insect damage, microbial deterioration and other factors especially when stored at high ambient temperatures and relative humidities. The detection of *Fusarium* species and fumonisins associated with cowpea seed was primarily investigated in this study. Secondly, the antifungal activity of extracts from three Rubiaceae plant species was evaluated against five *Fusarium* species. Column chromatography was used in an attempt to isolate potential compounds from the plant extracts.

Cowpea seed samples were obtained from a local market of a rural community in Mpumalanga Province, South Africa and were analysed for seed mycoflora and various fungal genera including, *Aspergillus* spp., *Fusarium* spp. (*Fusarium oxysporum. F. solani, F. subglutinans, F. verticillioides* and *F. proliferatum), Penicillium* spp., *Chaetomium spp., Chrysonilia* spp., *Cladosporium* spp., *Monascus* spp., *Phoma* spp., *Mucor* spp., *Alternaria alternata, Epicoccum spp.* and *Lasiodiplodia theobromae* were isolated from the seed samples. Some *Fusarium* species including *F. oxysporum*, *F.*

verticillioides, *F. subglutinans, F. solani* and *F. proliferatum* were cultured on maize patty media and analysed for fumonisin production. Fumonisin detection was conducted using the VICAM Affinity method. Only *Fusarium subglutinans* and *F. oxysporum* produced fumonisin B analogues ranging between 1.1 - 4.3 µg/g and 3.47 - 31.66 µg/g, respectively. Neither *Fusarium verticillioides, F. solani* nor *F. proliferatum* revealed any production of fumonisin B. Fumonisin B analogues were found to be present in all the cowpea seed samples with levels ranging between 6.2 - 59 µg/g.

Purified fumonisin B_1 was used to investigate the phytotoxic effects of the toxin *in vitro* and *in vivo* on cowpea seedling growth and emergence and also on the ultrastructure of the seed tissue. Surface-disinfected seeds were imbibed in sterile distilled water amended with FB_1 at various concentrations (10, 25, 50 and 100 µg/ml) and dry seeds and seeds imbibed in sterile distilled water served as the controls. Percentage germination was determined *in vitro* and *in vivo* according to the International Seed Testing Association (ISTA) rules. Root and shoot length was measured after 8 days. Compared to the controls all the concentrations significantly decreased seed germination and the highest concentrations of FB_1 (50 and 100 µg/ml) inhibited root and shoot elongation. Transmission electron microscopy (TEM) was used to study sections of the cotyledon and embryonic tissue of cowpea seed. Samples were treated with different concentrations of FB_1. Compared to the controls, the degree of damage to the vacuoles, cell walls and lipid bodies of cowpea seed tissue was directly proportional to the concentrations.

The efficacy of crude extracts from *Morinda citrifolia, Gardenia brighamii*, and *Psychotria capensis* was investigated against various *Fusarium* species by two methods. The microtitre plate doubling dilution method and bioautography method were used to test methanol, acetone, and dichloromethane extracts of the leaves for their antifungal activity. *Psychotria capensis* acetone extracts showed the best inhibition against *F. proliferatum* and *F. verticillioides* with an MIC of 1.5 mg/ml and an MFC of 1.5 mg/ml. The dichloromethane extracts exhibited no activity against the *Fusarium* species tested. Column chromatographic purification of methanolic extracts of the leaves of *P. capensis*

led to the isolation and identification of two compounds namely β-sitosterol and a carotenoid derivative.

Keywords: cowpea, *Fusarium* species, fumonisin, biological control, *Psychotria capensis*, *Gardenia brighamii*, *Morinda citrifolia*, phytotoxicity.

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LIST OF ABBREVIATIONS

AC	-	Acetone
ARC	-	Agricultural Research Council
DCM	-	Dichloromethane
DDT	-	Dichloro Diphenyl Trichloroethane
DMSO	-	Dimethyl Sulphoxide
DON	-	Deoxynivalenol
ELEM	-	Equine leukoencephalomalacia
EtOH	-	Ethanol
FB_1	-	Fumonisin B1
FB_2	-	Fumonisin B2
FB_3	-	Fumonisin B3
FHB	-	*Fusarium* Head Blight
GC	-	Gas Chromatography
HPLC	-	High Performance Liquid Chromatography
IARC	-	International Agency for Research on Cancer
INT	-	Iodonitrotetrazolium violet
ISTA	-	International Seed Testing Association
LC	-	Liquid Chromatography
MeOH	-	Methanol
MIC	-	Minimum Inhibitory Concentration
MFC	-	Minimum Fungicidal Concentration
MRC	-	Medical Research Council

MS	-	Mass Spectroscopy
NMR	-	Nuclear Magnetic Resonance
PBS	-	Preparation Buffer Solution
PDA	-	Potato Dextrose Agar
PPE	-	Porcine Pulmonary Edema
ppm	-	parts per million
PPRI	-	Plant Protection Research Institute
SFC	-	Supercritical Fluid Chromatography
TLC	-	Thin Layer Chromatography

CHAPTER 1

GENERAL INTRODUCTION

General introduction

1. 1. Background

Cowpea (*Vigna unguiculata* (L.) Walp.) is one of the most ancient human food sources and has probably been used as a crop plant since Neolithic times (Ehlers and Hall, 1997). A lack of archaeological evidence has resulted in contradicting views supporting Africa, Asia and South America as origin (Johnson, 1970; Tindall, 1983). Singh *et al*. (2003) reported that cowpea is of major importance to the nutrition and livelihoods of millions of people in less-developed countries of the tropics. Young leaves, green pods and green seeds are used as vegetables whereas dry seeds are used in a variety of food preparations (Nout, 1996; Nielsen *et al.,* 1997). Trading of fresh produce and processed cowpea foods and snacks provides rural and urban women with an opportunity for earning cash income. Cowpea is also a major source of protein, minerals and vitamins (Table 1.1) (Bressani, 1985). Accordingly, it can positively impact on the nutrition and health of poor people, particularly children.

In its fresh form young cowpea leaves, immature pods and peas are used as vegetables in West and Southern Africa. Several snacks and main meal dishes are prepared from the grain (Singh *et al*., 1997). Cowpea paste, which is prepared from dried peas, is the primary ingredient for the well-known Nigerian fried product “akara” (Bulgarelli *et al*., 1988). In Asia, green pods are eaten whereas in east and southern Africa the tender leaves are regularly picked and eaten like spinach (Singh *et al*., 1997). In Africa, the young leaves are sometimes dried for use in soups, while the haulms are fed to livestock. In advanced agriculture, cowpeas are used mostly for fodder and as cover crops, though black eye types are grown for dry seed on a large scale in California, USA (Allen, 1983).

Table 1. 1. Chemical composition of cowpea (%) (Kay, 1979; Tindall, 1983; Quass, 1995)

	Seeds	Hay	Leaves
Carbohydrate	56-66		8
Protein	22-24	18	4.7
Water	11	9.6	85
Crude fibre	5.9-7.3	23.3	2
Ash	3.4-3.9	11.3	
Fat	1.3-1.5	2.6	0.3
Phosphorous	0.146		0.063
Calcium	0.104-0.076		0.256
Iron	0.005		0.005

Cowpeas are susceptible to a wide range of pests and pathogens that attack the crop at all stages of growth and storage; including insects bacteria, viruses and fungi (Allen, 1983). Many fungi are serious parasites of seed primordial. Maturing and stored seeds and grains and their invasion by fungi can result in various damage including, reduced yields of seed in both quantity and quality, discolouration, decreased germinability, mycotoxin production and decay (Kritzinger *et al*., 2003; Castillo *et al*., 2004).
Mycotoxins, when ingested during the consumption of infected seed and other foodstuffs, can lead to serious health complications in animals as well as humans (Marasas, 1995).

Fumonisins are a group of naturally occurring mycotoxins produced by *Fusarium verticillioides*, *F. proliferatum* and other related species (Katta *et al*., 1997; Kedera *et al*., 1999; Tseng and Lui, 1999; Bacon *et al*., 2001; Miller, 2001). Of several fumonisins identified, only fumonisins B_1, B_2, and B_3 are produced in significant quantities under both culture and natural conditions (Marin *et al*., 1999; Aziz *et al*., 2005). Fumonisins B_1 and B_2 are structurally related mycotoxins known to be associated with outbreaks of equine leukoencephalomalacia (ELEM) (Bezuidenhout *et al*., 1988) and porcine pulmonary edema (PPE) in swine (Gelderblom *et al*., 1988). In addition, they are suspected carcinogens in human esophageal cancer (Sydenham *et al*., 1990; Rumbeiha and Oehme, 1997).

A wide variety of commodities have been analyzed for the presence of fumonisins including maize (*Zea mays*) (Fandohan *et al*., 2003), rice (*Oriza sativa*), millet (*Eleusine coracana*) (Amadi and Adeniyi, 2009), and cowpea seed (*Vigna unguiculata*) (Kritzinger *et al*., 2003). Houssou *et al*. (2009) also confirmed the presence of fumonisin mycotoxins associated with cowpea seeds from West Africa.

The problem of food and feed contamination with mycotoxins is of current concern and has received a great deal of attention in developing countries. The frequent incidence of these toxins in agricultural commodities has a potential negative impact on the economy of the affected region (Aziz *et al*., 2005; Hassan and Aziz, 1998). However, various methods have been applied for the control of mycotoxins including the use of fumigation. Seed fumigation with ethylene oxide and methyl formate was found to significantly reduce the incidence of fungi associated with toxigenic properties under storage conditions (Bankole, 1996).

Although, the use of chemicals of the control of plant disease can result in highly economic gains, it was highlighted that these compounds are neither environmentally friendly nor safe. Furthermore, the poor education background of the farmers often leads to misuse of pesticides. It was reported by Bankole and Adebanjo (2003) that hundreds people died in Nigeria as a result of consumption of cowpea treated with inappropriate pesticides. Consequently, the use of an alternative biocontrol option is more preferable whenever possible. Natural products derived from medicinal plants have proven to be an abundant source of biologically active compounds (Saadabi, 2006).

The potential of higher plants as source of antimicrobial agents is still largely unexplored. Among the estimated 250.000-500.000 plant species, only a small percentage has been investigated phytochemically and the fraction submitted to biological or pharmacological screening is even smaller. Historically pharmacological screening of compounds of natural or synthetic origin has been the source of innumerable therapeutic agents (Mahesh and Satish, 2008). These authors reported that, medicinal plants represent a rich source of antimicrobial agents. The antifungal properties of some plant extracts were investigated with the aim of finding alternatives to the use of chemical fungicides.

1. 2. Aims

- The main aim of this study was to investigate the occurrence of *Fusarium* species and fumonisins associated with cowpea seed.

- Secondly, the efficacy of plant extracts of the leaves of three Rubiaceae species was evaluated against five *Fusarium* species, most of which are associated with cowpea seeds.

1. 3. Objectives of the study

The specific objectives of this study were the:

1. Isolation and identification of *Fusarium* species from cowpea seed samples from a rural community in Mpumalanga Province and to investigate their potential for fumonisin production.

2. Detection and quantification of fumonisin B mycotoxins in cowpea seed samples from these rural communities.

3. Investigation of the phytotoxic effects of fumonisin B_1 on cowpea seed by assessing their effect on germination, root and shoot emergence (*in vitro);* germination and emergence in the greenhouse (*in vivo);* and effects on the ultrastructure of the seed tissue through electron microscopy.

4. Investigation of the antifungal activity of selected plant extracts against *Fusarium* species.

5. Isolation and identification of major compounds from selected plants.

1.4. Rationale and justification for the study

It is evident from the title of this dissertation that two broad and very different aspects are dealt with in this study, namely a mycological and mycotoxin investigation and an antifungal activity investigation. The central theme governing and linking the two investigations is the association of *Fusarium* species with cowpea seed. In the first part, the dissertation reports on the mycoflora, especially *Fusarium* spp. associated with cowpea seed. This section also includes the analysis of cowpea seed samples for fumonisin mycotoxins and also attempts to identify the *Fusarium* spp. responsible for toxin production in cowpea seeds. Although similar work has been done in a previous study (Kritzinger *et al*. 2003), new *Fusarium* associations could be recognized and more importantly new toxin producers on cowpea seed could also be identified. This information is important as few studies have been done concerning mycoflora and mycotoxins associated with cowpea seed samples from Africa and especially South Africa. The results will contribute positively to the current information of fungi and mycotoxins (specifically fumonisins) associated with cowpea seed. The study will either confirm known mycoflora or add new species to the existing list. Furthermore, it is important to obtain seed samples from various regions in South Africa in order to get a better understanding of the fungi and mycotoxins associated with the seed, and by doing so establish if a potential health risk exists amongst the people in rural communities who rely on cowpea seed as a main source of food. In Chapter 4, the phytotoxic effect of fumonisins on cowpea seed is carried out and this study is largely based on the first report of these phytotoxic effects by Kritzinger *et al*. (2006). This current study was done in order to confirm the findings as previously reported and to extend the germination and emergence work into the greenhouse as to access what the effects of the toxin would be in an *in vivo* situation.

Based on the fact that *Fusarium* species are indeed associated with cowpea seed and that some of these species are known to produce mycotoxins, including fumonisins, it is necessary to investigate ways to either prevent or control the contamination of seed by such species. Thus, the second part of the dissertation focuses on the biological control of *Fusarium* species known to be associated with cowpea seed. These species were selected based on previous studies on mycoflora associated with cowpea seed. Until recently, plant extracts were investigated primarily for there antimicrobial activity

against human fungal and bacterial pathogens. However, many studies are now focusing on the application of plant extracts against important plant pathogens that cause severe crop destruction and losses. In addition to *Fusarium* species producing mycotoxins, many of the species are associated with serious diseases on economically important crops. With this in mind and the fact that chemical control is no longer a viable option the need to find alternative means of control of these fungal pathogens is imperative. The first section of the antifungal investigation focuses on the anti-*Fusarium* activity of leaf extracts from three Rubiaceae species. In the second section an attempt was then made to isolate compounds from these species. Based on these results, further studies will be needed to be carried out to investigate the antifungal activity of the compounds isolated.

1. 5. Structure of thesis

Chapter 2. A concise review of the origin, taxonomy, distribution as well as importance of cowpea seed, *Fusarium* species and fumonisin mycotoxins, storage fungi and biological control is given.

Chapter 3. This chapter reports on the mycoflora and fumonisins associated with cowpea seeds from rural communities in Mpumalanga Province, South Africa. The investigation includes the identification and detection of species responsible for fumonisin production in cowpea seed.

Chapter 4. The phytotoxic effects of fumonisin B_1 on cowpea seed is dealt with in this chapter. The effects of FB_1 on germination as well as root and shoot elongation were conducted *in vitro*. The effect of the toxin on the ultrastructure of the cotyledon and embryonic tissue of the cowpea seed were also investigated with the aid of transmission electron microscopy.

The methodology of this chapter is very similar to that carried out by Kritzinger *et al.* (2006). However, the results of this chapter will provide valuable confirmation of the adverse effects of fumonisins on cowpea seed. In this study, in addition to the above-mentioned investigations,

experiments were carried out to access the effect of fumonisins on cowpea seed germination and emergence in the pot trial in the greenhouse.

Chapter 5. This chapter reports on the antifungal activity of crude extracts from three Rubiaceae plant species against five *Fusarium* species.

Chapter 6. Based on the results obtained in Chapter 5, this chapter deals with the isolation and identification of compounds from leaf extracts of *Psychotria capensis* and *Gardenia brighamii*.

Chapter 7. In this chapter, the findings from the research have been compared, interpreted and discussed as well as suggestions for future research have been made.

1. 6. Conference contributions

1. 35th Annual Conference of the South Africa Association of Botanists (SAAB), Stellenbosch University, 19-22 January 2009. **Antifungal activity of *Psychotria capensis* leaf extracts (Poster).** Kafua L., Kritzinger Q., Hussein A.

2. 4th International Seed Health Conference the Polish Phythopathological Society Section of Seed Pathology. 7-9 September 2009, Wroclaw, Poland. ***Fusarium* species and Fumonisins associated with cowpea seed: Occurrence, Phytotoxicity and biological control (Paper).** Kritzinger Q., Kafua L., Emamzadeh Yazdi S., Aveling T.A.S.

3. 36th Annual Conference of South Africa Association of Botanists (SAAB), North- West University, Potchefstroom, 11-19 January 2010. **Antifungal activity of *Gardenia brighamii* leaf extracts (Poster).** Kafua L., Kritzinger Q., Hussein A.

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CHAPTER 2

LITERATURE REVIEW

Literature review

2. 1. COWPEA: V*igna unguiculata* (L.) Walp.

2. 1. 1. Introduction

Cowpea, *Vigna unguiculata* (L). Walp. is indigenous to Africa (Davis *et al*., 1991). It was developed as a crop in West Africa or perhaps Ethiopia. These legumes are considered important in the livelihood of poor people in underdeveloped countries because that they are utilized as a food crop, cash crop and for animal consumption (Singh *et al*., 1997).

Cowpea was used in subsistence agriculture since ancient times as a pulse, leaf vegetable and fodder for cattle (van Wyk, 2005). The crop contributes significantly in the diets of people in developing countries of Africa, Latin America and Asia where it is used as a dietary protein to complement cereals (Phillips *et al*., 2003).

According to Singh and Eaglesfield (2000), cowpea seed can be grown under various production systems including rain fed and irrigated environments as well as in areas of poor soil in low rainfall regions. Cowpea varieties grown in the Sahel and on the periphery of the Sahara are drought and heat tolerant. Other cultivars are tolerant to acidic soils, particularly poor soil fertility, and shading by other crops (Summerfield and Roberts, 1985)

Furthermore, many of the species *unguiculata* produce multiple edible products, which provide food to subsistence farmers throughout the growing season as well as dry seeds that are easy to store and transport (Singh *et al*., 1997). Tender shoot tips and leaves of cowpeas can be consumed as soon as the plants reach the seedling stage whereas immature pods and immature seeds can be consumed during the fruiting stage. Harvested dry seed of all the *Vigna* crops can be consumed directly, and seeds of several crops are commonly used to make flour or produce sprouts. Furthermore, plant residues can be used as fodder for farm animals (Fery, 2002).

2. 1. 2. Origin and taxonomy

Many studies showed that cowpea seed originated from Africa, even though the place where the crop was first domesticated is uncertain (Kitch *et al*., 1998). Kitch *et al*. *(*1998) also reported that, the species *unguiculata* is thought to be West African Neolithic domesticated and whose progenitors were the wild weed species *dekindtiana* and *meusensis.* According to Sithole-Niang (2000), there is little archeological evidence to support the West and Central Africa origin of cowpea seed, because of the lack of major civilization in the sub-Sahara part of Africa. However, southern Africa is the center of genetic variety because the most ancient of wild cowpea occurs in Namibia from the West, across Botswana, Zambia, Zimbabwe and Mozambique to the east, and the Republic of South Africa and Swaziland to the south (Padulosi *et al*., 1997).

Cowpea (Fig. 2.1) is a dicotyledonous plant belonging to the order Fabales, family Fabaceae, sub-family Faboiddea, tribe Phaseoleae, sub-tribe Phaseolinae and genus *Vigna* (Singh *et al*., 1997). Allen (1983) reported that the genus *Vigna* comprises some 160 species mostly from Africa. Only seven of these species are cultivated, among which five are originated from Asia. Verdcourt (1970) subdivided the species into three subspecies (*unguiculata*, *catjang* and *sesquipedalis*). Hall (1997) mentioned that five subspecies of *Vigna unguiculata* are recognized.

Cowpea seed is commonly known in English as bachapin bean, black-eye bean or pea, catjang, china pea, cowgram, southern pea. In Afrikaans – akkerboon, swartbekboon, boontjie, koertjie, dopboontjie. In Zulu – imbumba, indumba, isihlumaya. In Tswana – Dina, nawa-ea-setswana. In Venda – munawa (plant), nawa (fruit). In Pedi – dinawa (plural), monawa, nawa. In Shangaan – dinaba, munaoa, tinyawa (Kay, 1979; Fox and Young, 1982; Tindall, 1983).

Figure 2.1. *Vigna unguiculata* plants (photo: K. Lodama)

2. 1. 3. Distribution

Cowpea seed was introduced from Africa to India between 2000 and 3500 years ago, probably at the same time as the introduction of sorghum (*Sorghum bicolor* L.) and millet (*Echinochloa frumentacea* Roxb.). According to Sithole-Niang (2000), cowpea cultivation in West and Central Africa covers more than eight million hectares. Nigeria is the largest producer followed by Niger, Brazil and India (Quass, 1995).

Furthermore, about two-thirds of the production and more than three-fourths of the area of production is spread over the vast Sudan savanna and sahelian zones of sub-Saharan Africa. It is also spread from Senegal going east through Nigeria and Niger, in Kenya and Tanzania, and from Angola across Botswana to Mozambique (Padulosi *et al*., 1997). Substantial quantities of cowpea are also produced in South America (largely in semiarid north-eastern Brazil), Asia, and the south-eastern and south-western regions of North America (Quass, 1995). Cowpeas have been grown in parts of southern Europe (Italy) before Roman times and attempts are being made to introduce the crop to parts of south-eastern Europe (Davis *et al*., 1986).

The main producing areas of cowpea in South Africa are: Limpopo (Bohlabela, Vhembe, Mopani, Capricom, Sekhukhune, and Waterberg); Mpumalanga (Gert Sibande, Nkangala, Ehlanzeni); North-West (Central, Bophirima, Southern) and KwaZulu-Natal (Umgungundlovu) (National Department of Agriculture, 2009).

2. 1. 4. Importance

Cowpea seed like other food legumes has high quality protein content. It is served as a natural protein to supplement other food crops (Hapton *et al*., 1997). According to Atachi *et al*. (1984) cowpea is often called the meat for the poor people, since its protein is cheaper than other protein sources. Its grain (seeds) contains approximately 24.8% protein and 64% carbohydrates and other nutrients needed by the body (Table 2.1) (Singh and Rachie, 1985). Cowpea seed also contain bioactive antioxidants such as tocopherols, vitamin C, carotenoids and phenolic compounds (Dobaldo *et al*., 2005). Phenolic natural antioxidants represent an important group of bioactive compounds in food, which may prevent the development of many diseases, including atherosclerosis and cancer (Formica and Regelson, 1995; Kahkonen *et al*., 1999).

Table 2. 1. Percentage nutrient content of mature cowpea seeds (Singh and Rachie 1985)

Protein	24.8%
Fat	1.9%
Fibre	6.3%
Carbohydrate	63.6%
Thiamine	0.00074%
Riboflavin	0.00042%
Niacin	0.00281%

Some cultivars of cowpea seed provide a fibre obtained from the peduncles (Kitch *et al*., 1998). This fibre is used to make fishing lines and also is considered as a source of pulp to make good quality paper (Summerfield and Roberts, 1985). Emechebe and Florini (1997) stated that the agronomical importance of cowpea cultivars with spreading interdeterminate or semi arid determinate bushy growth provides ground cover hence suppressing weeds and providing some protection against soil erosion (Singh and Rachie, 1985).

Maude (1996) indicated that cowpeas like other legumes can symbiose with nodular bacteria (rhizobia) present in most if not all tropical soils. Biological nitrogen fixation is advantageous to successive cereal crops in rotation or in association with cowpea in intercropping (Maude, 1996). The highest nitrogen fixation is an efficient way to deal with the shortage of expensive nitrogenous fertilizers in tropical countries (Singh *et al*., 1997).

Medicinally, cowpea has been identified by traditional healers in Zimbabwe to treat urinary shistosomiasis (bilharzia) (Nyazema, 1987). The decoction made from the seeds of cowpea and the roots of *Euclea divinorum* Hiern or *Terminalia sericea* Burch ex D C. is taken orally to treat this illness (Nyazema, 1987). Similarly, cowpea seeds and the roots of *Lannea edulis* (sond) Engl. can be used to treat blood in the urine and bilharzia (van Wyk and Gericke, 2000). Cowpea is used medicinally by grinding the seeds and mixed with oil to treat stubborn boils (Duke, 1990). The cooking liquor of the seeds is considered to be a potential remedy for the common cold (Siddhuraju and Becker, 2007). The leaves and seeds are applied as a poultice to treat swelling and infections, leaves are chewed to treat tooth ailments and powdered carbonized seeds are applied on insect stings (Brink and Belay, 2006). The same authors reported that the roots are used as an antidote for snakebites and to treat epilepsy, chest pain, constipation, and dysmenorrheal. Unspecified plant parts are used as a sedative in tachycardia (a rapid heart rate) and against various pains (Brink and Belay, 2006).

2. 1. 5. Constraints for cowpea cultivation and production

Previous studies from Cesse (1995), reported that cowpea is extremely susceptible to a wide range of pests and pathogens, which can cause serious damage to the crop at all stages of growth. These include insects, bacteria, viruses, and fungi (Hapton *et al*., 1997). Cowpea grain and fodder yields are very low in West Africa and some other world areas. Adam (1990) attributed these reduced yields to a number of reasons, including low density of cowpea and shading by cereals in intercropping systems and yields due to water shortage; attacks by pests and diseases; drought stress and low soil fertility and lack of inputs and infrastructure. Losses due to pest attack or disease can be as high as 90% (Davis *et al*., 1991). Major pests attacking cowpea plants include flower thrips (*Megalurothrips sjostedti*), pod borer (*Maruca virata*), and pod sucking bugs.

Storage weevils (*Callosobruchus maculatus*) damage stored cowpea seeds (Davis *et al*., 1991). Fungal diseases affecting cowpea include stem and root rots and leaf spot diseases. Viruses cause mosaic diseases and mottle symptoms in cowpea. The parasitic weed, *Striga gesneroides*, can severely damage cowpea plants.

Under poor storage conditions such as high humidity and temperature, deterioration of the seed occurs (Fig. 2.2) resulting in the occurrence of some fungal pathogens that may produce toxic secondary metabolites called mycotoxins (Frisvad *et al*., 2006; Richard *et al*., 2009). Fungi and insects have a significant role in the deterioration of quality in stored seeds. The rate of this deterioration increases when there is a presence of insects and fungi combined with a high relative humidity (R H) and temperature under storage conditions (Harrington, 1973; Delouche, 1980; Mills, 1986; Ellis, 1988).

Figure 2. 2. Deteriorated cowpea seed (Photo: Q. Kritzinger)

2. 2. Storage fungi and mycotoxins associated with cowpea

The loss of cowpea grains during storage due to microorganisms has long been a serious problem to growers (Law-ogbomo and Egharevba, 2006). Many fungi are serious parasites of maturing and stored seeds and their invasion can result in various damages including, reduced yields of seed both quantitatively and qualitatively, discolourations, decreased germinability, mycotoxin production and total decay (Embaby and Abdel-Galil, 2006). The Changes in the protein composition, reducing

and non-reducing sugars were observed in seeds of cowpea *(Vigna sinensis savi)* infested with either *Aspergillus nidulans or A.tereus* under different temperatures. Infection by *A. nidulans* was more deleterious than by *A. terreus* (Maheshwari and Mathur, 1987). The same authors reported that the biochemical content of cowpea seed was changed by *Fusarium oxysporum* f.sp. *tracheiphilum, Aspergillus flavus, A. niger* and *Macrophomina phaseolina* infection.

Cowpea seed samples collected from South Africa and Benin were contaminated with *Fusarium* species including *F. equiseti, F. graminearum, F. semitectum, F. proliferatum, F. chlamydosporum, F. sambucium,* and *F. subglutinans* (Kritzinger *et al*., 2003). *Alternaria alternata, Lasiodiplodia theobrome, Drechslera tetramera (Cochliobolus spicifer*) and *Fusarium verticillioides* (*Gibberella fujikuroi*) were isolated from cowpea seeds using the standard blotter and agar plate methods (Kumud *et al*., 2004). Emechebe and McDonald (1979) reported pathogens fungi detected on cowpea seed in Northern Nigeria from markets included *Ascochyta* spp., *Colletotrichum lindemuthianum, C. truncatum, Rhizoctonia solani, F. oxysporum, F. solani, Macrophomina phaseolina, Septoria vignae* and *Corticium rolfsii.*

Ibeh *et al*. (1991) reported that some isolates of *Apergillus* and *Fusarium* produced mycotoxins in dry beans, cowpea and lupine (*Lupinus sp*. L.). Habish (1972) found two cowpea samples to be naturally contaminated with fungal species including *Aspergillus* that produced aflatoxins in Sudan. Houssou *et al*. (2009) also confirmed the presence of aflatoxins in cowpea seed from West Africa. The contamination of cowpea seed with fumonisin mycotoxins will be discussed in 2.4.

2. 3. *Fusarium* spp. and fumonisin mycotoxins

2. 3. 1. *Fusarium* species

The genus *Fusarium* species contains filamentous ascomycete fungi with a worldwide distribution (Kerenyi *et al*., 2004). *Fusarium* species can parasitize cultivated plants and can also be found in the soil. It is found in normal mycoflora of commodities, such as rice (O*ryza sativa* L.), cowpea, soybean (*Glycine max* L.), and other crops (Pitt *et al*.,

2000). While most species are common at tropical and subtropical areas, some inhabit soil in cold climates. According to Yli Mattila *et al*. (2002) *Fusarium* species can grow successfully on a variety of substrates, and tolerate diverse environmental conditions. They have high level of intraspecific genetic and genotypic diversity. Some *Fusarium* species have a teleomorphic state (De Nijs *et al*., 1997; Kerenyi *et al*., 1997).

Besides being a common contaminant and a well-known plant pathogen, *Fusarium* species may cause various infections in humans. It is one of the emerging causes of opportunistic mycoses (Anaissie *et al*., 1988; Anaissie *et al*., 1989; Vartivarian *et al*., 1993; Guarro and Gene, 1995; Manfredini *et al*., 1995). *Fusarium* species are causative agents of superficial and systemic infections in humans. Furthermore, trauma is the major predisposing factor for development of cutaneous infections due to *Fusarium* strains. Infections due to *Fusarium* species are collectively referred to a fusariosis. The most virulent *Fusarium* species is *F. solani* (Mayayo *et al*., 1999). Disseminated opportunistic infections, on the other hand, develop in immunosuppressed hosts, particularly in neutropenic and transplant patients (Austen *et al*., 2001; Boutati *et al*., 1997; Vartivarian *et al*., 1993). Other infectious species, encountered to a lesser extent, are *F.* o*xysporum* and *F. verticillioides. Fusarium proliferatum* has also caused a small number of cases (Mayayo, 1999).

Fusarium species produce a variety of mycotoxins. Ingestion of grains contaminated with these toxins may give rise to allergic symptoms or be carcinogenic in long-term consumption (Pitt, 2000). Fumonisins are the mycotoxins produced primarly by *F. verticillioides* and *F. proliferatum* in maize (Pitt, 2000). The other group of mycotoxins such as zearalenone, trichothecenes, deoxynivalenol and others may also be produced by some *Fusarium* species (Schaafsma *et al*., 1998).

The four media used for growing *Fusarium* species for identification are: carnation leaf agar (Fisher *et al*., 1982), potato dextrose agar (Nelson *et al*., 1983), KCl medium and soil agar (Klotz *et al*., 1988). *Fusarium* species grow rapidly *in vitro* at 25 ^{o}C and produce woolly to cottony, flat, spreading colonies. The only slow- growing species is *F. dimerum*. From the front of *Fusarium* species, the colour of the colony may be white, cream, tan, salmon, cinnamon, yellow, red, violet, pink, or purple. From the reverse, it may be colourless, tan, red, dark purple, or brown (de Hoog *et al*., 2000).

Macroscopic and microscopic features, such as, colour of the colony, length and shape of the macroconidia, the number and arrangement of microconidia, and presence or absence of chlamydospores are key features for the differentiation of *Fusarium* species (de Hoog *et al*., 2000). A sclerotium, which is the organized mass of hyphae that remains dormant during unfavourable conditions, may be observed macroscopically and is usually dark blue in colour. On the other hand, sporodochium, the cushion-like mat of hyphae bearing conidiophores over its surface, is usually absent in culture. When present, it may be observed in cream to tan or orange colour, except for *F. solani*, which gives rise to blue-green or blue sporochia (de Hoog *et al*., 2000).

2. 3. 2. Fumonisins

Fumonisins were first isolated from maize crops in South Africa (Gelderblom *et al*., 1988). These mycotoxins have also been found in other commodities such as sorghum (*Sorghum bicolor* L. Moench) (da Silva *et al*., 2000) and cowpea seed (Kritzinger *et al*., 2003). Animal and human health problems related to these mycotoxins are almost exclusively associated with the consumption of contaminated maize or other maize products (Marasas, 2001). High doses of fumonisin B_1 (FB_1) cause leukoencephalomalacia (ELEM) in horses (Marasas *et al*., 1988; Wilson *et al*., 1992) and rabbits (Bucci *et al*., 1996) as well as pulmonary edema (PPE) in swine (Haschek *et al*., 1992; Haschek *et al*., 2001). Fumonisins were reported as common contaminants of maize-based foods and feeds in the United States of America, China, Europe, South America and Africa (Sydenham *et al*., 1991; Thiel *et al*., 1992, Doko and Visconti, 1994).

There are several studies reporting the toxic effects of FB_1 induced in the liver and kidney of rats and mice (Voss *et al*., 2001). Fumonisin B_1 is also a rodent carcinogen, which induces renal tumors in male rats and hepatic tumors in female mice (Gelderblom *et al*., 2001; Howard *et al*., 2001). Consumption of FB_1- contaminated maize has been linked to human oesophageal cancer in certain areas in China and South Africa (Sydenham *et al*., 1990; Chu and Li, 1994). Today FB_1 is classified as possibly carcinogenic to humans (class 2B) (IARC, 2002).

Fumonisins are suspected risk factors for oesophageal and liver cancer, neural tube defects and cardiovascular problems in populations consuming relatively large amounts of food made with contaminated maize and other products (Marasas, 2001; Missmer *et al*., 2006). The mechanisms where by FB_1 affects neural cells and the brain has been the aim of a number of studies. Increased levels of sphinganine in the forebrain and brainstem of rats treated with FB_1 were associated with a concomitant demyelination in the forebrain (Kwon *et al*., 1997). In addition, FB_1 seemed to inhibit protein synthesis, cause DNA fragmentation and cell death, and induce lipid peroxidation and cellular cycle arrest in rat c6 glioma cells (Mobio *et al*., 2000).

To date, a total of 28 fumonisin analogs have been characterised since 1988 and can be separated into four main groups, identified as the fumonisin A (FA_1, FA_2, FA_3, FAK_1), B (FB_1, FB_2, FB_3, FB_4), C (FC_1, FC_2, FC_3, FC_4), and P (FP_1, FP_2, FP_3) (Rheeder *et al*., 2002). However, the fumonisins B (FB) analogs, comprising the toxicologically important FB_1, FB_2, FB_3 and FB_4 are the most abundantly found in naturally contaminated foods and feeds (Rheeder *et al*., 2002).

Fumonisins B_1, B_2, B_3 and B_4 (Fig. 2.3) are found in fungal cultures or in naturally contaminated maize samples (Cawood *et al*., 1991). Fumonisin B_1 (FB_1) has the empirical formula $C_{34}H_{59}NO_{15}$ and is the diester of propane-1, 2, 3-tricarboxylic acid and 2-amino-12, 16-dimethyl-3, 5, 10, 14, 15-pentahydroxyeicosane (relative molecular mass: 721) (Gelderblom *et al.*, 1988).

According to the WHO (2000), FB_1 is a white hygroscopic powder, having the following characteristics: soluble in water, acetonitrile-water or methanol; is stable in acetonitrile-water (1:1), and at food processing temperature and to light but unstable in methanol. Several analytical methods have been reported for detection of fumonisins, including high performance liquid chromatography (HPLC) of fluorescent FB_1 derivative (Ross *et al*., 1991), thin layer chromatography (TLC) (Plattner *et al*., 1990; Syndenham *et al*., 1990) and liquid chromatography (LC), mass spectroscopy (MS), post-hydrolysis gas chromatographic and immunochemical methods (WHO, 2000; WHO, 2001).

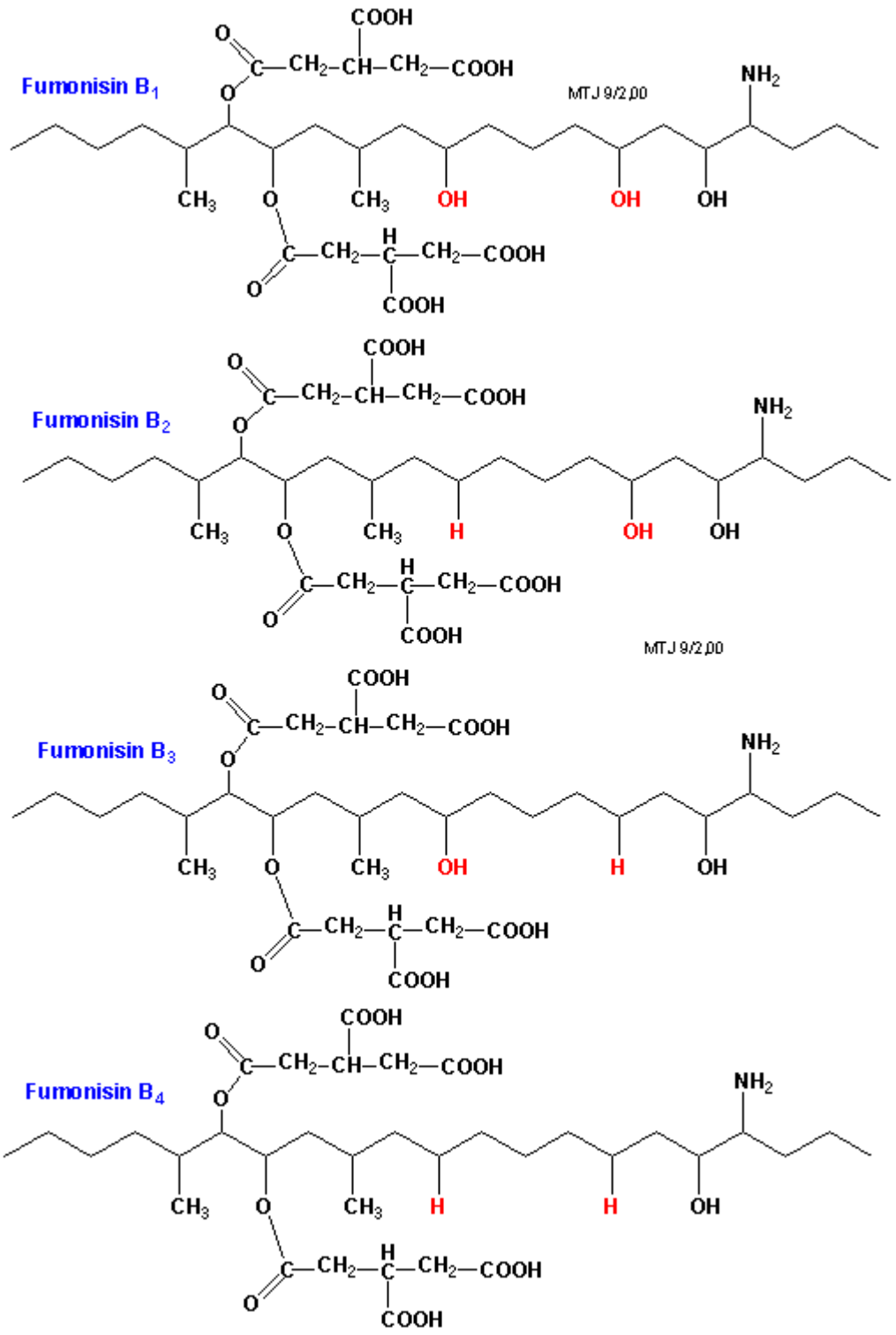

Figure 2. 3. Chemical structures of fumonisins B analogues (Cawood *et al.*, 1991)

Five toxic fungal secondary metabolites (mycotoxins) are considered to be economically and toxicologically important worldwide: aflatoxin, ochratoxin, deoxynivalenol (DON = vomitoxin) and derivatives, zearalenone (ZON) and derivatives, and fumonisins (FB_1, FB_2). The last three of these, DON, ZON, and fumonisins, are produced by various *Fusarium* species (Charmley *et al.*, 1994).

Fandohan *et al*. (2003) investigated the infection of maize by *Fusarium* species and contamination with fumonisin in Africa, and concluded that this genus comprises several toxigenic species with *F. verticillioides* and *F. proliferatum* being the most prolific producers of fumonisins.

Fumonisins are produced by several *Fusarium* species (Rheeder *et al*., 2002) including:

- *F. verticillioides* (Sacc.) Nirenberg,
- *F. proliferatum* (Matsushina) Nirenberg,
- *F. nygamai* (Burgess & Trimboli),
- *F. anthophilum* (A. Braun) Wollenweber,
- *F. dlamini* (Marasas, Nelson & Toussoun),
- *F. napiforme* (Marasas, Nelson & Rabie),
- *F. thapsinum* (Klittich, Leslie, Nelson & Marasas),
- *F. globosum* (Rheeder, Marasas & Nelson),
- *F. sacchari* (Buttler& Khan),
- *F. fujikuroi* (Wollenw),
- *F. subglutinans* (Wollenw & Reinking),
- *F. andiyazi* (Marasas, Rheeder, Lamprecht, Zeller & Leslie) and
- *F. polyphialidicum* (Marasas, Nelson, Toussoun & Van Wyk).

2. 3. 3. Phytotoxic effects of fumonisins

Fusarium species are widespread throughout the world and produce phytotoxins such as the fumonisins, fusaric acid, enniatin, and moniliformin (Abbas *et al*., 1984; Burmeister and Plattner, 1987).

Fumonisins have been shown to have phytotoxic properties towards duckweed *(Lemna minor* L.) (Vesonder *et al*., 1992), tomato cultivars (Mirocha *et al*., 1992), maize callus cultures (van Asch *et al*., 1992), and jimsonweed (Abbas *et al*., 1992). Doehlert *et al*. (1994) reported that high levels of fumonisin in maize seed may have deleterious effects on seedling emergence and phytotoxic effects on maize seedling growth. Kritzinger *et al*. (2006) confirmed that the higher concentrations of FB_1 (50 and 100 µg/ml) significantly decreased seed germination and inhibited root and shoot elongation of

cowpea seedlings. Abbas *et al*. (1992) investigated the physiological and ultrastructural effects of FB_1 on jimsonweed leaves and found that the toxin caused rapid, light-dependent cytoplasmic and chloroplast disruption, through an unknown mechanism.

FB_1–induced lesions in *Arabidopsis* are similar to pathogen-induced lesions in many respects, including deposition of phenolic compounds and callose, production of reactive oxygen intermediates (ROIs), accumulation of camalexin, and expression of pathogenesis-related (PR) genes (Stone *et al*., 2000). This possible mechanism action of FB_1 is supported by the following evidences: (i) FB_1 is able of interacting with the hydrophobic region of the membrane, as shown by the toxin-induced changes in membrane fluidity (Gutierrez-Najera *et al*., 2005), (ii) FB_1 can be inserted into liposome bilayers (Yin *et al*., 1996) and interacts with polar and non-polar regions of cholesterol and taurocholate in monomolecular films (Mahfoud *et al*., 2002).

2. 4. *Fusarium* and fumonisins associated with cowpea seed

In South Africa, six *Fusarium* species (*F. chlamydosporum*, *F. equiseti*, *F. graminearum*, *F. sambucinum*, *F. scirpi*, and *F. subglutinans*) were identified to be associated with cowpea seed (Kritzinger *et al*., 2003). *Fusarium oxysporum* was also reported an important pathogen causing *Fusarium* wilt of cowpea in Brazil (Ehler and Hall, 1997). It is thought that the high susceptibility of cowpea seeds to pest or *Fusarium* is associated with inadequate defenses, either constitutive or induced (Singh and Rachie, 1985).

Houssou *et al*. (2009) found *F. equiseti*, *F. oxysporum* and *F. semitectum* as potential pathogens in *Fusarium* infected cowpea seeds. Ramachandran *et al*. (1982) reported that *F. equiseti* caused a necrosis in cowpea and the progress of disease to the stem resulted in plant death. Shama *et al*. (1988) analysed cowpea seeds commonly used by producers and observed the presence of *F. oxysporum*, *F. verticillioides*, *F. semitectum*, *F. solani* and *F. equiseti* as components of the mycoflora of the seeds, located mainly in the cotyledons, causing rot or giving rise to abnormal plantlets with damage to the plumule. Jindal and Thind (1990) detected *F. equiseti* and *F. semitectum*, in wrinkled and withered seeds. Houssou *et al*. (2009) studied the natural infection of cowpea by

toxigenic fungi and mycotoxin contamination in West Africa, and reported that 96 samples of cowpea seed were fumonisin positive with negligible or very low levels of contamination with fumonisin B_1 (0.010, 0.012 and 0.068 µg/g). However, Kritzinger *et al*. (2003) confirmed also that cowpea seed from South Africa showed the presence of fumonisin B_1 at concentrations ranging between 0.12 and 0.61 µg/g. The same author also reported that *F. proliferatum* was responsible for fumonisin productions in cowpea seeds.

2. 5. Control of fungi contamination on seed

The control of cowpea diseases is very important as a complementary technology to boost cowpea production. Various approaches have been used over many decades to control cowpea diseases in the field and under storage conditions, including breeding for resistant varieties, chemical treatment, and biological control (Ofuya, 1997).

2. 5. 1. Chemical control

Fungicides have been used extensively to control plant diseases. One of the major disadvantages of fungicides is that they cause environmental pollution and leave residue in agricultural soil and on products (Soytong and Ratanacherdchai, 2005). In addition, the risk of developing resistance by microorganisms and the high cost-benefit ratio are other disadvantages of synthetic pesticide usage (Brent and Hollomon, 1998).

The combinations of top quality seed and cultural management practice along with the use of fungicides provides a valuable means of controlling diseases to many crops (Sbragia, 1975; Baldwin and Rathmell, 1988). Synthetic chemicals are widely used in the control of plant diseases and stored products (Kordali *et al*., 2009). Synthetic pesticides can also cause environmental pollution owing to their slow biodegradation (Barnard *et al*., 1997; Misra and Pavlostathis, 1997).

Other alternative approaches including physicochemical methods (Samarajeewa *et al*., 1990) as well as fungicide usage have been undertaken to reduce or prevent contamination of food and feed with mycotoxins. However, most physicochemical methods are not feasible at small scale farm level. Fungicide usage is more feasible but

the effects of fungicides on non-target organisms coupled with their poor biodegradability are unwelcome. The misuse and prohibitively high cost of chemicals for resource poor farmers in developing countries and the identification of different putative races of pathogen making breeding for host plant resistance difficult have necessitated the need to look for alternatives (Emechebe and Florini, 1997).

2. 5. 2. Phytochemical control using plant extracts

During recent years, scientists have focused on the increase of food production for the fast expansion of world population. Unfortunately, substantial yield losses occur due to insects and plant diseases caused by fungi, bacteria and viruses (Fletcher *et al*., 2006). Microorganisms play a central role in deterioration of stored foods (Credland *et al*., 1986). Fungi are significant destroyers of stored foodstuffs, rendering them unfit for human consumption and affecting their nutritive value (Cherry *et al*., 2005). Quite often the growth of toxigenic fungi and mycotoxins produced in food and grains stored for long period of time present a potential hazard to human and animal health (Soliman and Badeaa, 2002).

Fungi and bacteria have unfavourable effects on quality, safety and preservation of food. It is therefore necessary to search for control measures that are cheap, ecologically sound and environmentally safe to eliminate or reduce the incidence of these economic important pathogens.

In recent years much attention was given to the use of non- chemical compounds applied as seed treatment against seed-borne pathogens. Plant extracts have played a significant role in the inhibition of seed-borne pathogens and in the improvement of seed quality and field emergence of plant seed (Nwachukwu and Umechuruba, 2001). Shah *et al*. (1992) reported that *Argemone mexicana* (L.) seed extract was effective in eliminating most of seed-borne fungi of cowpea but not against *Alternaria alternata*, *Curvularia lunata*, *Mucor* spp. and *Macrophomina phaseolina*. Leaf extracts of *Delonix regia* JPG., *Pongamia glabra* Vent. and *Acacia nilotica* L. significantly inhibited spore germination, mycelial growth and spore production of *Alternaria helianthi*, *Macrophomina phaseolina* and *Fusarium solani* from sunflower seeds (*Helianthus annuus* L.) (Thiribhuvanamala and Narasimhan, 1998). Parimelazhagan and Francis

(1999) reported reduction in the radial growth of *C. lunata* associated with rice when treated with leaf extracts of *Clerodendrum viscosum* L., which also increased seed germination and root and shoot lengths of rice.

In recent research Akinbode and Ikotun (2008) found that the incidence of *Colletotrichum destructivum* was reduced significantly to 18.6% on cowpea seeds treated with 10% aqueous extracts of leaves of *Moringa oleifera* Lam., *Vernonia amygdalina* Del. and *Annona muricata* L. for 18 hours. The essential oil of lemon grass (*Cymbopogon citratus* L.) was reported to control seed mycoflora of maize and cowpea in storage (Adegoke and Odesola, 1996). According to Amadioha (2003) *Piper nigrum* L. and *Ocimum sanctum* L. could be used as pesticides of plant origin to control *Colletotrichum lindemuthianum* of cowpea in the field. Adandonon *et al*. (2006) found that *M. oleifera* combined with *Trichoderma* resulted in the control of damping-off in cowpeas caused by *Sclerotium rolfsii*. Furthermore, Velluti *et al*. (2003) reported that essential oils from cinnamon (*Cinnamomum osmophloeum* Kaneh.), clove (*Syzygium aromaticum* L.) and oregano (*Origanum vulgare* L.) had a significant inhibitory effect on the growth of *Fusarium proliferatum* at 20 ^{o}C (1000 µg essential oil /g), while at 500 µg/g only cinnamon and oregano were effective. The authors confirmed that cinnamon, oregano and palmarose oils had a significant inhibitory effect on FB_1 production by the three strains of *F. proliferatum* at 0.995 water activity at 20 and 30 ^{o}C.

2. 6. Literature cited

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CHAPTER 3

FUSARIUM SPECIES AND FUMONISINS ASSOCIATED WITH COWPEA SEEDS FROM RURAL COMMUNITIES IN MPUMALANGA PROVINCE, SOUTH AFRICA

Fusarium species and fumonisins associated with cowpea seeds from rural communities in the Mpumalanga Province, South Africa

Abstract

A study was carried out to investigate the storage mycoflora and the presence of fumonisin mycotoxins on cowpea seed obtained from a local market of a rural community in Mpumalanga, South Africa. Five samples of two hundred seeds each were analysed for storage mycoflora. The seeds from each sample were divided into two groups namely, untreated and surface-disinfected. Surface-disinfection was done by soaking the seeds in 1% sodium hypochlorite for 1 min, and then rinsed three times with sterile distilled water. Seeds were plated out on potato dextrose agar (PDA), and incubated at 25 ˚C for seven days. Thirteen different fungal genera were recorded including *Aspergillus* spp., *Fusarium* spp. (*F. oxysporum, F. solani, F. subglutinans, F. verticillioides,* and *F. proliferatum), Chaetomium spp., Chrysonilia* spp., *Monascus* spp., *Mucor* spp., *Alternaria alternata, Epicoccum spp., Cladosporium* spp., *Lasiodiplodia* spp., *Penicillium* spp., *Phoma* spp. and *Rhizopus* spp.

Fumonisin detection was conducted using the VICAM Affinity method. For the five cowpea seed samples, the investigation revealed the presence of fumonisin B analogues in all the seed samples within a wide range of concentrations from 6.2 – 59 µg/g. *Fusarium oxysporum, F. solani, F. subglutinans, F. verticillioides, F. proliferatum* isolates were inoculated and grown on maize patty medium and analysed for fumonisin production. *Fusarium subglutinans* and *F. oxysporum* isolates produced fumonisin B analogues with levels ranging between 1.1 - 4.3 µg/g and 3.47 - 31.66 µg/g, respectively. Neither *Fusarium verticillioides, F. solani* nor *F. proliferatum* revealed any production of fumonisins.

Keywords: *Fusarium* spp.; cowpea seed; mycoflora; fumonisins.

3. 1. Introduction

Fusarium (a large genus of filamentous fungi) was introduced by Link in 1809, and is now approaching its third century as a genus that contains many plant pathogenic fungi. The members of this genus can cause diseases in plants, humans and domesticated animals (Leslie *et al*., 2005). Most species are harmless saprobes and are relatively abundant members of the soil microbial community. One of the most important effects of post harvest decay of fruit and vegetables and especially of seed and feed deterioration by fungi is the induction of mycotoxicoses (Amadi and Adeniyi, 2009). Mycotoxins when ingested during the consumption of infected seed and other foodstuffs can lead to serious health complications in animals as well as humans. The main toxins produced by these *Fusarium* species are fumonisins and trichothecenes (Miller, 2001).

Fumonisin B_1 (FB_1), (Fig. 3.1) is related to the late 1988 discovered toxins, fumonisins, which are produced mainly by *Fusarium verticillioides* (older synonym is *Fusarium moniliforme*) and *Fusarium proliferatum* (Marasas *et al*., 2004). It has been claimed that *F. napiforme, F. anthophilum, F. dlamini* and *F. nygamai* can also produce FB_1 (Marasas, 2001). Fumonisin B_1 has been reported as a natural contaminant in maize and maize based food from many parts of the world, such as the USA, Canada, South Africa, Nepal, Australia, Thailand, Philippines, Indonesia, Mexico, France, Italy, Poland and Spain (EHC, 2000).

Among the different fumonisin analogues isolated and characterized, FB_1 is known to cause a unique neurotoxic syndrome called equine leukoencephalomalacia (ELEM) in horses (Rheeder *et al*., 2002). According to Nelson *et al*. (1994), fumonisin B_1 is hepato-carcinogenic to rats and is also associated with pulmonary edema in swine. Jones *et al*. (2001) reported a high incidence of human liver cancer and esophageal cancer in regions of South Africa and China related to ingestion of mouldy corn infected by *F. verticillioides*. Furthermore, the International Agency for Research on Cancer (IARC) evaluated FB_1 as a possible carcinogen to humans (Group 2B) (IARC, 2002).

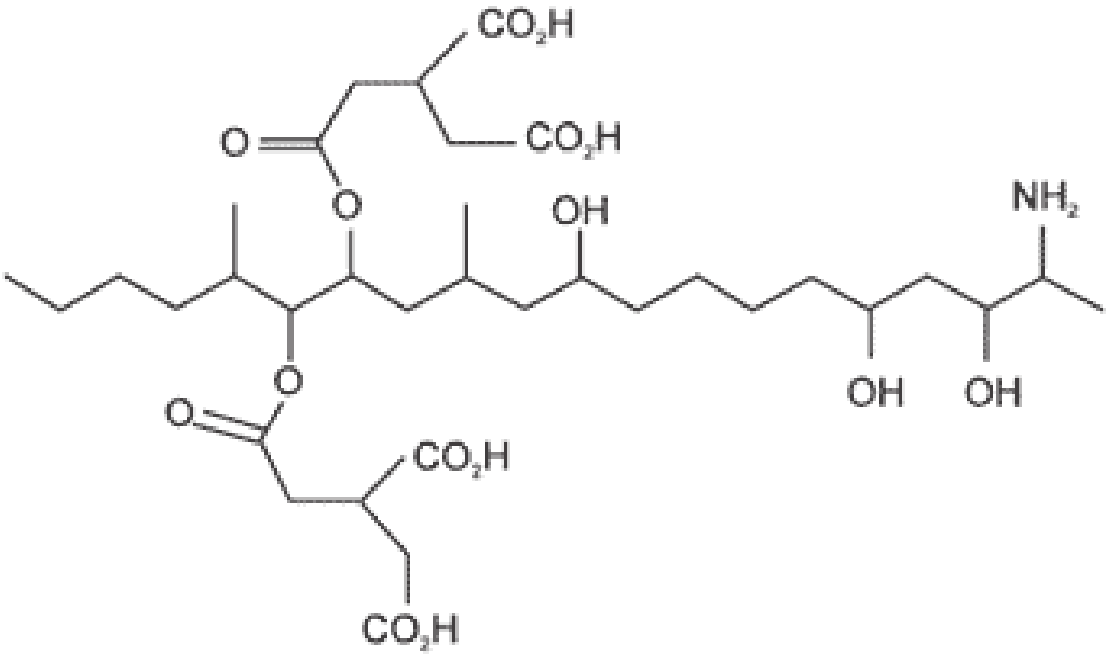

Figure 3. 1. Chemical structure of fumonisin B_1 (Nelson *et al.*, 1991)

Cowpea (*Vigna unguiculata* L.) is a widely grown legume food crop of the tropics. Like other legumes, cowpea seeds contribute to the level of dietary protein in starchy tuber-based diets through their relatively high protein content (25%), and their quality, by forming complementary mixtures with cereals (Phillips and Baker, 1987). Produced on more than 30 million acres in hot, drought-prone regions of the tropics and subtropics, cowpea not only provides nutritious food for millions of people and their livestock, but also generates income for rural producers, traders, and small-scale urban food vendors, while at the same time enhancing soil fertility (Kitch *et al*., 1997). About two-thirds of the production and more than three-fourths of the area of production is spread over the vast Sudan Savanna and Sahelian zones of Sub-Saharan Africa (Ehlers and Hall, 1997). The main producing areas of cowpeas in South Africa are in Limpopo (Bohlabela, Vhembe, Mopani, Capricon, Sekhukhune, and Waterberg); Mpumalanga (Gert Sibande, Nkangala, Ehlanzeni); North-West (Central, Bophirima, Southern) and KwaZulu-Natal (Umgungundlovu) provinces (National Department of Agriculture, 2009). Because of its superior nutritional attributes, versatility, adaptability, and productivity, cowpea was chosen by the US National Aeronautical and Space Administration as one of few crops worthy of study for cultivation in space stations (Ehlers and Hall, 1997).

Fusarium was one of 23 fungal genera identified from cowpea seed by Houssou *et al.* (2009). *Fusarium* infection on cowpea seed was also observed by Kritzinger *et al.* (2003) and Embaby and Abdel-Galil (2006). It was observed that fungal species identified from cowpea seed produced mycotoxins including aflatoxins (*Aspergillus* spp.) (Habish, 1972), fumonisins (*Fusarium proliferatum*) (Kritzinger *et al.*, 2003); aflatoxins and fumonisins (*Aspergillus flavus* and *Fusarium* spp.) (Houssou *et al.*, 2009). However, apart from Kritzinger *et al.* (2003) and Houssou *et al.* (2009) information is limited with respect to the natural contamination of cowpea seeds by fumonisins.

This chapter investigates the presence of *Fusarium* species and fumonisins associated with cowpea seeds obtained from a local market in Mpumalanga Province. *Fusarium* species isolated were also investigated for fumonisin production.

3. 2. Materials and methods

3. 2. 1. Collection of seeds

Five cowpea seed samples were collected from a local market in the locality of Noas (Nkomazi East), Mpumalanga Province in November 2007. These seeds were the produce of small-scale farmers residing in the area. The five samples (Table 3.1) were taken to the laboratory and analyzed for the presence of mycoflora. These seeds were stored in brown paper bags in a cold room (3 ^{o}C) until use.

Table 3. 1. Mass of cowpea seed samples collected from a local market in Nkomazi East, Mpumalanga

Sample numbers	Mass
1	605 g
2	610 g
3	315 g
4	315 g
5	315 g
Total	2.159 g

3. 2. 2. Mycoflora analysis

For each sample, 200 seeds were selected randomly and half of the number (hundred) was surface-disinfected using sodium hypochlorite (1% for 1 min) and thereafter rinsed three times with distilled water. The other one hundred seeds were untreated. There were four replicates of 25 seeds per sample. The seeds were directly plated out onto 90 mm Petri dishes containing Potato Dextrose Agar (PDA) with five seeds per Petri dish, one in the centre of the plate and one in each quadrant. The Petri dishes were sealed and incubated at 25˚C for seven days and then analyzed for fungal growth (Fig.3.2). Fungi were examined and identified with the aid of a stereomicroscope. Fungi were identified to genus level and possibly to species level (Watanabe, 1994). Special emphasis was placed on the *Fusarium* species isolated. *Fusarium* isolates were sub-cultured on to PDA plates and sent for verification at the ARC-Biosystematics Division, Pretoria, South Africa. The isolates were deposited in the culture collection at the ARC-Biosystematics Division.

Figure 3. 2. Fungal growth from cowpea seed after seven days of incubation (Photo: K. Lodama)

3. 2. 3. Fumonisin analysis

Maize patty medium: Eight *Fusarium oxysporum*, two *F. solani*, two *F. subglutinans*, two *F. verticillioides* and two *F. proliferatum* isolates from cowpea seed samples were grown on maize patty medium in duplicate according to the method of Alberts *et al*. (1993). Maize patty medium was prepared in 90 mm Pyrex petri dishes by adding 25 g of finely ground maize kernels to 25 g of water. The petri dishes were autoclaved for 1 h at 121 ^{o}C and 120 kPa on two consecutive days. *Fusarium* species suspensions were prepared in 100 ml of sterile distilled water from cultures grown for 7-9 days. The maize patty media were inoculated with 1 ml of the suspension, and inoculated at 25 ^{o} C for 21 days or until all of the media was completely covered by the fungus.

Fumonisin extraction and detection from cowpea seed and maize patty media: The determination of fumonisins was done at Nooitgedacht Research Centre, Department of Agriculture and Land Administration in Ermelo, Mpumalanga. Detection of fumonisin B analogues was carried out using the VICAM Affinity method, based on the procedure described by Duncan *et al*. (1998). Briefly, 50 g of sample mixed with 5 g of salt was extracted with 100 ml methanol-water (8:2) by blending. The mixture was homogenized for 5 min in an Omni-mixer (Du point Instrument Sorvall, model 17105) at a medium velocity. The extract was filtered through Whatman 2V filter paper and 10 ml of the extract was diluted to 50 ml with 0.1% phosphate buffer saline (PBS) (Vicam, 1998). The diluted extracts were then filtered into a 10 ml cuvette and the filtrate was applied to a FumoniTest immunoaffinity column. The column was then washed with 10 ml PBS-0.1% and eluted with 1 ml methanol-water (8:2) into a clean glass cuvette. One ml Developer A / Developer B mixture was added to the 1 ml sample eluate, mixed and read in a the VICAM fluorometer after a 4 min delay time. Each sample was tested in duplicate. This method only detected fumonisin B and not specifies analogue (i.e. FB_1, FB_2, and FB_3).

3. 3. Results and discussion

The present chapter provides information on the mycoflora and fumonisin analysis of cowpea seeds obtained from a local market of a rural community in Mpumalanga Province in South Africa. This study complements the investigation conducted by Kritzinger *et al*. in 2003

Mycoflora analysis: The results of the fungi isolated from the untreated and surface-disinfected seed are tabulated in Table 3.2. Data in this table shows that the untreated seed had a higher percentage of fungal infection. On the other hand, the disinfected seed led to a lower percentage of fungal infection and there were significant differences in the total composition of mycoflora. Similar results were obtained by Tseng *et al*. (1995), Ruiz *et al*. (1996), El-Nagerabi and El-Shafie (2000) Castillo *et al*. (2004), and Kritzinger *et al*. (2003). Results in this chapter show that, like other crops, cowpea may be infected by fungi belonging to the genera *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp. as reported by Seenappa *et al*. (1983) and Kritzinger *et al*. (2003). Embaby and Abdel-Galil (2006) also reported that *Fusarium oxysporum*, *Fusarium* spp., *Alternaria*, *Epicoccum*, *Trichoderma harzianum*, and *Aspergillus niger* were the most frequent fungi isolated from cowpea seeds.

In this study, 13 identified fungal genera were isolated from the cowpea seed with the members of the fungi imperfecti group predominanting, including *Aspergillus* spp., *Fusarium* spp. *Penicillium* spp., *Chaetomium* spp., *Chrysonilia* spp., *Cladosporium* spp., *Monascus* spp., *Phoma* spp., *Mucor* spp., *Alternaria alternata, Epicoccum* spp. *Rhizopus* spp. and *Lasiodiplodia* spp. (Table 3.2). For the two methods (surface-disinfected and untreated), *Aspergillus flavus, A. niger, Alternaria alternata, Fusarium* spp. and *Mucor* spp were present in all the samples. Of all fungi identified, *Cladosporium* spp. was the least dominant (Table 3.2).
Of the *Fusarium* species isolated from the samples *F. oxysporum, F. solani, F. subglutinans, F. verticillioides* and *F. proliferatum* were positively identified. These findings concur with Kritzinger *et al*. (2003) who reported the isolation of *F. equiseti, F. semitectum F. proliferatum, F. subglutinans, F. graminearum*, and *F. scirpi* from

cowpea seed. Rodrigues and Menezes (2005) observed a total of 211 *Fusarium* species on cowpea seed by means of the blotter test and slide cultures.

Analysis of cowpea seeds commonly used by producers showed the presence of *F. oxysporum*, *F. verticillioides*, *F. semitectum*, *F. solani* and *F. equiseti* as components of the mycoflora of these seeds, located mainly in the cotyledons, causing rot or giving rise to abnormal plantlets with damage to the plumule (Shama *et al*., 1988). Jindal and Thind (1990) found *F. equiseti* and *F. semitectum* to be associated with wrinkled and withered cowpea seed. *Aspergillu*s spp., *Mucor hiemalis, Macrophomina phaseolina*, *Rhizopus oryzae, Alternaria longissima, Cochliobolus pallescens, Botryodiplodia theobromae* and *Colletotrichum* species were found to be associated with cowpea seeds from Ibadan, in Nigeria (Amadi and Oso, 1996).

Table 3. 2. Fungal genera and species isolated from five cowpea seed samples obtained from rural communities in Nkomazi East, Mpumalanga

	1		2		3		4		5	
Fungi species	*D*	*U*	*D*	*U*	*D*	*U*	*D*	*U*	*D*	*U*
Alternaria alternata	**3**	**4**	**2**	**5**	**1**	**4**	**4**	**5**	**2**	**5**
Aspergillus flavus	**6**	**7**	**5**	**8**	**5**	**7**	**4**	**6**	**2**	**5**
A. niger	**5**	**5**	**3**	**4**	**4**	**6**	**5**	**8**	**3**	**7**
Chaetomium spp.			**4**	**4**	**5**	**5**	**4**	**3**	**6**	**8**
Chrysonillia spp.					**3**	**5**	**4**	**5**	**5**	**7**
Cladosporium spp			**1**	**4**					**4**	**6**
Epicoccum spp.					**3**	**4**	**2**	**5**	**3**	**9**
Fusarium spp.					**2**		**1**			
F. oxysporum	**1**	**1**	**1**	**1**		**2**		**2**		
F. solani					**1**		**1**			
F. subglutinans		**1**				**1**				
F. verticillioides							**1**			**1**
F. proliferatum			**2**							
Lasiodiplodia spp.	**2**	**8**	**3**	**5**						
Monascus spp.	**3**	**4**	**3**	**4**	**3**	**8**	**2**	**6**		
Mucor spp	**3**	**5**	**2**	**4**	**5**	**7**	**2**	**5**	**2**	**2**
Penicillium spp.			**2**	**2**				**8**	**4**	
Phoma spp.			**3**	**3**	**3**	**5**	**1**	**4**		
Rhizopus spp.	**2**	**3**	**4**	**4**	**2**	**4**			**4**	**6**
(Others) Unidentified	**12**	**17**	**14**	**18**	**10**	**12**	**7**	**10**	**9**	**11**
Total fungi	**37**	**55**	**49**	**66**	**47**	**70**	**38**	**67**	**44**	**67**

Each value is the number of fungal species isolated from 100 seeds

- **D** : Disinfected
- **U** : Untreated

Fumonisin analysis: The results of the fumonisin B analogues detected from cowpea seed samples in this study are recorded in Fig. 3.3. All five cowpea seed samples revealed the presence of fumonisins B analogues within a wide range of concentrations from 6.2 – 59 µg/g. The highest level of fumonisin B (59 µg/g) was recorded for sample 3, while sample 1 showed the lowest level (6.2 µg/g) (Fig. 3.3). Fumonisin B_1 was detected in naturally infected cowpea seed with a mean level of 0.03 µg/g in Benin (Houssou *et al*. 2009). Cowpea seed samples from South Africa revealed the presence of fumonisin B_1 at concentrations ranging between 0.12 to 0.61 µg/g (Kritzinger *et al*., 2003).

Eight *F. oxysporum*, two *F. subglutinans*, two *F. verticillioides*, two *F. solani* and two *F. proliferatum* isolates were inoculated onto maize patty medium and analysed for fumonisin production. Only *F. oxysporum* (five isolates) and *F. subglutinans* (two isolates) produced fumonisin B analogues with levels ranging between 3.47 - 31.66 µg/g and 1.1 – 4.3 µg/g, respectively. *Fusarium verticillioides*, *F. proliferatum* and *F. solani* isolates did not produce fumonisins. Our results are in accordance with other studies. Embaby and Abdel-Galil (2006) also detected the presence of fumonisins in legume seeds contaminated by *F. oxysporum* by thin layer chromatography (TLC).

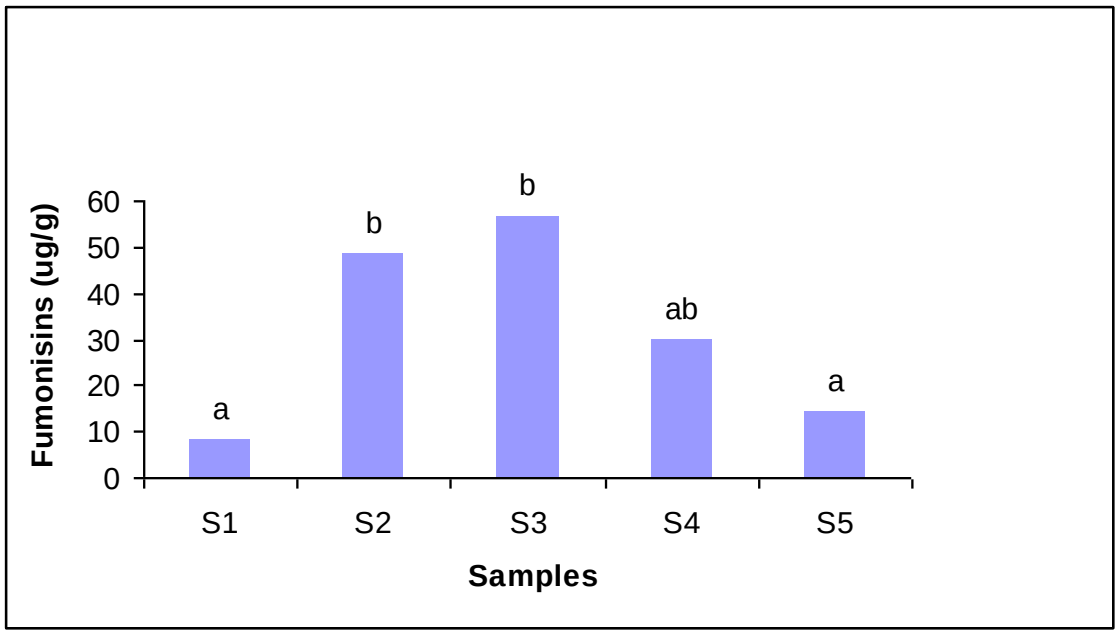

Figure 3. 3. Fumonisins concentration (µg/g) detected in cowpea seed samples from a local market, in Mpumalanga by using VICAM Affinity method

The value of the bars shows the concentration of fumonisins detected in cowpea seed (average of 2 replicates) and the bars not followed by the same letter are significantly different (P=0.05) according to the student's *t* test.

In the natural occurrence of *Fusarium* and subsequent fumonisin presence in maize in Benin, it was found that 68% of samples were infected by *Fusarium verticillioides* and 31% by *Fusarium proliferatum* (Fandohan *et al.*, 2003). The same authors showed that most of the samples were fumonisin positive with levels ranging up to 12 mg/kg.

The ability of strains of *F. oxysporum* to produce fumonisins has not been widely studied. Isolates of *F. oxysporum* and *F. oxysporum* var. redolens from root lesions of Eastern white pine have been reported to produce fumonisin B analogues in some cases (Abbas *et al*., 1995). Sewran *et al*. (2005) also reported that *F. oxysporum* detected in maize from Korea has the ability to produce fumonisin C analogues. *Fusarium subglutinans* isolated from maize and sorghum produced fumonisin B_1 (Desjardins *et al*., 2000).

This study represents the first report on the natural infection of *Fusarium subglutinans* in cowpea seeds and their potential for fumonisin B production. Further work is needed to identify the specific fumonisin B analogues produced by the *Fusarium* species isolated in this study, and to confirm the identification of *Fusarium oxysporum* as well as *Fusarium subglutinans* for there fumonisin producing ability through further analytical methods. If this study is compared to the first report carried out by Kritzinger *et al*. (2003), it is evident that many fungal genera have been isolated from cowpea seed previously. However, from these samples the following genera were isolated (*Alternaria alternata*, *Chrysonillia* spp., *Epicoccum* spp., *Lasiodiplodia* spp., *Monascus* spp. *and Rhizopus* spp.) which were not present in the former study. The following *Fusarium* species were also not isolated from cowpea seed in the previous study (*Fusarium oxysporum*, *F. subglutinans*, *F. solani*). Thus, this investigation has yielded valuable confirmation of known fungi associated with cowpea seed, but also new fungal associations. Furthermore, fumonisin B analogues were found to be present in the seed samples tested. This complements the previous studies in which cowpea seed samples tested positive for fumonisin contamination. In the study conducted by Kritzinger *et al*. (2003), *F. proliferatum* was identified as the species responsible for fumonisin production, but in this study *F. subglutinans* and *F. oxysporum* were identified as the species producing fumonisin mycotoxin. When considering the results from this study as well as the previous reports (Kritzinger *et al*., 2003; Houssou *et al*., 2009), it is important to mention that further cowpea seed samples from rural communities in Mpumalanga as well as other provinces in South Africa need to be analysed in order to generate sufficient data to gain further insight into the extent of *Fusarium* infection and fumonisin contamination with the seed. This will provide important information to ascertain if a potential health risk exists amongs the communities that rely on this crop primarily as a source of food.

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CHAPTER 4

PHYTOTOXIC ACTIVITY OF FUMONISIN B_1 ON COWPEA SEED

Phytotoxic activity of fumonisin B_1 on cowpea seed

Abstract

Fusarium species, including (*F. oxysporum, F. solani, F. subglutinans, F. verticillioides*, and *F. proliferatum)* have been isolated from cowpea seed, and some of these species are known to produce fumonisin B_1 mycotoxins. Purified fumonisin B_1 was used to investigate the phytotoxic effects of the toxin *in vitro* and *in vivo* on cowpea seedling growth and also the ultrastructure of the seed tissue. Surface-disinfected seeds were imbibed in sterile distilled water amended with FB_1 at various concentrations (10, 25, 50 and 100 µg/ml). Percentage germination was determined according to the Internationl Seed Testing Association (ISTA) rules. All the concentrations significantly reduced the germination of the seed varying between 6.6% for 100 µg/ml and 10.2% for 50 µg/ml. The two highest concentrations (50 and 100 µg/ml) significantly inhibited root and shoot elongation. In FB_1– treated embryonic and cotyledon tissue, the degree of damage to vacuoles, cell wall, lipid bodies of cowpea seed tissue was directly proportional to the concentrations. From these results it was seen that FB_1 could prevent or interfere with the normal development and functioning of the cell wall, vacuoles and lipid bodies. As these destructive effects are seen at the high concentrations (50 and 100 µg/ml), FB_1 may play a negative effect on germination of the seed as well as root and shoot elongation.

Keywords: phytotoxicity, fumonisin B_1; *Fusarium* spp.; cowpea seed; germination; ultrastructure.

4. 1. Introduction

Fungi and bacteria are the most important pathogens in terms of distribution, diversity and total damage to plants in field as well as in storage (Strobel, 1982). These pathogens can, in part, produce phytotoxic compounds that are responsible for disease symptoms. Until in the early 1960s reports on phytotoxins appeared only sporadically, and many of these were focused on the biological activity of phytotoxins rather than

their chemistry or mode of action (Lamprecht *et al*., 1994). Recently, new techniques in chromatography and spectroscopy have improved the isolation and characterization of phytotoxins (Strobel, 1982).

The pathogenic nature of certain species of fungi to plants has been observed virtually since the beginning of agriculture. These pathogens often produce metabolites that show toxic effects when ingested (Apsimon *et al*., 1994). Toxigenicity of *Fusarium* species is known to be a major agricultural problem. Futhermore, *Fusarium* is a phytopathogenic fungus with a global distribution, capable of infecting a wide range of crop plants (Jurado *et al*., 2006).

In addition some *Fusarium* species can produce a number of mycotoxins such as fusaric acid, fusarins (Marasas *et al*., 1984) and fumonisins (Gelderblom *et al*., 1988). Since their description in 1988, fumonisins have been found in *Fusarium verticillioides* infested maize (*Zea mays*) crops and products in many parts of the world (Sydenham *et al*., 1991, 1992). Until now, several fumonisins (FB) have been isolated and categorized. Only FB_1, FB_2 and FB_3 are the ones present in naturally contaminated foods (Soriano *et al*., 2005).
In several cases, FB_1 has been reported as an animal and plant toxin which is responsible for several diseases, such as leukoencephalomacia in horses; pulmonary oedema and hydrothorax in pigs and liver cancer in rats (Gelderblom *et al*., 1988; Harrison *et al*., 1990). In South Africa, fumonisins are believed to be responsible for the high level of human oesophageal cancer and also in some areas of Transkei (Rheeder *et al*., 1992).

Phytotoxic properties of fumonisins have been widely studied by various authors (van Asch *et al*., 1992; Abbas *et al*., 1993; Doehlert *et al*., 1994; Kritzinger *et al*., 2006). The phytotoxic effect of FB_1 on monocot crops, including barley (*Hordeum vulgare* L.) maize, rice *(Oryza sativa* L.), sorghum (*Sorghum bicolour* L.) and wheat (*Triticum aestivum* L.), showed that they were visibly harmfully affected by FB_1 (Abbas and Boyette, 1992). In maize and tomato (*Lycopersicon esculentum* Mill.) seedlings, fumonisins reduced root and shoot length and dry mass in a dose-dependent manner (Lamprecht *et al*., 1994). These authors also reported that in a detached leaf assay, fumonisin application caused necrotic patches. In addition, amylase production in maize

endosperm (specifically low pI amylases) was inhibited, suggesting that FB_1 may interfere metabolically with germination (Doehlert *et al*., 1994).

Cowpea, *Vigna unguiculata* (L.) is a crop that belongs to the class of foods known as legumes or pulses and these seeds are susceptible to fungal contamination especially when stored under poor conditions together with high relative humidities and high temperatures (Seenappa *et al.*, 1983). Research has shown that *Fusarium* species including *Fusarium oxysporum*, *F. solani*, *F. semitectum*, *F. sporotrichioides*, *F. equiseti* and *Fusarium proliferatum* are potential pathogens for cowpea seed (Kritzinger *et al*., 2003; Rodrigues and Menezes, 2005; Houssou *et al*., 2009). Some of these species have the ability to produce fumonisins. In South Africa, four samples of cowpea seed were determined to be fumonisin positive (Kritzinger *et al*., 2003).

This chapter reports on the effect of FB_1 toxin on (i) cowpea seed germination; (ii) root and shoot elongation as well as emergence in the greenhouse; and (iii) ultrastructural effects on cowpea seed tissue with the use of transmission electron microscopy (TEM). The investigation in this chapter is similar to that of the investigation carried out by Kritzinger *et al*. in 2006. The expected results of this study will provide important confirmation of the deleterious effects that the toxin has on cowpea seed germination and development. It is important to mention that this study includes an *in vivo* (glasshouse) study that was not carried out in 2006.

4. 2. Materials and methods

4. 2. 1. Seed material and treaments

Seed material: Cowpea seeds (cultivar IT 85F-867-5) used in this investigation was obtained from Ecolink, Nelspruit, South Africa. Three replicates of 100 seeds were used for each treatment. The seeds were surface disinfected with 1% of sodium hypochlorite for 1 min and thereafter rinsed three times with sterile distilled water.

Toxin: Pure FB_1 mycotoxin (batch A/03, 10.10 mg) was supplied by the PROMEC Unit, Medical Research Council (MRC), Tygerberg, South Africa.

Seed treatment: Twenty ml of methanol was added to 10.10 mg of FB_1. The required amount of FB1 was added to 50 ml sterile distilled water to yield final concentrations of 10, 25, 50, 100 µg/ml. Two controls were included, namely, control imbibed and control dry. Each treatment had four replicates with the exception for the dry control. The seeds were left to imbibe in the various solutions for 10 hours before placing them in paper towels for germination trials.

4. 2. 2. *In vitro* and *in vivo* germination trials

Germination *in vitro trial*

Seeds were placed in moist paper towels equidistant apart (Fig. 4.1). Paper towels were then rolled up and placed individually in polythene bags, put in plastic buckets, and maintained at 25 °C in incubator for 8 days. The percentage germination, root and shoot elongation were determined after 8 days according to the International Seed Testing Association (ISTA) rules. The entire experiment was repeated twice.

Figure 4. 1. The arrangement of cowpea seed on germination paper (Photo: K. Lodama)

Germination *in vivo* trial

The soil type used in this study was commercial pasteurised potting mix, and this potting soil mix was distinguished from others by having a high content of organic material.

Cowpea seeds (according to treatments as previously mentioned in 4.2.1) were placed in the potting soil a depth of 3 cm from the top in pots in the greenhouse. Four different treatments plus two controls (imbibed and dry) replicated four times each were conducted using completely randomized block design. To ensure sufficient moisture of the soil 30 ml of water was applied every day. Seed germination and shoot length were recorded for each treatment 8 days after sowing. The entire experiment was repeated twice.

4. 2. 3. Transmission electron microscopy

Representative seeds from each treatment (as described in 4.2.1) were removed 10 hours after imbibition. Seeds were dissected and the embryonic axes and cotyledon tissue were removed. Small pieces of sample were fixed in 2.5% glutaraldehyde 2.5 % glutaraldehyde (plus 2.5 % Formaldehyde) in 0.075 M phosphate buffer (pH 7.4) for two hours at room temperature. Seeds were rinsed three times in 0.075 M phosphate buffer for 10 minutes and post fixed in 0.5% aqueous osmium tetroxide for two hours. Thereafter, seeds were rinsed three times with distilled water in a fume hood and dehydrated in ethanol series (30%, 50%, 70%, 90%, 100%, 100%, and 100%) for 10 minutes in each. Samples were then stored in 100% ethanol for a one days. Seed were infiltrated with Quetol 651 epoxy resin in ethanol for one hour and infiltrated with pure Quetol for four hours and embedded. Thereafter, thin sections materials were made with a diamond knife and viewed with the transmission electron microscope (JEOL 2100 F).

4. 3. Results and discussion

4. 3. 1. Effect of FB_1 on seed germination, root and shoot elongation (*in vivo* and *in vitro*)

The germination of the seeds varied between 6.6% for the 100 µg/ml treated seeds and 78.8% for the imbibed control (Fig. 4.2). There was a significant difference ($P \leq 0.05$) in germination between treatments showed (Fig. 4.2). The results showed that all the concentrations significantly decreased seed germination. The phytotoxic effect of fumonisins has previously been shown in maize seedlings where the reduction of roots and shoots length was noted (Doehlert *et al*., 1994; Lamprecht *et al*., 1994). The significant negative correlation between fumonisin content and germination may indicate that fumonisins inhibit seed germination. The phytotoxic effects of FB_1 was exhibited on emerged *Striga hermonthica* and *S. asiatica* plants when FB_1 was applied at concentrations of 250, 500 and 1000 µg/ml (Kroschel and Elzein, 2004).

It is evident that seed germination was affected by toxins. Seed germination decreased with increasing toxin concentrations (Fig. 4.2). A similar pattern was seen by Kritzinger *et al*. (2006) where all the toxin concentrations significantly decreased seed germination and the two highest concentrations (50 and 100 µg/ml FB_1) inhibited root and shoot elongation. The similar correlation is found in this study where FB_1 exhibited inhibitory effects on root and shoot growth of seedlings (Fig. 4.3). The 50 µg/ml and 100 µg/ml concentrations showed a distinctive decline in both root and shoot growth when compared to the control (Fig. 4.3). It was observed that the 100 µg/ml concentration FB_1 toxin was found to be the most potent treatment in the inhibition of shoot growth of seedlings.

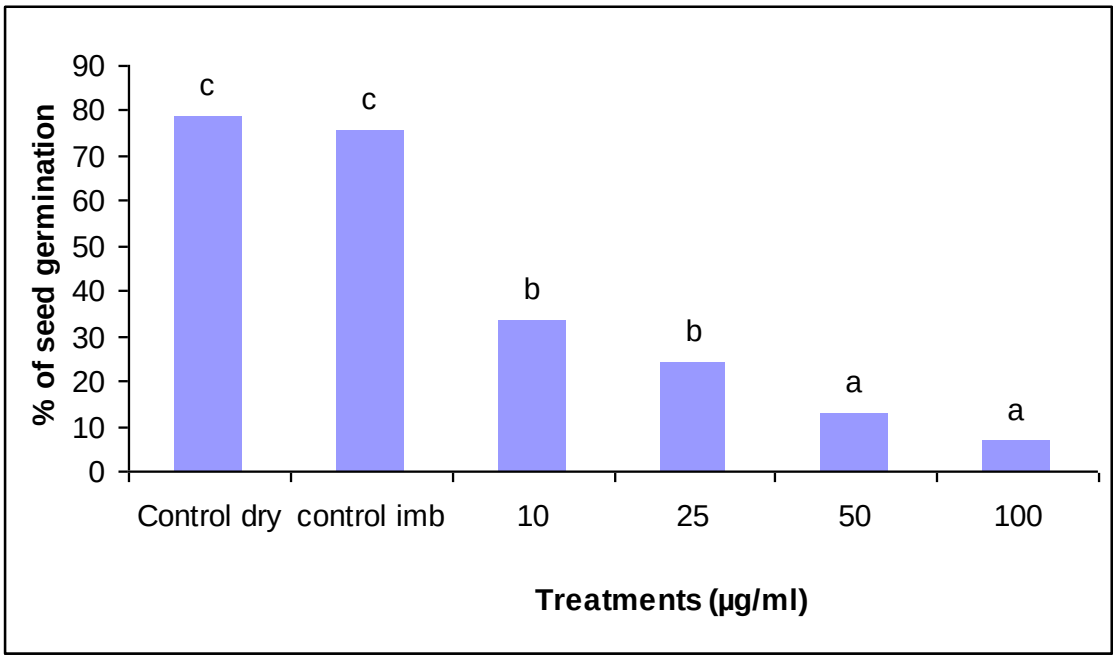

Figure 4. 2. Effect of different concentrations of FB_1 on cowpea seed germination (*in vitro*)

The value of the bars shows the percentage of seed germination and the bars not followed by the same letter are significantly different (P=0.05) according to the student's *t* test.

The results from the emergence in the greenhouse (Fig. 4.4 and 4.5) showed that fumonisin B_1 concentrations of 25, 50, and 100 µg/ml significantly decreased germination of the seeds and also retarded the elongation of the shoots. From these results it was concluded that fumonisin B_1 had a very significant detrimental effect on the emergence of the greenhouse seedlings.

These results from the *in vitro* and *in vivo* investigations showed that higher concentrations of FB_1 in cowpea seed could have a negative impact on the ability of cowpea seeds to germinate, and consequently to emerge in the field.

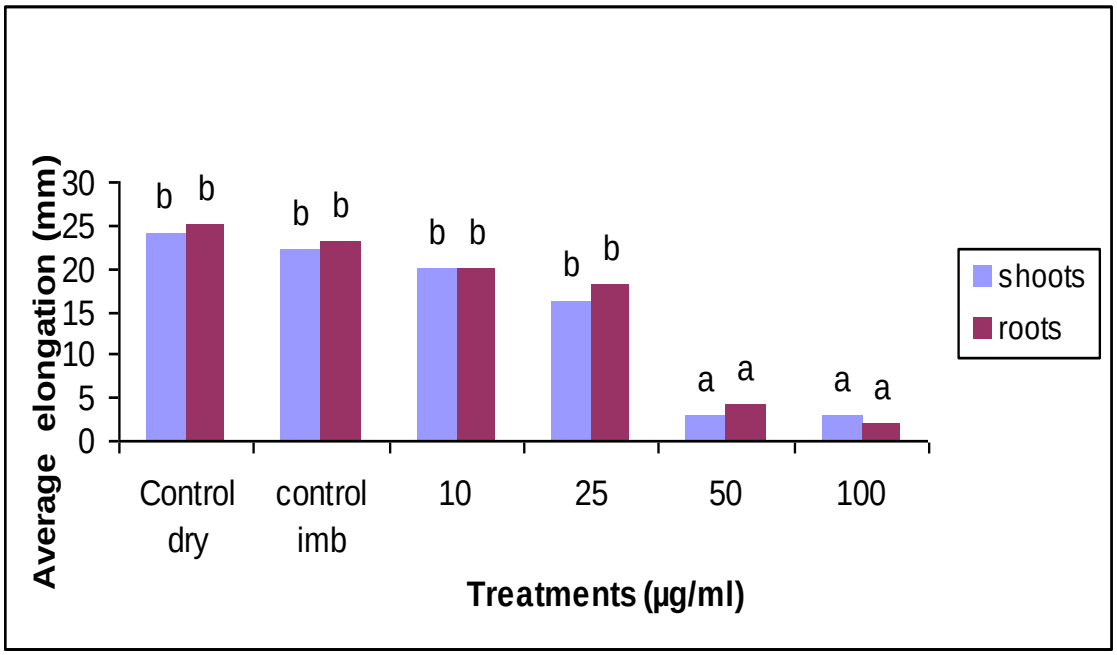

Figure 4. 3. Effect of different concentrations of FB_1 on shoot and root elongation of cowpea seed (*in vitro*)

The value of bars shows the average elongation of roots and shoots and the bars not followed by the same letter are significantly different (P=0.05) according to student's t test.

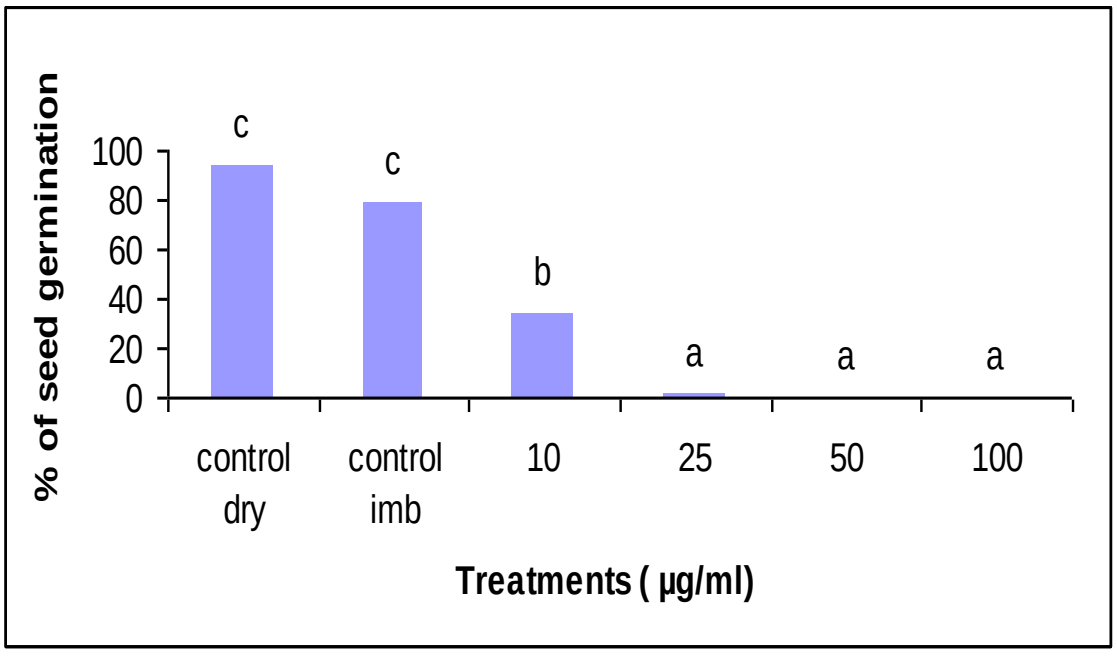

Figure 4. 4. Effect of different concentrations of FB_1 on cowpea seed germination (*in vivo*)

The value of the bars shows the percentage of seed germination and the bars not followed by the same letter are significantly different (P=0.05) according to the student's t test.

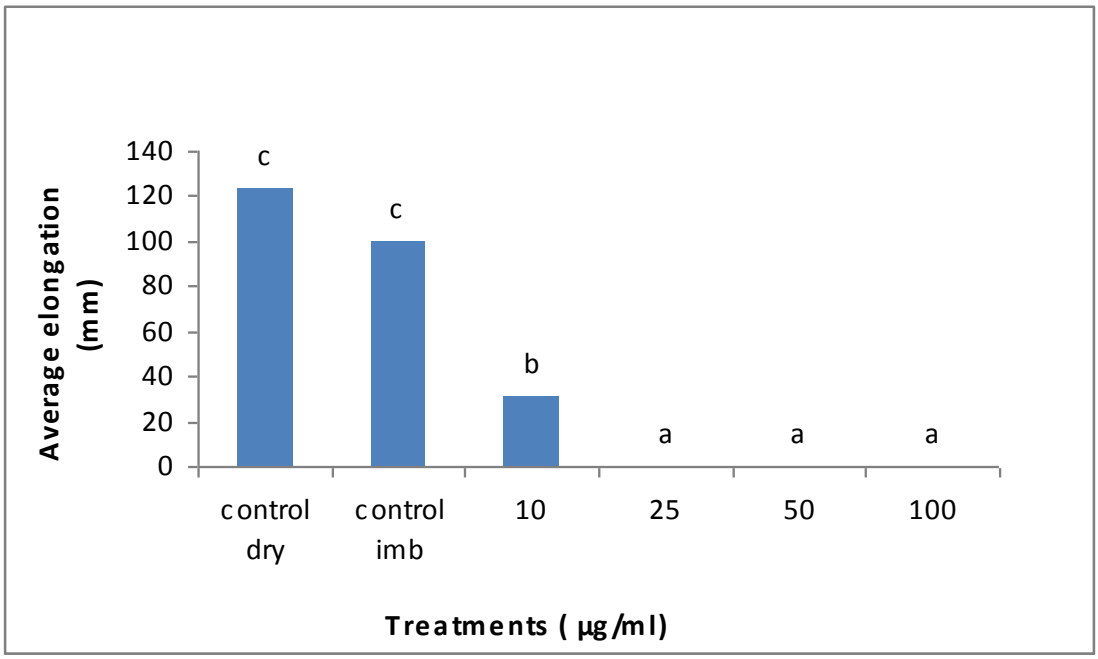

Figure 4. 5. Effect of different concentrations of FB_1 on shoot elongation of cowpea seed (*in vivo*)
The value of the bars shows the average of shoot elongation and the bars not followed by the same letter are significantly different (P=0.05) according to student's t test.

4. 3. 2. Effect of FB_1 on the ultrastructure of cowpea seed tissue

The TEM ultrastructure studies indicate that there were dramatic differences between the control and the toxin treated embryonic and cotyledon tissue of cowpea seed (Fig. 4.6 and 4.7). In the lower concentrations, 10 and 25 µg/ml (Fig. 4.6, E, F and Fig. 4.7, A, B) and both controls dry and imbibe (Fig. 4.6, A, B, C and D), the cell wall, lipid bodies, protein bodies and vacuoles can be seen clearly. However, from the 50 and 100 µg/ml concentration there are many black spots along the cell walls and inside the protein and lipid bodies (Fig. 4.7, C, D, E and F). It was also observed that the higher concentrations showed an abundance of lipid bodies and the colour became darker. In the lower concentration (10 and 25 µg/ml) treatments and both controls the lipid bodies are white in colour (Fig. 4.6, C, D and E and Fig. 4.7, A). In the higher concentation (50 and 100 µg/ml) treatments the plasma membrane separated from the cell wall, the vacuole and protein bodies became irregular in size and discolouration occurred (Fig. 4.7, C, D, E, and F).

From these results it can be seen that FB_1 could prevent or interfere with the normal development and functioning of the cell wall, protein bodies, vacuoles and lipid bodies. Furthermore, these destructive effects of FB_1 specifically for the high concentrations as seen in the ultrastructure may play a negative effect on germination of the seed and root and shoot elongation. The results of this study are in agreement with a similar investigation done by Kritzinger *et al*., in 2006 on the effect of fumonisins on the ultrastructure of cowpea seed. It was reported that the higher concentration (100 µg/ml) of FB_1-treated embryonic tissues of cowpea seed caused destructive effects and the plasma membrane separated from the cell wall and irregular sizes vacuoles formed due to the contraction of the protoplasm (Kritzinger *et al*., 2006). The interference of the metabolism of the sphingolipids by FB_1 could play a role in the alterations noted in the ultrastructure of the toxin-treated seeds. The toxin has interfered with the cell anatomy in some of the treated tissue and this interference could be responsible for the negative impact on the germination of the seeds as well the growth of the seedlings.

The phytotoxicity of FB_1 is largely due to its effect on the disruption of sphinganine metabolism (Soriano *et al*., 2005). Soriano *et al*. (2005) also reported that fumonisin-induced disruption of sphingolipid metabolism is an important part in the cascade of events leading to altered cell growth, differentiation and cell injury observed *in vitro* and *in vivo*. This is because FB_1 is a competitive inhibitor of both sphinganine and fatty acyl co-enzyme A of sphingosine N-acetyltransferase (ceramide synthase) (Riley *et al*., 1998). The mechanism of action is according to the four following events; (i) inhibition of ceramide biosythesis, (ii) increase of free sphingonine and sphingosine, (iii) reduced acylation of sphingosine derived from complex sphingolipid turn over and the degradation of dietary sphingolipids, an increase in sphingoid base degradation of dietary sphingolipids, and (iv) inhibit the plasma membrane H+-ATPase from maize embryos (Kroschel and Elzein, 2004).

Fumonisin toxicity is based on the structural similarity to the sphingoid bases; sphingosine and sphinganine. This enzyme catalyzes the acylation of sphinganine in the biosynthesis of sphingolipids and also the deacylation of dietary sphingosine and the sphingosine that is released by the degradation of complex sphingolipids (ceramid, sphingomyelin and glycosphingolipide) (Wang *et al*., 1991). Sphingolipids are basically important for the membrane and lipoprotein structure and also for cell regulation and

communication (second messenger for growth factors) (Riley *et al*., 1996). Sphingolipids are found primarily in plasma membranes of all eukaryotes, some prokaryotes, and in all foods (Gutierrez *et al*., 2004).

As observed in this investigation, high concentrations of FB_1 inhibited the germination of cowpea seed, reduced root and shoot elongation and caused destructive effects on embryonic and cotyledon tissue. It will therefore be useful to determine the concentration of fumonisins, if any, in cowpea seed samples before sowing.

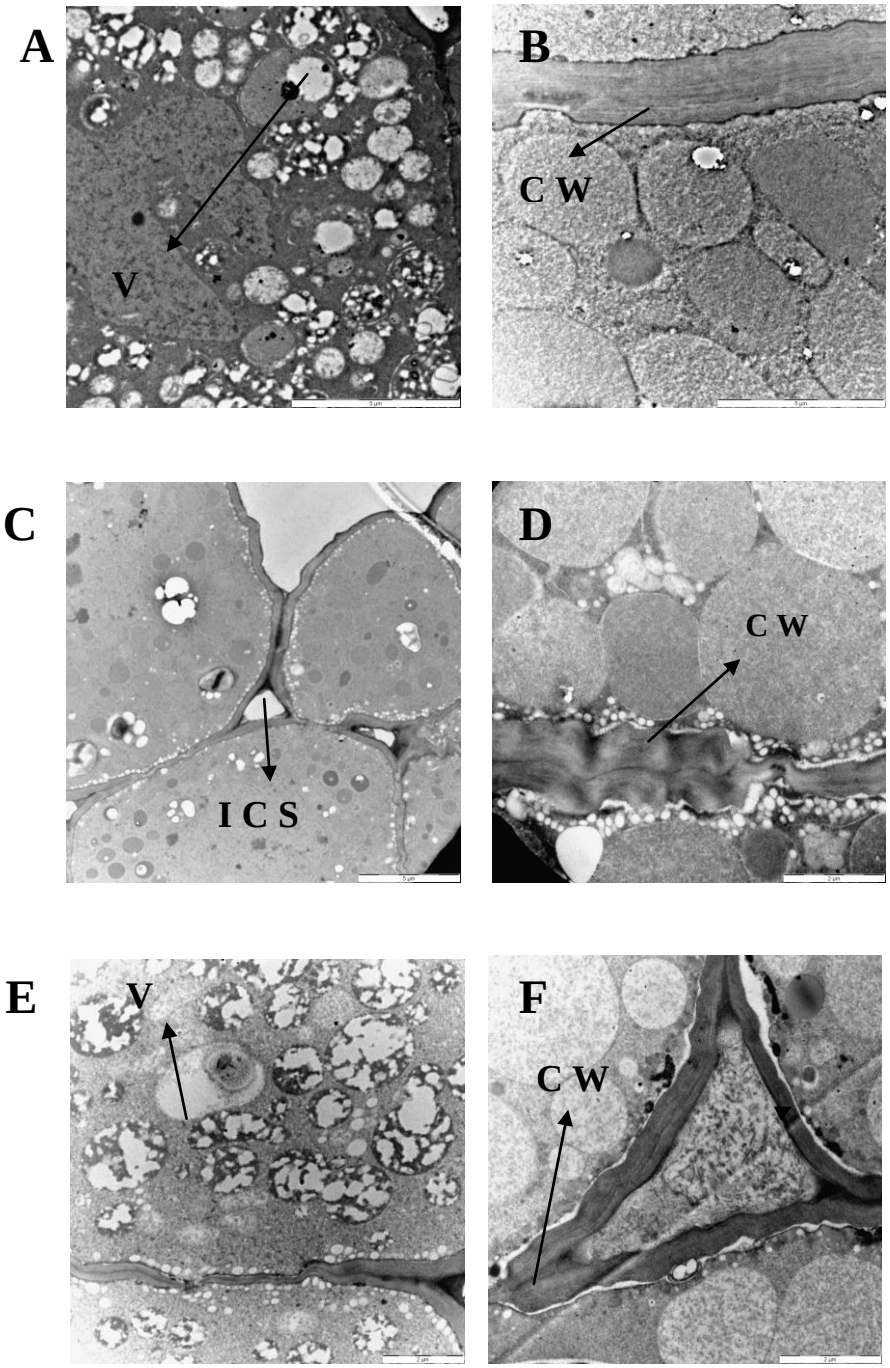

Figure 4. 6. Ultrastructural effects of FB_1 on cowpea seed tissue. TEM micrographs of the embryonic and cotyledon axes of cowpea seed (a) control dry embryonic tissue; (b) control dry cotyledon; (c) control imbibe embryonic tissue; (d) control imbibe cotyledon; (e) embryonic tissue treated with 10 µg/ml FB_1; (f) cotyledon tissue treated with 10 µg/ml FB_1. V = vacuole, CW = cell wall, ICS = intercellular spaces. Bar = 2 µm

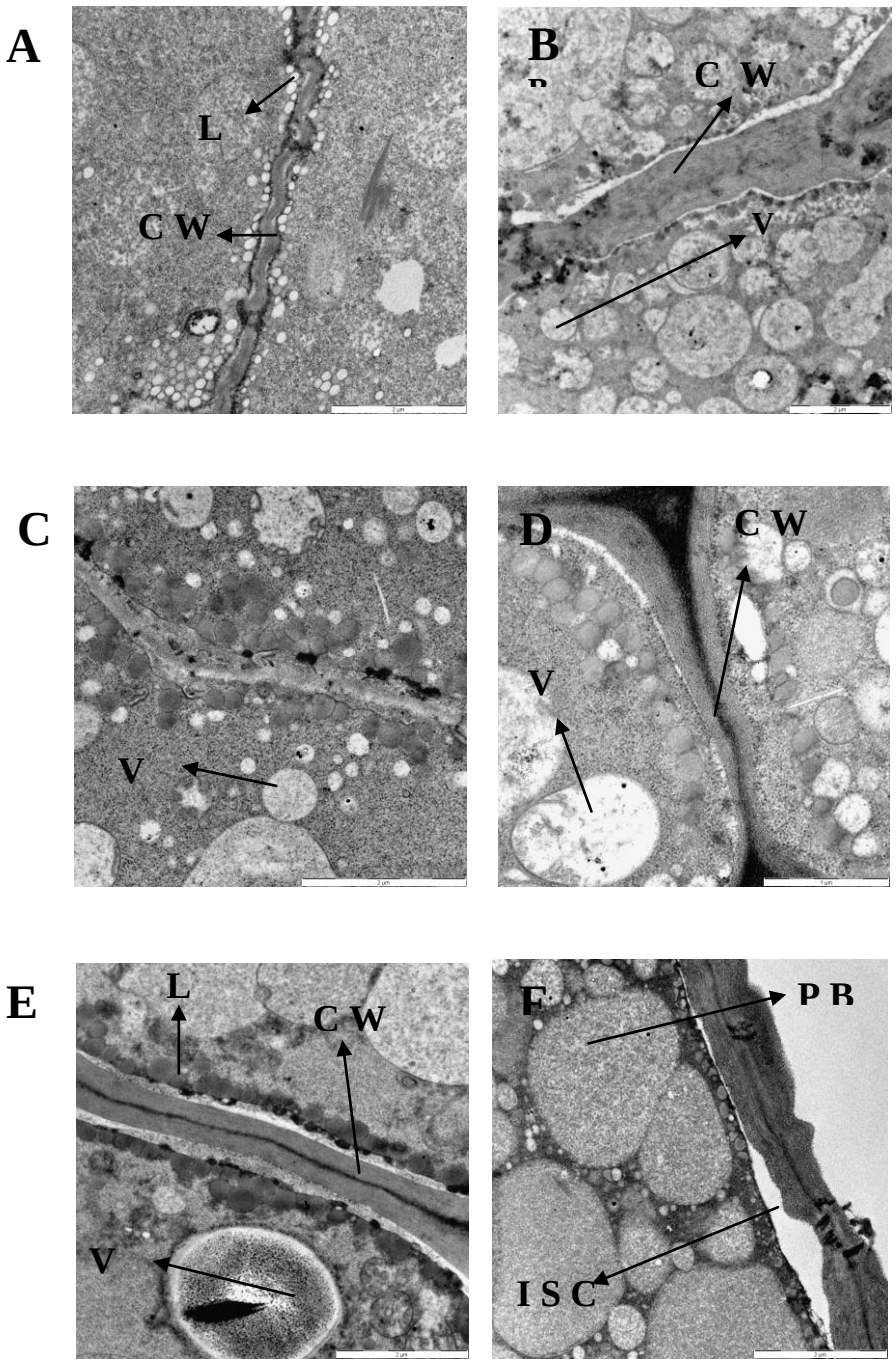

Figure 4. 7. Ultrastructural effects of FB_1 on cowpea seed tissue. TEM micrographs of embryonic and cotyledon axes of cowpea seed (a) embryonic tissue treated with 25 µg/ml FB_1; (b) cotyledon tissue treated with 25 µg/ml FB_1; (c) embryonic tissue treated with 50 µg/ml FB_1; (d) cotyledon tissue treated with 50 µg/ml FB_1; (e) embryonic tissue treated with 100 µg/ml FB_1; (f) cotyledon tissue treated with 100 µg/ml FB_1. V = vacuole, CW = cell wall, ICS = intercellular spaces, L = lipid, PB = protein bodies. Bar = 2 µm

4. 4. Acknowledgements

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CHAPTER 5

ANTIFUNGAL ACTIVITY OF LEAF EXTRACTS FROM THREE RUBIACEAE SPECIES ON VARIOUS *FUSARIUM* SPECIES

Antifungal activity of leaf extracts from three rubiaceae species on various *Fusarium* species

Abstract

Acetone, methanol, and dichloromethane crude extracts of the leaves of *Psychotria capensis*, *Gardenia brighamii*, and *Morinda citrifolia* were studied as potential sources of antifungal agents for selected *Fusarium* species *(F. verticillioides, F. oxysporum, F. graminearum, F. proliferatum,* and *F. nygamaii).* Certain *Fusarium* spp. are known to produce fumonisin B_1, which can adversely affect the health of animal, humans and plant. In all cases the acetone and methanol extracts exhibited moderate activity to all *Fusarium* species tested with minimum inhibitory concentrations (MIC's) of 1.5 mg/ml for *P. capensis* against *F. proliferatum, F. verticillioides* and *F. nygamai* and *G. brighamii* against *F. oxysporum*.

Keywords: antifungal activity, *Fusarium* species, *Psychotria capensis*, *Morinda citrifolia*, *Gardenia brighamii*.

5. 1. Introduction

In recent years, scientists have focused on the increase of food production needed for the fast expansion of world population. Unfortunately, substantial yield losses occur due to insects and plant diseases caused by fungi, bacteria and viruses (Fletcher *et al*., 2006). Legume seed deterioration during storage is a major constraint to profitable grain legume crop production in Nigeria and in some other semi- humid tropic regions, since it causes considerable loss (Essien *et al*., 2008). Hal and Harman (1991) estimated post harvest loss due to fungal attacks to be approximately 30-80% in grain legumes in semi-arid Africa. Fungi and bacteria have also unfavourable effects on quality, safety and preservation of food. Synthetic chemicals are widely used in the control of plant diseases. However, these chemicals may cause toxic residues in treated products (Isman, 2000). Synthetic pesticides can also cause environmental pollution owing to their slow biodegradation (Misra and Pavlostathis, 1997). In addition, the risk of

developing the resistance by micro-organisms and the high cost-benefit ratio are other disadvantages of synthetic pesticide usage (Brent and Hollomon, 1998).

The alternative choice may be the use of botanical fungicides that are easily biodegradable and safe, with minimal environmental impact and danger to consumers (Fawecett and Spencer, 1970). Plants have, and continue to be sources of antifungal agents (Hostettman *et al*., 2000). Many plant species therefore contain antifungal compounds which can be developed into commercial products. Several studies have shown that natural products are capable of inhibiting fungal toxins (Dubey and Dwivedi, 1991).

Volatile compounds from plants, especially essential oils, have antimicrobial, fungicidal and insecticidal activities (Wilson *et al*., 1997). Suleiman *et al*. (2008) reported the effect of aqueous leaf extracts on a spot fungus (*Fusarium* sp) isolated from cowpea. Fandohan *et al*. (2004) reported significant inhibitory effect of essential oil from seeds of the neem tree (*Azadirachta indica*) on the growth of *Fusarium verticillioides* and fumonisin contamination in maize.

The present chapter investigates the antifungal activity of selected plants (*Psychotria capensis*, *Gardenia brighamii*, and *Morinda citrifolia*) against various *Fusarium* species.

5. 2. Description and medicinal uses of selected plants

5. 2 .1. *Morinda citrifolia* (noni)

5.2.1.1. Description and geographical distribution

The genus *Morinda* (Fig. 5.1) (Rubiaceae), including the species *Morinda citrifolia* L. commonly called Noni or Indian mulberry, is a small evergreen tree or shrub of Polynisian origin and widely distributed in Southern Asia and the Pacific Island (McClatchey, 2002). The plant is a bush or small tree, 3–10 m tall, with abundant wide elliptical leaves (5–17 cm length, 10- 40 cm width). The small tubular white flowers are

grouped together and inserted on the peduncle. The petiole leaves ring-like marks on the stalks and corolla is greenish-white (Dixon *et al*., 1999; Chan-Blanco *et al*., 2006). *Morinda citrifolia* has been introduced into an environment in which they did not evolve and thus frequently have no natural enemies to limit their reproduction and spread. *Morinda citrifolia* has a long history of use as a medicinal plant in the parts of Southeast Asia, Polynisia, and Australia and is considered to be the second most important medicinal plant in the Hawaiian Island (Tabrah and Eveleth, 1996).

Figure 5. 1. *Morinda citrifolia* (noni) (www.resorthealth.com/noni_products/index.html)

5.2.1.2. Chemical constituents of the genus *Morinda*

The first record of the chemical studies on the genus *Morinda* goes back to 1907 when Barroweliff and Tutin carried out a chemical examination of *M. longiflora* and reported hydroxymethoxymethylanthraquinone, a monoethyl ether of alizarin, formic, acetic, butyric, palmitic and citric acids, a hydrocarbon hentriacontane, and an alcohol morindanol from the leaves and roots of *M. longiflora* (Barroweliff and Tutin, 1907). Oesterle (1908) isolated an anthraquinone derivative, morindin from alcoholic and aqueous extracts of the wood of *M. citrifolia*. In the same year, the same group isolated morindin and trihydroxanthraquinone monomethyl ether along with a wax-like substance with molecular formula $C_{18}H_{28}O_5$ melting at 124. 5º C from the bark extract

of *Morinda citrifolia* (Oesterle and Tisza, 1908). Burnett and Thomson (1968) isolated 2-hydroxyanthraquinone, alizarin, alizarin 1-methyl ether, rubiadin, rubiadin1-methyl ether, xanthopurpurin, alizarin 2-methyl ether, 1-hydroxy-2-methylanthylanthraquinone, 2-methylanthraquinone, 2-methoxy-anthraquinone and 1-methoxy-2-methylanthraquinone from roots and stems of *M. umbellata*. The roots also yielded munjistin, lucidin, glycosides of rubiadin and rubiadin 1-methyl ether (Burnett and Thomson, 1968).

5.2.1.3. Medicinal and pharmacological properties

Traditionally almost every part of the plant (*M. citrifolia*) is reported as a general medicinal healing agent for the treatment of a variety of human ailments such as diabetes, high blood pressure, arthritis, aging, heart remedy, headache, gastrointestinal and liver ailments. A broad range of therapeutic effects, including antibacterial, antiviral, antifungal, antitumor, antihelmintic, analgesic, hypotensive, anti-inflammatory and immune enhancing effects are attributed to its various parts (Su *et al*., 2005). The roots are used as a cathartic (a purgative medicine) and febrifuge (remove fever) and applied externally to relieve pain in gout. Leaves are reported as tonic and febrifuge. Fruits are used for throat complaints and dysentery (Sastri, 1962). Leaves and stem bark are given to cure tuberculosis (Jayaweera, 1982).

Noni is a traditional food and medicine in many tropical ereas including the pacific Islands, Southeast Asia, India, and the Philippines. Ethnobotanical data is plentiful on the uses of fruit, leaves, and roots of the plant. Ancient Hawaiians used noni fruit for both internal and topical applications (Hiramatsu *et al*., 1993; Koyama *et al*., 2001; Taloua *et al*., 2001; Sadeghi-Aliabadi *et al*., 2004). The stem, bark, root, leaves, and fruits of the plant have been used traditionally by islanders as medicines to treat a broad range of diseases, including diabetes, hypertension, and cancer (Saludes *et al*., 2002). The fruit juice of *M. citrifolia* contains a polysaccharide-rich substance called noni-ppt that has been reported to have antitumor activity in the Lewis lung peritoneal carcinoma model (Saludes *et al*., 2002).

5. 2. 2. *Psychotria capensis*

5.2.2.1. Description and geographical distribution

Psychotria capensis (Eckl) Vatke var, (Fig. 5.2) commonly known as Lemoenbos (African); izele, isithitibala (Zulu); umgomo-gono (Xhosa) is the largest species in the Rubiaceae family (Hamilton, 1989; Taylor, 1996). The same authors (Taylor, 1996) reported that the genus *Psychotria* is one of the largest genera of flowering plants with estimated 1000 to 1650 species distributed worldwide. The genus *Psychotria* (Rubiaceae) is widespread in Brazil and contains several species of chemical interest (Elisabetsky *et al*., 1997). At least 11 species of *Psychotria* are native from the state of Rio Grande do Sul (Dillenburg and Porto, 1985; Henriques *et al*., 2004). The plant is an evergreen shrub or small tree, 3 – 8 m tall, with a slender stem, horizontal branches and pale brown bark. The leaves are shiny, light to dark green above and paler below, smooth and leathery. They are fairly large, 70–150 x 15–60 mm, elliptic to obovate, opposite and often drooping (Nepokroeff *et al*., 1999).

Figure 5. 2. ***Psychotria capensis*** **(Photo: K. Lodama)**

5.2.2.2. Chemical constituents of the genus *Psychotria*

The order Rubiales is characterized by the presence of iridoids and alkaloids of mixed biosynthetic pathway (Judd, 1999). Tannins are not characteristic of this taxon (Dahlgren, 1980), although these compounds were found in *Psychotria douarrei* (Davis *et al*., 2001). The genus *Psychotria* is known as a source of monoterpene indole alkaloids, and a chemical survey for alkaloids and iridoids in 15 Brazilian species of *Psychotria* showed the presence of monoterpene indole alkaloids in 14 of them (Lopes *et al*., 2004). *Psychotria leiocarpa* presents N,β-D-glucopyranosyl vincosamide (GPV), an N-glycosylated indolic alkaloid, as its major alkaloid, representing up to 2.5% of leaves' dry weight. Moreover, in the above mentioned chemical survey, *P. leiocarpa* was the only *Psychotria* species shown to accumulate small amounts of the iridoid glucosides asperuloside and deacetylasperuloside (Henriques *et al*., 2004). *Psychotria* species often accumulate indole alkaloids, and this trait may be important to chemosystematics, since this genus is taxonomically complex due to a lack of conspicuous morphological differentiating features (Nepkroeff *et al*., 1999; Lopes *et al*., 2004).

5.2.2.3. Medicinal and pharmacological properties

A number of *Psychotria* species have yielded bioactive extracts. Some examples include antibiotic activity in extracts from *P. microlabastra* (Kham *et al*., 2001) and *P. capensis* (McGaw *et al*., 2000) (Africa), antiviral activity in *P. serpens* (Kuo *et al*., 2001) (China) and antiviral/antifungal and anti inflammatory activities found in *P. hawaiiensis* (Locher *et al*., 1995) and *P. insularum* (Dunstan *et al*., 1997) (Central America), respectively.

Active molecules produced by *Psychotria* species include naphtoquinones (Hayashi *et al*., 1987), peptides (Witherup *et al*., 1994), benzoquinones (Solis *et al*., 1995), pigments (Glinski *et al*., 1995) and alkaloids (Beretz *et al*., 1985). Perhaps the best known compound isolated from *Psychotria* species is the alkaloid emetine. Emetine is an isoquinoline alkaloid extracted from *P. ipecacuanha* (ipecac) bark, a plant used by traditional communities as a stimulant and “antidote to opium” (Giorgetti *et al*., 2007) and in the treatment of intoxication due to its emetic effect (Hasegawa *et al*., 2002).

Synthethic analogs of emetine, which have less adverse effects, are currently used in the treatment of amoebiasis (Mangana-Garcia and Arista-Viveros, 2008). Emetine is cytotoxic, inhibiting protein synthesis, and may have applications in drug-induced apoptosis (Moller *et al*., 2007).

Another well known *Psychotria* species is the one used in the preparation of the hallucinogenic drink "ayahuasca", *P. viridis*. The decoction is prepared using the plant in combination with the vine *Banisteriopsis caapi* (Malpighiaceae). *Psychotria viridis* and *B. caapi* are rich sources of the proto-alkaloid dimethyltryptamine (DMT) and the carboline harmine, respectively (Freedland and Mansbach, 1999). Both substances are psychoactive, and the two have a strong synergism when administered together, possibly due to inhibitory effects of harmine on monoamine oxidase, a DMT detoxifying enzyme (Buckholtz and Boggan, 1977). The recent popularization of the ayahuasca in the United States and Europe has raised several debates, from mental health issues to conflicts on drug abuse *versus* religious freedom (Santos *et al*., 2007; Tupper, 2008).

Several South American *Psychotria* species are used as medicinal plants by Amazon native populations (Sanz-Biset *et al*., 2009). An ethnobotanical survey identified species used as painkillers by "caboclos", traditional rural communities from the state of Pará, Brazil, which comprises a large fraction of the Amazon rainforest. The extracts of *P. colorata* showed analgesic activity, and preliminary tests pointed to alkaloids as major responsibles for the effect (Elisabetsky *et al*., 1995). Further chemical investigations demonstrated the presence of several pyrrolidinoindoline and quinoline alkaloids (Verotta *et al*., 1998), with hodgkinsine, previously isolated from *Hodgkinsonia frutescens* (Rubiaceae) (Fridrichsons *et al*., 1974), as a major component. Hodgkinsine is a potent analgesic, with results comparable to morphine in murine models (Kodanko *et al*., 2007; Amador *et al*., 2000).

Psychotria capensis is a shrub, which has been used for gastric complaints. Other *Psychotria* species are known for their antimalaria, anti-emetic and antimicrobial activity (Jayasinghe *et al*., 2002; Badenhorst, 2004). Several *Psychotria* species are used medicinally for pain-related purposes. The crude extract of *P. microlabastra* has

been reported for their antimicrobial (Khan *et al*., 2001), antioxidant and antimutagenic properties (Fragoso *et al*., 2008).

5. 2. 3. *Gardenia brighamii*

5.2.3.1. Description and geographical distribution

Gardenia brighamii H. Mann., (Fig. 5.3) (Rubiaceae) is an evergreen (perennial) shrub, which is 2–8 m tall. The thick, glossy, dark green leaves are opposite, oval or narrow, 3-5 cm long, and 1–1.5 cm large. Leaf arrangement is a whorl. The waxy, highly fragrant white flowers, 2–5 cm across, are commonly borne singly in the leaf axes (Kobayashi and Kaufman, 2006). The genus *Gardenia* consists of more than 80 species spread among the tropical forests of certain regions of the world. Seventeen species of this genus occur in Thailand (Suvatti, 1978). *Gardenia brighamii* has a distribution in tropical regions of Asia, Africa, and the Pacific. Three endemic species are present in Hawaii all which are rare in their native habitat (Koob, 1999).

Figure 5. 3. *Gardenia brighamii* (Photo: K. Lodama*)*

5.2.3.2. Chemical constituents of the genus *Gardenia*

There are a number of reports on the isolation of triterpenes (Reddy *et al*., 1975), iridoid glycosides (Watanabe *et al*., 1994), quinic acid derivatives (Nishisawa *et al*., 1988), a keto fatty acid (Mahmood *et al*., 1991) and flavones (Gunatilaka *et al*., 1982). Some *Gardenia* species have been investigated (*G. sootepensis*, *G. brighamii*, *G. augustus*; *G. carinata*) for their chemical constituents. The flowers were found to contain β-sitosterol, a highly conjugated diterpene carboxylic acid, 7,4'-dihydroxyflavone and a long chain aliphatic compound (Davies *et al*., 1992), while the fruits contained a quinic acid lactone (quinide) (Liu and Mabry, 1982). Mues *et al*. (1979) isolated a new sesquiterpene together with benzoic acid, 4-hydroxy-3-methoxybenzoic acid, 4-hydroxy-3,5-dimethoxybenzoic acid, 5,7,4'-trihydroxy-6-methoxyflavone (from G. sootepensis). Marco *et al*. (1988) reported other long-chain compounds such as, 5,7,3'-trihydroxy-6,4',5'-trimethoxyflavone.

5.2.3.3. Medicinal and pharmacological properties

Gardenia species have been used in traditional medicine for the treatment of inflammation, jaundice, headache, fever and hypertension (Aburada *et al.*, 1976; Tseng *et al.*, 1995). Its pharmacological actions such as a protective activity against oxidative damage, cytotoxic effect, anti inflammatory activity and fibrolytic activity have been reported (Aburada *et al.*, 1976; Tseng *et al.*, 1995; Jagedeeswaran *et al.*, 2000). Crocetin, a major component of *Gardenia* fruits, was found to be a potent inhibitor of tumour promotion via antioxidant activity (Hsu *et al.*, 1999). Geniposide, an iridoid glucoside isolated from *Gardenia* fruits, showed an antithrombotic effect due to the suppression of platelet aggregation (Suzuki *et al.*, 2001). The fruit of *G. jasminoides* was reported to have antiangiogenic activity (Eun-Hee *et al*., 2003).

Gardenia species have been used in folklore for the remedy of a variety of diseases, including malaria (Weenen *et al.*, 1990; Gakunju *et al*., 1995), fever, hypertension, and ulcers of a skin (Tseng *et al*., 1995). According to Thai traditional medicine the alcoholic extract of bark is used externally for relieving pains and paralysis of limbs (Chang and But, 1987).

5. 3. Materials and methods

5. 3. 1. Plant material collection and extraction

The three plants were chosen on basis of traditional knowledge used and previous scientific studies (Aburada *et al*., 1976; McGaw *et al*., 2000; Badenhorst, 2004). The leaves of *P. capensis* and *G. brighamii* were collected during November 2007, in the Manie van der Schijff Botanical garden at the University of Pretoria, South Africa. The leaves of *Morinda citrifolia* were collected in February 2009 from a local market in Kinshasa in the Democratic Republic of Congo. Voucher specimens were made and deposited in the H.G.W.J. Schweickerd Herbarium (PRU), University of Pretoria.

All the leaves were separated from the stems and dried at room temperature. The dried material is prefered as fewer problems are associated with the large–scale extraction of dried rather than fresh plant material (Eloff, 1998). The dried leaves from the three plants were ground with a laboratory grinder (IKA AII Basic, IKA Works). The ground plant part was then separately subjected to consecutive extraction with three different solvents namely, dichloromethane, acetone, and methanol. These solvents represent different polarities in order to extract a wide range of constituents.

The ground plant parts from each plant species were individually extracted by adding 20 g of finely ground plant material with 150 ml of dichloromethane (DCM). The mixture was placed on the magnetic stirrer for over night and then the mixture was filtrated. This was repeated three times. The filtered solution was then evaporated to dryness using a rotary evaporator (Buchii). The procedure was repeated using the same plant material but with acetone and methanol.

5. 3. 2. Fungal isolates

The five *Fusarium* species cultures of (*F. verticillioides* (PPRI No 7259)*; F. oxysporum* (PPRI No 8287)*; F. graminearum* (PPRI No 7723)*; F. proliferatum* (PPRI No 6349) and *F. nygamaii* (PPRI No 4572) were supplied by the Biosystematics Division,

Mycology Unit, Plant Protection Research Institute, Agricultural Research Council, (ARC) Roodeplaat, in South Africa.

5. 3. 3. Antifungal assays

5. 3. 3. 1. Bioautography

The Thin Layer Chromatography (TLC) technique was used and developed with different solvent-systems. For *Psychotria capensis*, three plates were used: (i) hexane-ethyl acetate (3:7), (ii) ethyl acetate-hexane (7:3), and (iii) dichloromethane-methanol (9:1). For *Gardenia brighamii*, two plates were used (i) dichloromethane-methanol (95:5), and (ii) hexane-ethyl acetate (3:7). For *Morinda citrifolia*, two plates were used with the same solvent system, (i) dichloromethane-methanol (90:10). Methanol leaf extracts of *P. capensis; M. citrifolia* and *G. brighamii* were made up to stock concentrations of 10 mg/ml. These extracts were spotted quantitatively on silica-TLC plates, and the plates were then air-dried. Two cultures of *Fusarium* species (*F. proliferatum* and *F. verticillioides*) were prepared in fresh malt extract broth for spraying onto the TLC plate.

After the extracts dried completely, the plates were put in the TLC-tank and left until the solvent was about 1 cm from the top of the plates. The plates were then taken out of the tank and the solvent front marked with pencil line. A fine sprayer was used to spray the fungal suspension of five day old *F. proliferatum* and *F. verticillioides* cultures onto the plates. The plates were incubated at 25 ^{o}C for three days and then sprayed with an aqueous solution of P-iodonitrotetrazolium violet (INT) as growth indicator. The experiment was repeated twice.

5. 3. 3. 2. Microtitre plate doubling dilution method

The serial microtitre plate doubling dilution was used to test methanol, acetone, and dichloromethane extracts of the leaves of the selected plants against five *Fusarium* species for their antifungal activity. The microdilution assay, one of the most commonly used to determine the minimum inhibitory concentration (MIC) values of plant extracts for antifungal and antimicrobial susceptibility, is also a simple procedure for testing a small number of isolates (Tanaguchi and Kubo, 1993). Additionally, it has also the

advantage that the same rows can be taken for minimum fungicidal concentration (MFC). Masoko *et al*. (2005) reported that the serial dilution microplate method worked well with fungi after slight modifications and found good results from *Terminalia* species which had antifungal properties. Malt extract broth was prepared and sterilized. Stock solution subcultures of the fungi were made by transferring 50 ml of this broth medium in each of five sterilized conical flasks and transferring fungi with a sterilized inoculation loop into the prepared broth and leaving these flasks for six days on an electric shaker at 25^{o} C. Subcultures for the experiment were then further prepared from these stock solutions by preparing 50 ml of malt extract broth in each of five sterilized conical flasks. A volume of 0.5 ml of the fungal stock solutions was then transferred into these flasks with a sterile pipette. The flasks were again put on an electric shaker for six days, at 25^{o}C.

A 50 mg/ml stock solution (10% DMSO) of each extract was prepared by dissolving 50 mg of each dried extract in 100 µl DMSO and 900 µl broth. Sterile microtitre assay trays containing 96 round- bottom wells were employed. To all the wells of the plate, 100 µl of malt extract broth was added. The first wells of row A, were divided into the four groups as (i) A 1 – 3, 100 µl of acetone extract was added; (ii) A 4 – 6, 100 µl of methanol extract; (iii) A 7 – 9, 100 µl of dichloromethane extract; and (iv) A 10-12 were used for the controls. To well A 10, 100 µl of the antifungal drug Virikop (EFEKTO) (see Appendix 8.1 for preparation) was added as the positive drug control. To well A 11, 100 µl of 10% DMSO was added as the solvent control and 100 µl of nutrient broth was added to the last well A 12 as a medium/ sterility control. A series of dilutions were then made by taking 100 µl of the wells in the first row (row A) and transferring it to the second row of wells (row B), where it was mixed and then 100 µl of row B was then again transferred to row C. This method of creating doubling dilutions was repeated up to row H, where 100 µl was taken out of row H's cells and discarded to keep the volumes in all wells equal. The final concentration for each well was follows: A = 12.5, B = 6.25, C = 3. 125, D = 1.5625, E = 0.78125, F = 0.390625, G = 0.1953125, H = 0.0976562 mg/ml.

The optical density of the prepared six day old fungal cultures were determined and diluted with malt extract broth until the optical density was about 0.2. To all the wells in the plate, except column 12 (sterility control), 100 µl of the diluted (if necessary) fungal cultures were added. With every plate, another fungal culture was used, and for five

Fusarium species, each species was tested on one plate. The plates were then covered and incubated at 25^{o} C in the dark for three days.

Iodonitrotetrazolium chloride (INT) was prepared to a concentration of 0.2 mg/ml. To all the wells (except columns 3, 6, and 9) 40 µl of the prepared INT was then added. The MIC (Minimum Inhibitory Concentration) of the different extracts for the fungi was then recorded according to the colour changes of the INT. The MIC's was taken to be the lowest concentration of the extract that didn't turn purple in the presence of INT.

At the particular concentration of every extract that represented the MIC, 50 µl of the no-INT- added columns were transferred to three wells of a new labelled microtitre plate (to serve as triplicates). To all the wells 150 µl of malt extract broth was added and 40 µl of INT added to that. The microtitre plate was then incubated for 24 hours again and the colour changes observed. Where the INT turned purple, the fungi was only temporarily inhibited by the plant extract. Where the INT did not turn purple, that particular concentration of the extract produced total inhibition of the fungi, killing it and was therefore the fungicidal concentration or MFC (Minimum Fungicidal Concentration). The experiment was done in duplicate.

5. 4. Results and discussion

In this study, the antifungal activity of leaf extracts from three Rubiaceae species against five *Fusarium* species were evaluated by two methods.

The methanolic extracts from *G. brighamii*, *P. capensis* and *M. citrifolia* tested against *F. proliferatum* and *F. verticillioides* showed inhibition on TLC plates that could be seen by the clear zone which indicates activity of the plant extracts against the *Fusarium* species tested (Fig. 5.4, Fig. 5.5 and Fig. 5.6). From those Figures the results showed that, the methanolic extracts of *Gardenia brighamii* presented the best inhibition against *F. proliferatum* and *F. verticillioides* (Fig. 5.4).

The results obtained in the microdilution assay of three solvents (acetone, methanol, and dichloromethane) from the leaf extracts of *P. capensis*, *M. citrifolia*, and *G.*

brighamii against the five *Fusarium* species are shown in Table 5.1. The bioassay showed that, the acetone extracts gave the best results over the other two extracts (methanol and dichloromethane), as indicated by the MIC of 1.5 mg/ml given in Table 5.1. *Fusarium verticillioides* and *F. proliferatum* were most affected when compared to the other species (*F. oxysporum*, *F. nygamii*, *F. graminearum*).

The results of this chapter confirms that *M. citrifolia*, *P. capensis*, and *G. brighamii* crude extracts showed antifungal effects on the five *Fusarium* species. The inhibitory effect varied with the different solvent and the acetone extracts showed the most activity against the *Fusarium* species tested. Only dichloromethane extracts exhibited no activity against *Fusarium* species tested. This observation may be attributed to the nature of the biological active compounds between solvent and plant. The lack of antifungal activity observed for the dichloromethane extract suggests that the active metabolites of these three Rubiaceae species are not lypophilic (dichloromethane). All of the parts of *M. citrifolia* (root, bark, leaf, bud, and fruit) have been used to treat a wide range of health problems (Pawlus and Kinghorn, 2007). The result from this study showed that the leaf extract of *M. citrifolia* inhibited the growth of five *Fusarium* species tested in the microdilution method. However, there are very few documented reports regarding the antifungal activity of *M. citrifolia*. Jainkittivong *et al*. (2009) reported antifungal activity of *M. citrifolia* fruit extract against *Candida albicans*, and confirmed that the fruit extracts from this plant had an antifungal effect and the inhibitory effect varied with concentration. Khuntia *et al*. (2010) reported the antifungal activity of *M. citrifolia* leaf extracts by using the agar infusion method of which activity was based on the measurement of the diameter of the inhibition zones.

Gardenia species have been reported to have biological and pharmacological activities (Tseng *et al.,* 1995). Lee *et al*. (2009) reported that the ethanolic extracts of *G. jasminoides* Ellis exerted protective activities against potential gastritic diseases. There are no reports regarding antifungal activity of *G. brighamii*.

The genus *Psychotria* contains over 200 species; many of them are used for traditional medicinal properties (Nepokroeff *et al*., 1999). Elisabetsky *et al*. (1995) reported that an ethnopharmacological survey showed that home remedies prepared with flowers, fruits and roots of *P. colorata* are used by Amazonian caboclos as pain killers. Similarly, Elisabetsky *et al*. (1990) showed that the leaf and flower extracts of *P. colorata* have marked opioid-like analgesic activity. Khan *et al.* (2001) reported that the methanol

extracts of leaves, root and stem bark of *P. microlabastra* showed a broad spectrum of antibacterial activity. There are also no reports on antifungal activity of *P. capensis*.

This study represents the first report of the antifungal activity of these three Rubiaceae species by using the microtitre plate doubling dilution method. The findings as reported in the present chapter indicate a strong potential antifungal activity of crude extracts from *P. capensis, G. brighamii* and *M. citrifolia*. However, some limitations should be mentioned. Although, the leaf extracts from the three Rubiaceae species exhibited antifungal activity, it should be noted the present testing was limited to only the crude extracts. Studies to evaluate the applicability to testing the pure compounds obtained from *P. capensis* and *G. brighamii* against *Fusarium* species is necessary. The evaluation of the cytotoxicity of these extracts also requires further research.

Table: 5. 1. Minimum Inhibition Concentration (MIC) and Minimum Fungicidal Concentration (MFC) in mg/ml of Rubiaceae leaf extracts

***Fusarium* spp.**	**Plant extracts**										**Control ***
		P. capensis			*G. brighamii*			*M. citrifolia*			
		AC	MeOH	DCM	AC	MeOH .	DCM	AC	MeOH	DCM	
F. oxysporum	**MIC**	3.2	6.25	n/o	1.5	3.2	n/o	3.2	3.2	n/o	3.2
	MFC	6.25	6.25	n/o	3.2	6.25	n/o	6.25	3.2	n/o	3.2
F.proliferatum	**MIC**	1.5	3.2	n/o	3.2	6.25	n/o	1.5	3.2	n/o	1.5
	MFC	1.5	6.25	n/o	3.2	6.25	n/o	3.2	6.25	n/o	3.2
F. verticillioides	**MIC**	1.5	3.2	n/o	3.2	6.25	n/o	3.2	6.25	n/o	1.5
	MFC	1.5	6.25	n/o	6.25	12.5	n/o	6.25	12.5	n/o	3.2
F. nygamaii	**MIC**	1.5	3.2	n/o	6.25	6.25	n/o	6.25	3.2	n/o	3.2
	MFC	6.25	6.25	n/o	6.25	6.25	n/o	12.5	6.25	n/o	6.25
F. graminearum	**MIC**	6.25	6.25	n/o	3.2	6.25	n/o	3.2	3.2	n/o	3.2
	MFC	6.25	6.25	n/o	3.2	12.5	n/o	6.25	6.25	n/o	3.2

AC = Acetone, MeOH= Methanol, DCM = Dichloromethane, *= Chemical control VIRIKOP, n/o= not observed

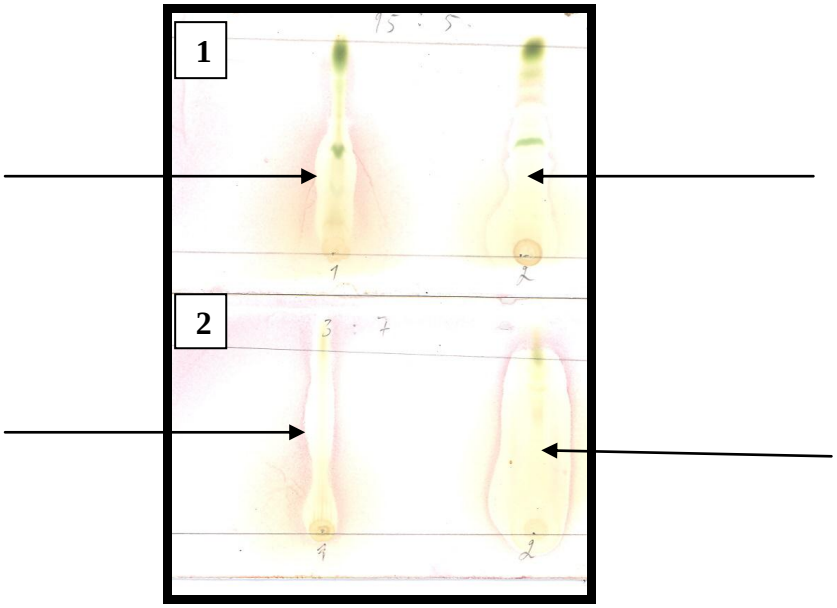

Figure 5. 4. Chromatogram of methanolic *G. brighamii* leaf extracts separated by: (1) dichloromethane: methanol (95:5), (2) hexane ethyl acetate (3:7) and sprayed with *F. proliferatum* (1) and *F. verticillioides* (2) and a growth indicater INT. Clear zones (shown with arrows) indicate antifungal activity

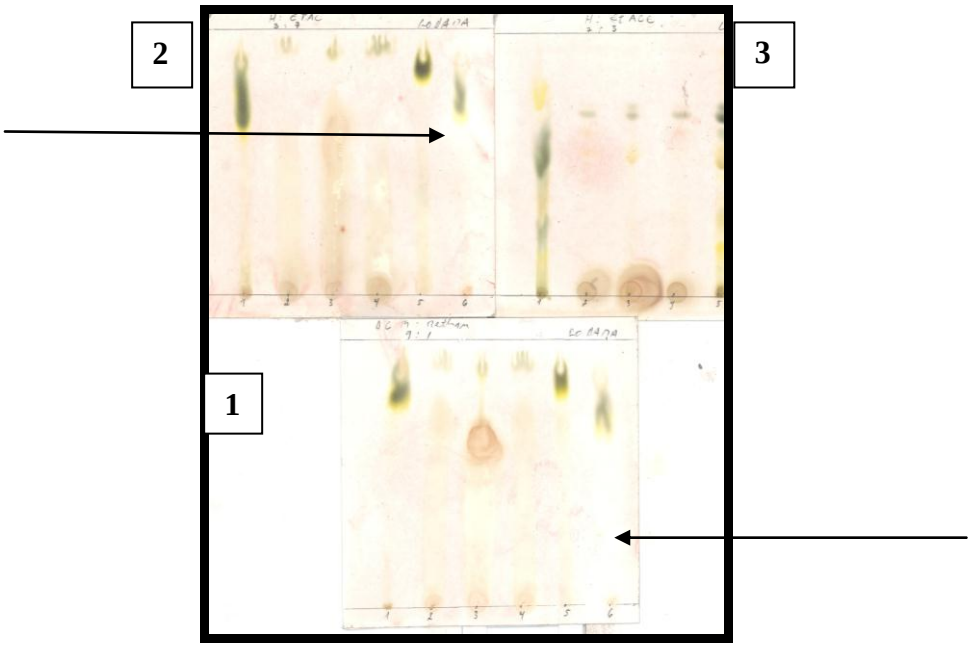

Figure 5. 5. Chromatogram of methanolic *P. capensis* leaf extracts separated by: 1) hexane: ethyl acetate 3:7, 2) hexane: ethyl acetate 7:3, 3) dichloromethane: methanol 9:1 and sprayed with *F. proliferatum* and a growth indicater INT. Clear zones (shown with arrows) indicate antifungal activity

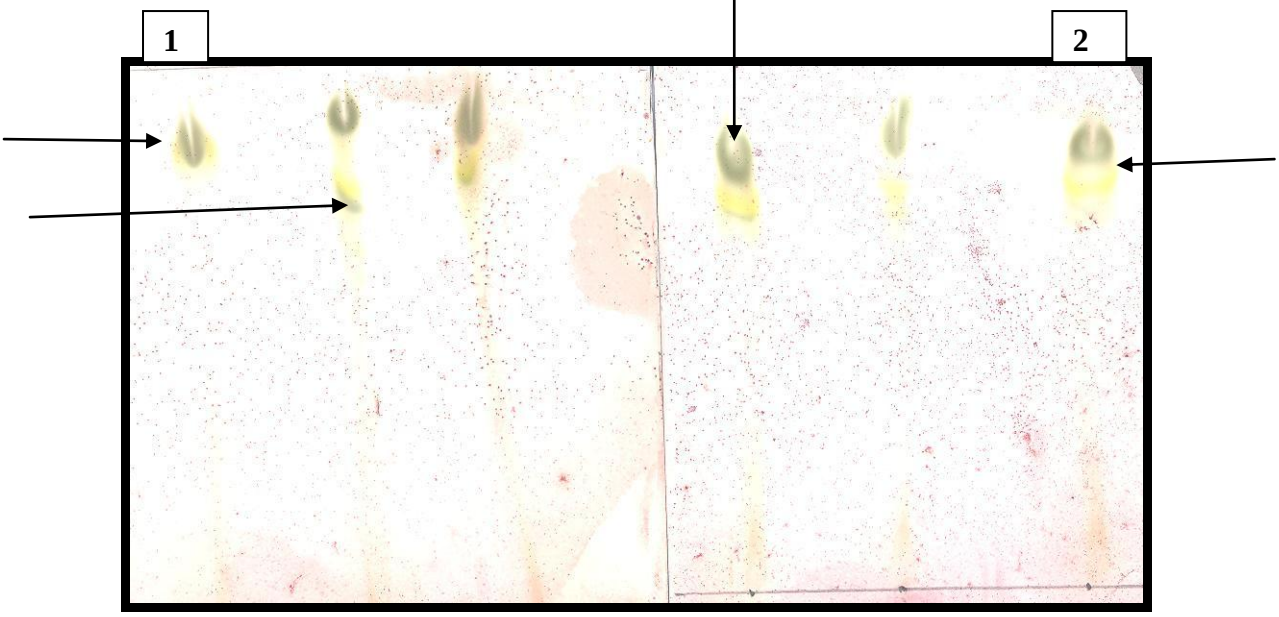

Figure 5. 6. Chromatogram of methanolic *M. citrifolia* leaf extracts separated by dichloromethane: methanol (90:10), and sprayed with *F. verticilloides* (1) and *F. proliferatum* (2) and a growth indicater INT. Clear zones (shown with arrows) indicate antifungal activity

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CHAPTER 6

ISOLATION AND IDENTIFICATION OF COMPOUNDS FROM *PSYCHOTRIA CAPENSIS* AND *GARDENIA BRIGHAMII*

Isolation and identification of compounds from *Psychotria capensis* and *Gardenia brighamii*

Abstract

Based on the results of the previous experiments (bioautography and microtitre plate doubling dilution method), the efforts were directed towards the isolation and identification of compounds from *Psychotria capensis* and *Gardenia brighamii* leaf extracts, which could be responsible for the reported bioactivities such as, antimicrobial, antiviral and antifungal activity. Routine chromatographic techniques, silica gel and sephadex column as well as thin layer chromatography (TLC) were used for isolation and purification while nuclear magnetic resonance spectroscopy (NMR) was used for compound identification. Two compounds were isolated from *P. capensis*, namely β-sitosterol and a carotenoid derivative and from G. *brighamii* one unknown compound was isolated.

Keywords: isolation; compounds; *Psychotria capensis*; *Gardenia brighamii*.

6. 1. Introduction

Plant extracts are habitually composite mixtures containing hundreds or thousands of diverse constituents (Hamburger and Hostettmann, 1991). The division and purpose of the active mechanism in medicinal plant extracts make available solutions in studying their pharmacological, pharmacokinetic and toxicological mechanisms (Sun and Sheng, 1998). The natural products in general and medicinal plants in particular, are chemical substances with potential therapeutic applicability (Elisabetsky, 1986). There are intense needs for novel antifungal and antibacterial compounds applicable to food and feed preservatives. These compounds must have high activity against fungi and bacteria and low biotoxicity to human beings and animals (Lida *et al*., 1999).

Chromatographic techniques such as thin layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC) are frequently used for the analysis of

medicinal plant medicines (Wen *et al*., 2004). Column chromatography is one of the most frequently used techniques in the isolation of natural plant constituents. In principle, plant constituents are distributed between the solid phase (for example silica gel or sephadex) and mobile phase, which comprises an eluting solvent. In silica gels the separation of compounds from each other in an extract is based on number of factors including the polarity of compounds, hence compounds are eluted from the column with solvent systems of differing polarity (Quincy *et al*., 1964). Silica gels constitute polar ends which interact strongly with polar compounds and they are eluted later from the column. (Hanai, 2003). In Sephadex, the separation of constituents in an extract depends on the size of the molecules and the solvents systems (McCoy and Carter, 1968).

Psychotria capensis and *G. brighamii* crude leaf extracts exhibited good antifungal activity against the *Fusarium* species tested (Chapter 5). The purpose of this chapter was to isolate and identify compounds from *P. capensis* and *G. brighamii* leaf extracts. If succesfull, these compounds could be inverstigated further to for their antifungal activity. Due to the limited supply of plant material of *M. citrifolia*, this plant was not investigated for potential compounds.

6. 2. Materials and methods

6. 2. 1. Plant material

Approximately 1.0 kg *P. capensis* and 2.0 kg of *G. brighamii* leaves were collected during November 2007 from Manie van der Schijff Botanical garden at the University of Pretoria.

6. 2. 2. Preparation of extracts

Plant extracts exist as mixtures with many different compounds. It is therefore of critical importance to find a suitable extractant for the special work. For the current study, the dried plant material (50 g) was ground to fine powder. Each plant material was extracted with ethanol overnight at room temperature. The solvent was then removed and replaced with fresh ethanol. This procedure was repeated twice for each

plant material. Extracts were filtered and concentrated to dryness using a rotary evaporator (Buchi) under reduced pressure.

6. 2. 3. Isolation from *Psychotria capensis*

Thirty five grams of extract from *P.capensis* was applied to a silica gel column (Fig. 6.4) using a hexane: ethyl acetate mixture of increasing polarity and then methanol. Thirty-six fractions were collected and combined into 16 majors fractions based on the TLC profile. Fraction 9 was chromatographed on Sephadex column using methanol as eluent, and three major fractions were obtained. From those three fractions two pure compounds were isolated. The isolation procedure is represented in Fig. 6.1.

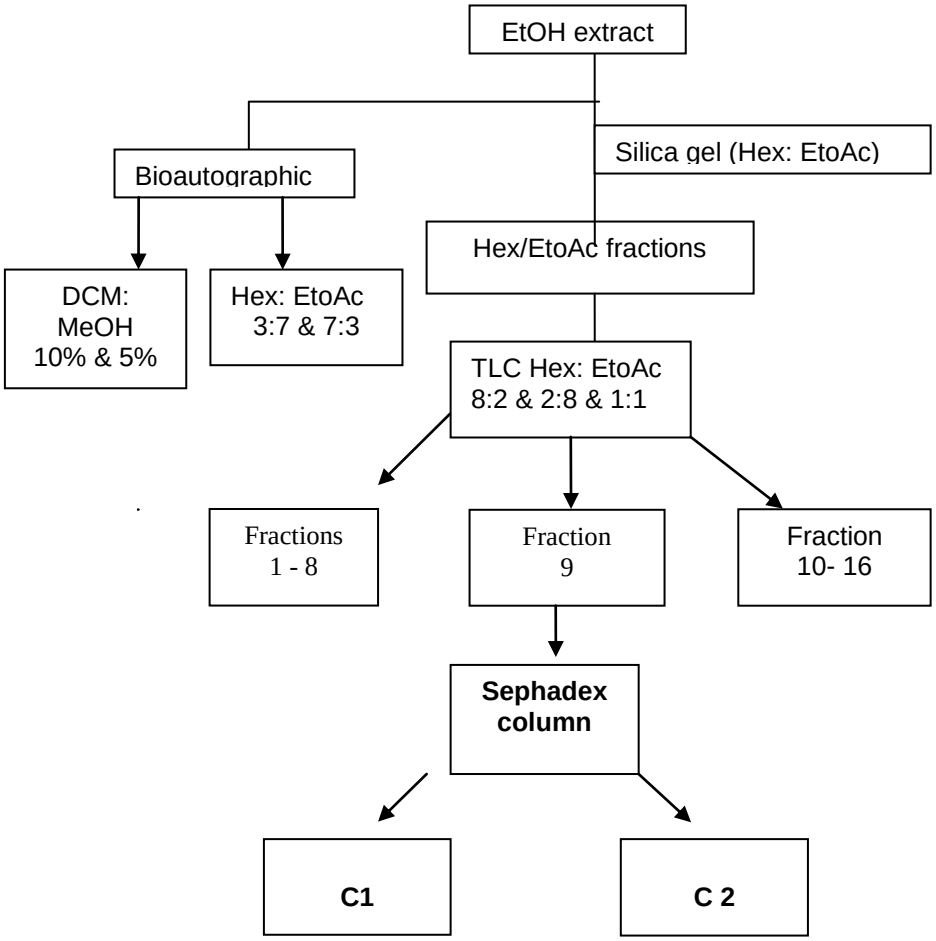

Figure 6. 1. Isolation scheme of *P. capensis* compounds

The preliminary isolation of active constituents from the ethanol extract using silica gel column chromatography resulted in 16 major fractions. These fractions were reconstituted in an appropriate organic solvent, and developed by TLC and sprayed with vanillin spray reagent (Fig. 6.2 and 6.3). These TLC analyses enabled the pooling of the fractions with containing similar constituents and also make it easy to target the major constituents.

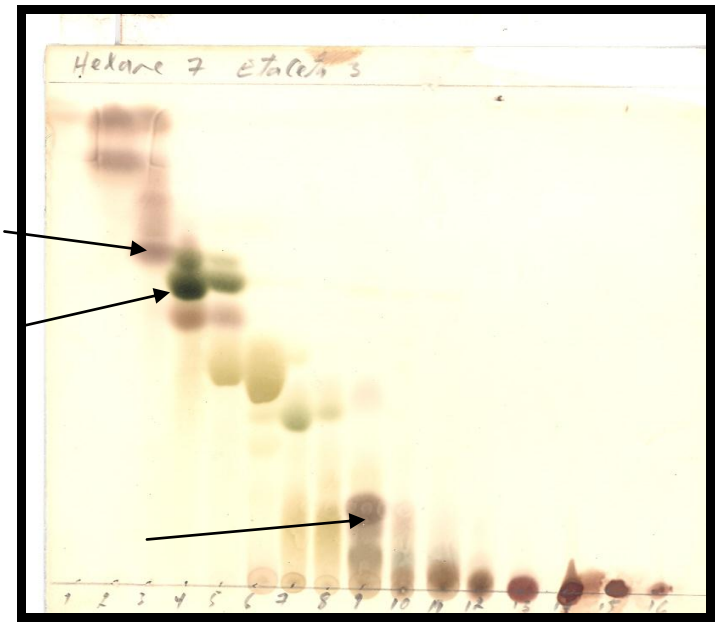

Figure 6. 2. TLC plate with 16 fractions developed in hexane: ethyl acetate (7:3) and sprayed with vanillin. Arrows indicate compounds of interest.

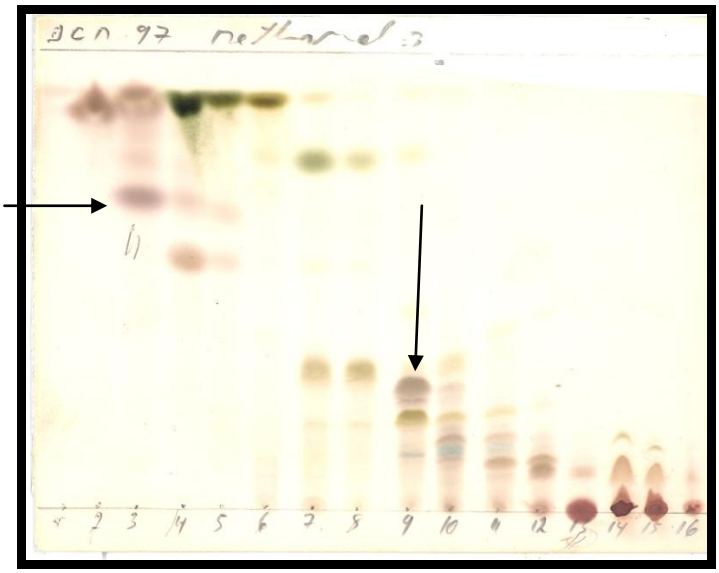

Figure 6. 3. TLC plate with 16 fractions developed in dichloromethane: methanol (97:3) and sprayed with vanillin. Arrows indicate compounds of interest.

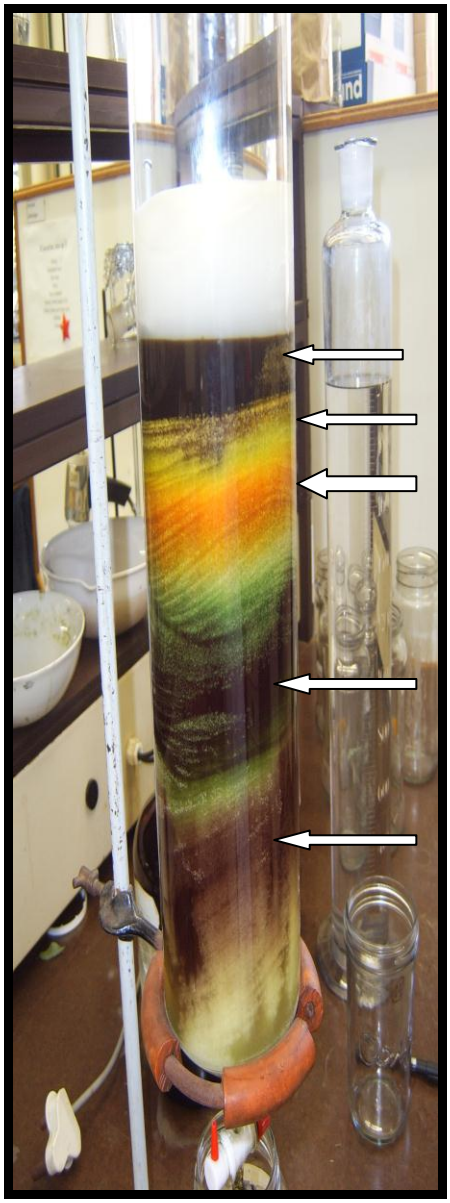

Figure 6. 4. Silica gel column chromatography of an ethanolic extract of *P. capensis.* The arrows indicate the separation of different phases of the extract with different solvent systems in the column.

6. 2. 4. Isolation from *Gardenia brighamii*

A 120 g of extract was applied to a silica gel column (Fig. 6.6) using hexane: ethyl acetate mixture of increasing polarity, and then methanol as solvents. Forty-two fractions (Fig. 6.5) were collected for analysis. The isolation procedure is represented in Fig. 6.7.

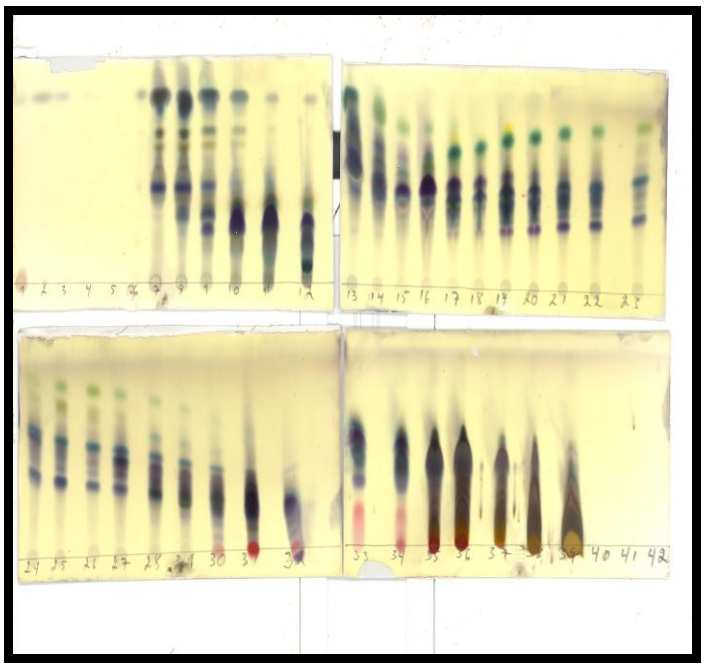

Figure 6. 5. TLC plate with 42 fractions developed in dichloromethane: methanol (99.05:05) and sprayed with vanillin dissolved in H_2SO_4

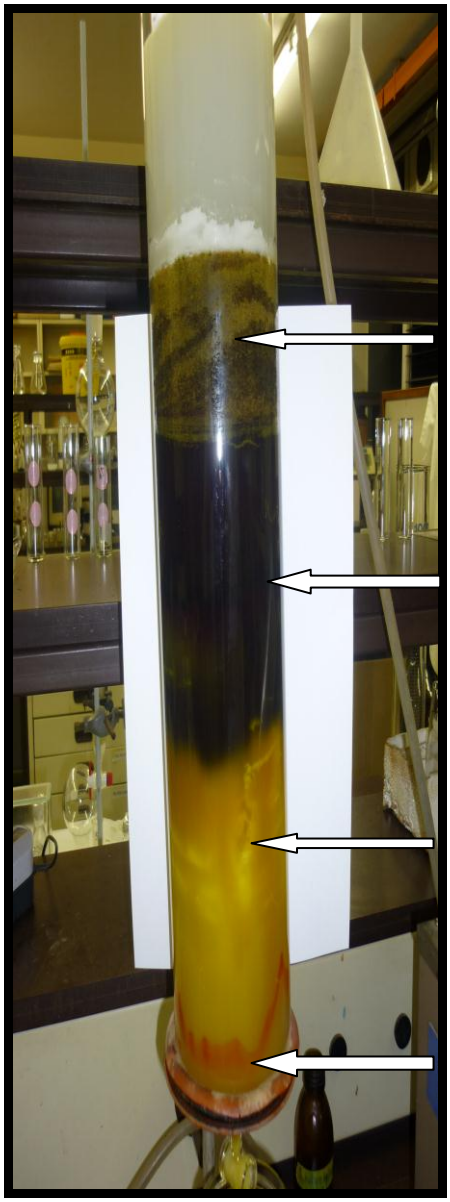

Figure 6. 6. Silica gel column chromatography of a methanolic extract of *G. brighamii*. The arrows indicate the separation of different phases of the extract with different solvent systems in the column.

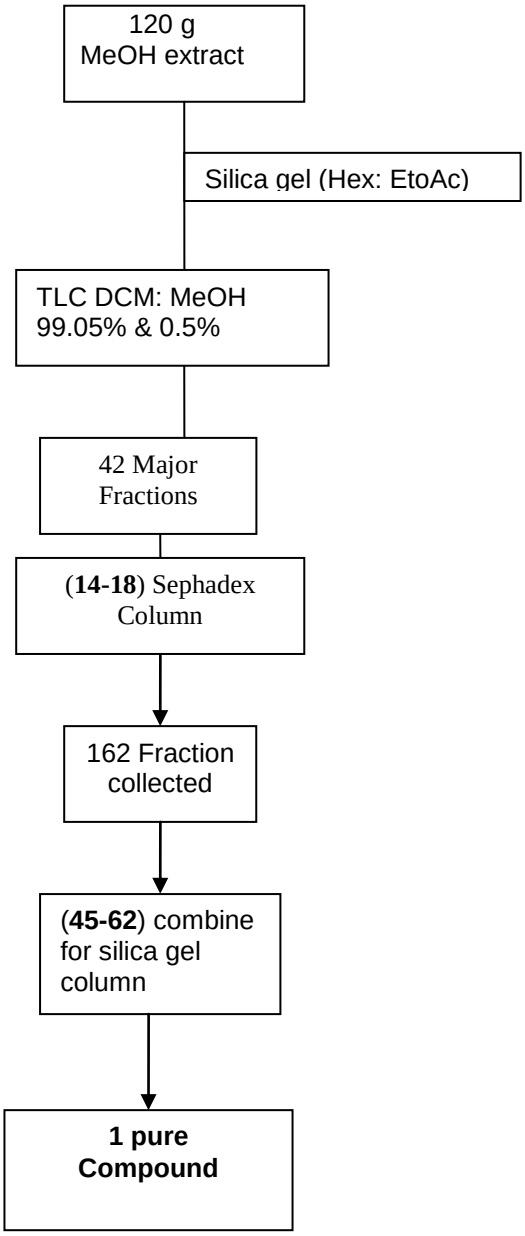

Figure 6. 7. Isolation scheme of *G. brighamii* compounds

6. 3. Results and discussion

The ethanolic extract of *P. capensis* was chromatographed on a silica gel column which resulted in 36 fractions. These fractions were developed by bioautographic TLC and sprayed with vanillin. Of these fractions obtained using solvents of different polarity, two pure compounds were obtained (Fig. 6.8) and the elucidation of chemical structures involved using a combination of different techniques including nuclear magnetic resonance spectroscopy (NMR).

The compound was purified as needle crystals from the non-polar fraction of *P. capensis* extract and identified on the basis of ^{1}H and ^{13}C NMR data. The ^{1}H NMR data showed signals at 5.39 (^{1}H, H-6), 3.56 (^{1}H, H -3), and six methyl signals at 1.05 (s, H-Me - 19, 0.96 (d, J = 6.5 Hz , Me- 21), 0.89 (t, J = 7.4 Hz, Me - 29), 0.87 (d, J = 6.7 Hz, Me - 26), 0.85 (d, J = 6.7 Hz, Me - 27), 0.72 (s, Me - 8) and showed ^{13}C NMR signals at 141.2 (s, C - 5), 122.1 (d, C - 6), and 72.2 (d, C - 3) in addition to 26 signals of the rest of the skeleton. The data obtained correlated with those published by Moghaddam *et al*. (2007) and Priyanka *et al.* (2009) and the compound was identified as β–sitosterol (Fig. 6.9) (see Appendix 8.2).

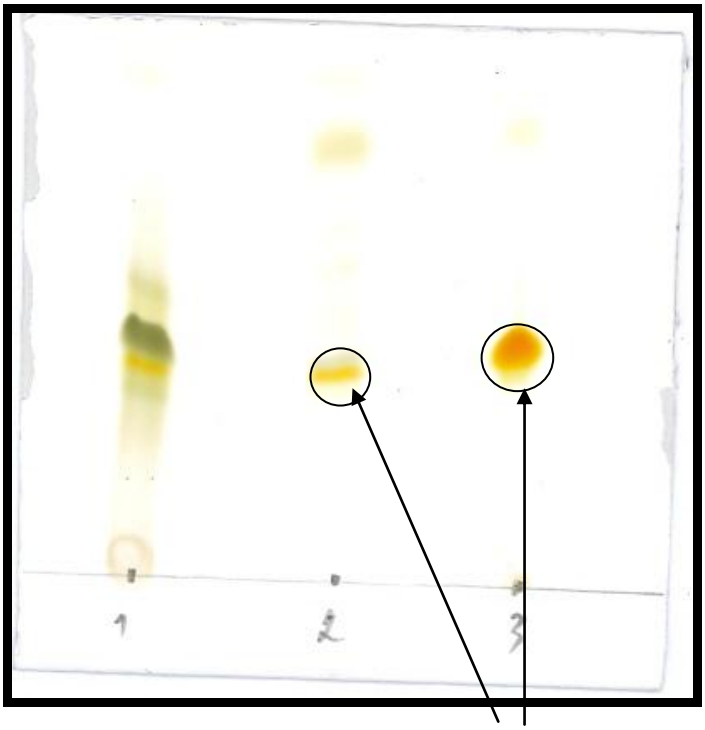

Figure 6. 8. Chromatogram showing the two pure compounds (1, 2) isolated from *P. capensis*. Solvent system: hexane: ethyl acetate (7:3)

The compound β-sitosterol (Fig. 6.9), as confirmed using NMR analysis is one of hundreds of plant-derived "sterol" compounds (including sterols and sterolins), that have structural similarity to the cholesterol made in our bodies (Bouic *et al.*, 1996). The most prevalent phytosterols in the diet are β-sitosterol, campesterol and stigmasterol (Pegel *et al.*, 1997). The same authors reported that β-sitosterol is a natural micro-nutrient plant fat which is found in the cells and membranes of all oil producing plants, fruits, vegetable, grains, seeds , roots and trees.

These compounds are ubiquitous throughout the plant kingdom, and they appear to have important immunomodulatory and anti-inflammatory activities in human and animal physiology (Pegel *et al.*, 1997). Nutrition researchers at the University of Buffalo (USA) have found in a study that β-sitosterol reduced the number of breast-cancer cells grown in a laboratory setting by 66 % within 5 days, compared to controls. β-sitosterol has an amazing array of scientifically acknowledged benefits for key areas of health. Health benefits which can all be supported by published studies in international journals show β-sitosterol to be: antihyperglycemic, antidiabetic, antibacterial, antimicrobial, improve blood parameters, anti-inflammatory, anti-pyretic (reducing fever), beneficial for the uterus, anti-ulcer (Pegel *et al.*, 1997).

Furthermore, Aderiye (1989) found that the compound β-sitosterol extracted from yam peel (*Ipomoea batatas* L.) exhibited antifungal activity on TLC bioassay and also confirmed the inhibitory effect of the compound β-sitosterol on spore germination and germ-tube elongation of *Aspergillus niger* and *Botryodiplodia theobromae*.

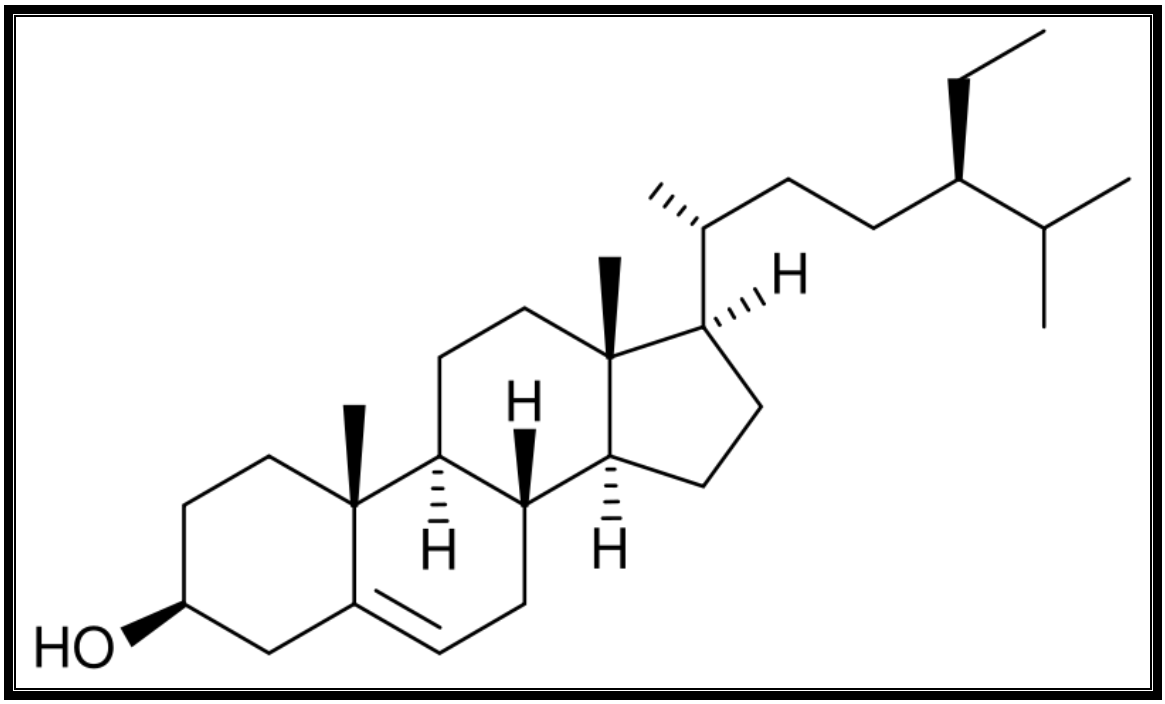

Figure 6. 9. Chemical structure of β-sitosterol

The partial structure of the carotenoid derivative (Fig. 6.10) was confirmed using NMR analysis (see Appendix 8.2). Carotenoids are the natural compounds that were discovered during the 19th century. In 1831, Wachen Roder H. proposed the term "carotene" for the hydrocarbon pigment he had crystallized from carrot (*Daucus carota* L.) roots (Sandmann, 1994). Some carotenoids have shown interesting biological properties, such as anticarcinogenetic effects, anti-inflammatory effects and radical scavenging activity (Maeda *et al*., 2005).

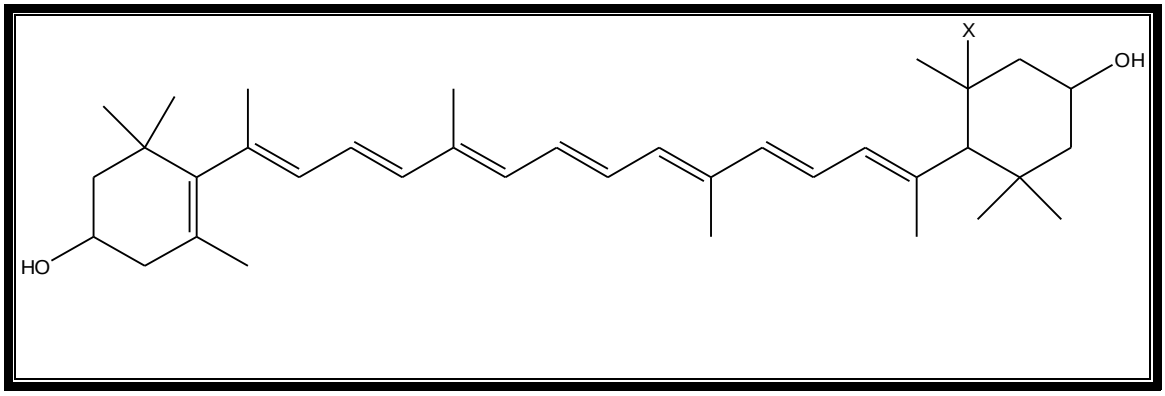

Figure 6. 10. Proposed chemical structure of the carotenoid derivative compound

Carotenoids are antioxidants with considerable pharmaceutical potential. More than 600 carotenoid structures are known but their availability is limited owing to practical difficulties associated with chemical synthesis and isolation from microorganisms or plant tissue (Sandmann *et al*., 1999). The compound C14-carotenoid derivative (Fig 6.11) was isolated from the coat of *Bixa orellana* L. by chromatography (CC, TLC, HPLC) and the structure elucidated by means of spectroscopy (UV-visible, MS, 1H and 13 C NMR) (Mercadante *et al*., 1997). When comparing the ^{1}H and ^{13}C NMR data between the proposed chemical structure of the carotenoid derivative in Fig. 6.10 and the one identified by Mercadante *et al*. (1997) (Fig. 6.11), they look similar. Further work is required to isolate and identify of the final chemical structure of the carotenoid derivative from *P.capensis* and the compound isolated from *G. brighamii*. Due to insufficient yield, β-sitosterol and the carotenoid derivative were not tested for antifungal activity and therefore further work should be carried out to ascertain whether the antifungal nature of the crude extracts could be ascribed to the compounds isolated.

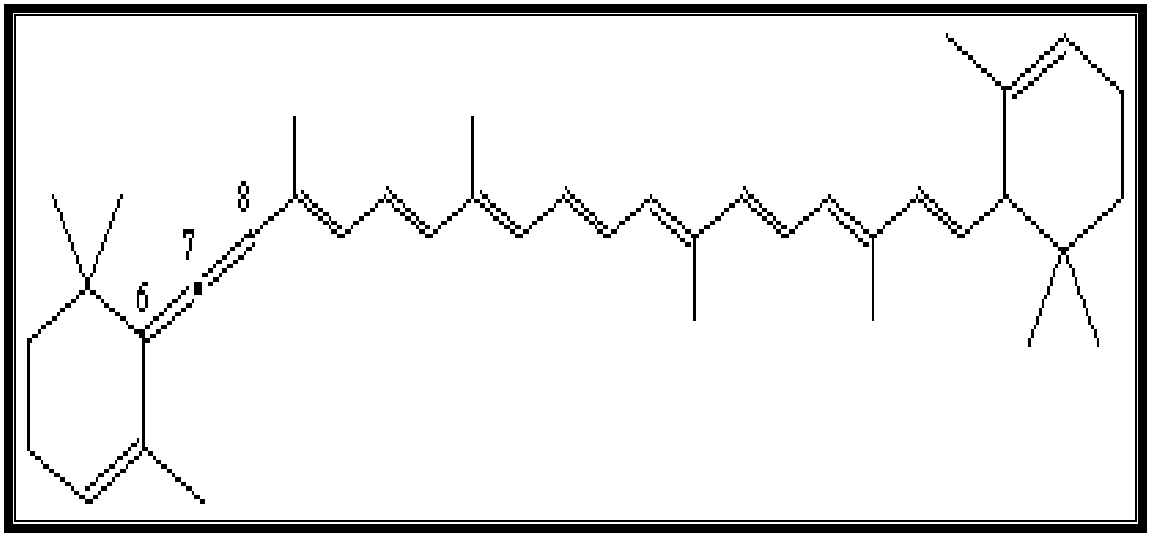

Figure 6. 11. Chemical structure of a carotenoid derivative (Mercadante *et al*., 1997)

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CHAPTER 7

GENERAL DISCUSSION

General discussion

Cowpea [*Vigna unguiculata* (L.) Walp.] is the most important source of vegetable protein in the daily diets of rural and urban communities and mainly used in the dry savannas of West and Central Africa and parts of East as well as Southern Africa (Singh and Rachie, 1985). It is also a source of rich minerals and vitamins in the daily diets and thus it positively impacts on the health of humans. The addition of even a small amount of cowpea ensures nutritional balance of the diet by enhancing the protein quality through synergistic effect of high protein and high lysine from cowpea and high methionine and high energy from the starchy foods (Singh *et al*., 1985). Furthermore, cowpea may be used also for improving soil fertility and parasitic weed control (Nielsen *et al*., 1997)

These legumes in many tropical and subtropical countries suffer great losses in storage due to diseases and attack by fungi as well as insect pests. Infections cause deterioration of the grain, poor germinating ability and make it unfit for human or animal consumption (Singh *et al*., 1997). Certain fungi that colonise crops in the field and post-harvest storage can produce mycotoxins, which pose a potential threat to human and animal health.

In this study, five cowpea seed samples were obtained from a local market in a rural community of the Mpumalanga province and were subjected to mycoflora analysis and fumonisin detection. The results from the mycoflora analysis showed that *Fusarium* species including *F. oxysporum*, *F. solani*, *F. subglutinans*, *F. verticillioides* and *F. proliferatum* were found associated with cowpea seed. Other fungal species including *Aspergillus flavus* and *A. niger* as well as *Penicillium* spp, *Monascus* spp, *Phoma* spp, *Rhizopus* spp and *Alternaria alternata* were also recorded. These results are in agreement with the findings of Kritzinger *et al*. (2003), Rodrigues and Menezes (2005), and Houssou *et al*. (2009) who found similar mycoflora to be associated with cowpea seed.

Among the species of *Fusarium* detected in cowpea seeds some of the species are known to produce fumonisin mycotoxins for example *F. verticillioides* and *F.*

proliferatum. The contamination of feedstuffs with fumonisins can pose a serious threat to animal and human health (Gelderblom *et al*., 1993). Furthermore, they are also known to be phytotoxic (Lamprecht *et al*., 1994). All five samples analysed in this study revealed the presence of fumonisin B analogues at concentrations ranging from 6.2 to 59 µg/g. Fumonisins have previously been detected in cowpea seed samples from Benin and South Africa (Kritzinger *et al*., 2003).

In this study, some *Fusarium* species including *F. verticillioides, F. proliferatum, F. oxysporum, F. subglutinans* and *F. solani* isolated from cowpea seed were grown on maize patty media and analysed for fumonisin production. The results revealed that *F. subglutinans* and *F. oxysporum* produced fumonisin B analogues with levels ranging between 1.1 - 4.3 µg/g and 3.47 - 31.66 µg/g, respectively. Though achieved with different crops, Ramirez *et al.* (1996) recorded the natural occurrence of fumonisins and their correlation to *Fusarium* contamination in maize hybrids grown in Argentina. Our results confirm the natural infection of fumonisin B analogues on cowpea seed and show the potential of *F. oxysporum* and *F. subglutinans* isolated from cowpea seed to produce fumonisin B mycotoxins. This is in contrast to the first report of fumonisins associated with cowpea seed, where Kritzinger *et al.* (2003) reported that *F. proliferatum* was responsible for the fumonisin production.

Furtherwork is needed to identify the specific fumonisin B analogues produced by *Fusarium* species isolated in this study, and to confirm the identification of *F. oxysporum* as well as *F. subglutinans* as well as their fumonisin producing ability.

Since it is known that fumonisin B analogues are found in cowpea seed, it was necessary to investigate the phytotoxic effects of fumonisin B_1 (FB_1) on cowpea seed. Cowpea seeds were treated with purified FB_1 at different concentrations (10, 25, 50 and 100 µg/ml). The results indicated that all the FB_1 concentrations significantly decreased seed germination and the highest concentrations (50 and 100 µg/ml) decreased root and shoot elongation. Similar results were found by Kritzinger *et al*. in (2006). The results from the emergence in the greenhouse showed that seeds treated with fumonisin B_1 at concentrations of 25, 50, and 100 µg/ml significantly decreased germination of the

seeds and also retarded the elongation of the shoots. From these results it is concluded that fumonisin B_1 has a significant detrimental effect on the emergence of the seedlings. In this study, in an attempt to understand the toxic effect of FB_1 on the cowpea seed germination and seedlings, the embryonic and cotyledon tissues of the seed were treated with different concentration of FB_1 (10, 25, 50, and 100 µg/ml) and the ultrastructural changes caused by this toxin were studied by using transmission electron microscopy (TEM). The negative effects of FB_1 were observed especially for the highest concentrations (50 and 100 µg/ml) with discolouration and irregulation size of lipids, vacuoles, and cell wall. Hence, it can be advantageous to determine the concentration of fumonisins in cowpea seed samples before sowing.

Due to the concerns about insecticide and fungicide residue on grains, and threats to human health and the environment, awareness of risks has increased interest in finding safer insecticides or alternative stored-product protectants to replace synthetic chemicals (Silver, 1994). One such alternative is the use of natural plant products that have fungitoxic activity. In this study, three Rubiaceae species including, *Psychotria capensis*, *Morinda citrifolia* and *Gardenia brighamii* were selected for antifungal activity against five *Fusarium* species. The results showed that the acetone and methanol leaf extracts from *P. capensis* were the most active against *F. proliferatum* and *F. verticillioides* and the dichloromethane extract from all three plants were not active against all tested *Fusarium* species. The serial microtitre plate doubling dilution of the three Rubiaceae species investigated showed inhibitory activity against all *Fusarium* species tested, with the MIC ranging from 1.5 to 6.2 mg/ml. The occurrence of clear zones on TLC plates (bioautography) confirmed the efficacy of plant extracts against *Fusarium* species tested.

Solvents of different polarity were used to separate the compounds responsible for the inhibitory activity of *P. capensis* and *G. brighamii*. Two pure compounds were isolated from *P. capensis*, namely β-sitosterol and a carotenoid derivative, and from *G. brighamii*, a triterpene derivative, which were identified using a combination of different techniques including nuclear magnetic resonance spectroscopy (NMR). The triterpene derivative and the compound from *G. brighamii* will undergo further investigation to determine their chemical structures and identify them. Further work

will also include antifungal bioassays of the three compounds isolated in this investigation to determine whether the compounds indeed show antifungal activity.

The results of this study confirm that some naturally occurring botanical materials have positive deterrence against *Fusarium* species. Of all the materials tested, the extracts from *P. capensis* demonstrated the strongest activity against *Fusarium* species. The bioactivity of *P. capensis* was established by Jayasinghe *et al*. (2002) and Badenhorst (2004). The former reported on the antimalaria activity, while the latter indicated the anti-emetic and antimicrobial activity of *Psychotria* spp. *Morinda citrifolia* and *G. brighamii* also reduced the development of *Fusarium* species. The use of plant material to control pest and diseases in stored products and or crops may become an important supplement or alternative tool to use of synthetic chemicals.

The successful isolation of bioactive compounds from indigenous medicinal plants will validate indigenous knowledge adding value to plants and support plant conservation and knowledge preservation. It may also contribute to research and development in the production of new pesticides for the treatment of legume seed and other grains during storage from various infectious. The results of this study suggest promising potential for the use of *P. capensis*, *M. citrifolia*, and *G. brighamii* against *Fusarium* species. These materials are safe, less expensive than synthetic, and can be easily used by farmers and agricultural communities. Also, they are environmental friendly, in such way they can lead to lower pollution from chemicals and contribute to sustainable development.

However, some limitations should be mentioned. Although, the leaf extracts from the three Rubiaceae species exhibited antifungal activity, it should be noted that the present investigation was limited only to crude extracts. The evaluation of the cytotoxicity of these extracts also requires further research. In continuation of this work, it is needed that the chemical structures of the isolated compounds (the carotenoid derivative from *P. capensis* and the compound from *G. brighamii*) be identified. As mentioned in the General Introduction (Chapter 1), this study reports on two different aspects. In the first part of the dissertation it was reported that certain *Fusarium* species were isolated from cowpea seed samples. Some of these species were tested for their potential to produce fumonisin B mycotoxins and potential fumonisin-producing *Fusarium* species were identified from cowpea seeds. With this information in mind and the fact that most

Fusarium species are associated with serious diseases on economically important crops including cowpea, the second part of the dissertation investigated the possible use of plant extracts, as an alternative to chemicals, to prevent and / or control *Fusarium* species. The first section of the antifungal investigation revealed that certain crude extracts of the three Rubiaceae species showed slight inhibitory activity against the *Fusarium* species tested. In the second section an attempt was then made to isolate compounds from two of these species. Three compounds (two identified and one unknown) were isolated but further studies will need to be carried out to determine the antifungal activity of the isolated compounds.

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CHAPTER 8

APPENDIX

Appendix

8. 1. Preparation of Virikop (Fungicide)

Virikop is a broad-spectrum antifungal produced by EFEKTO. The active ingredient is copper oxychloride. The stock solution was obtained mixed 1 mg of Virikop added 100 µl of DMSO- 100% and 900 µl of sterized distilled water to make 1.0 mg/ml.

8. 2. Analysis NMR spectra of the Compounds

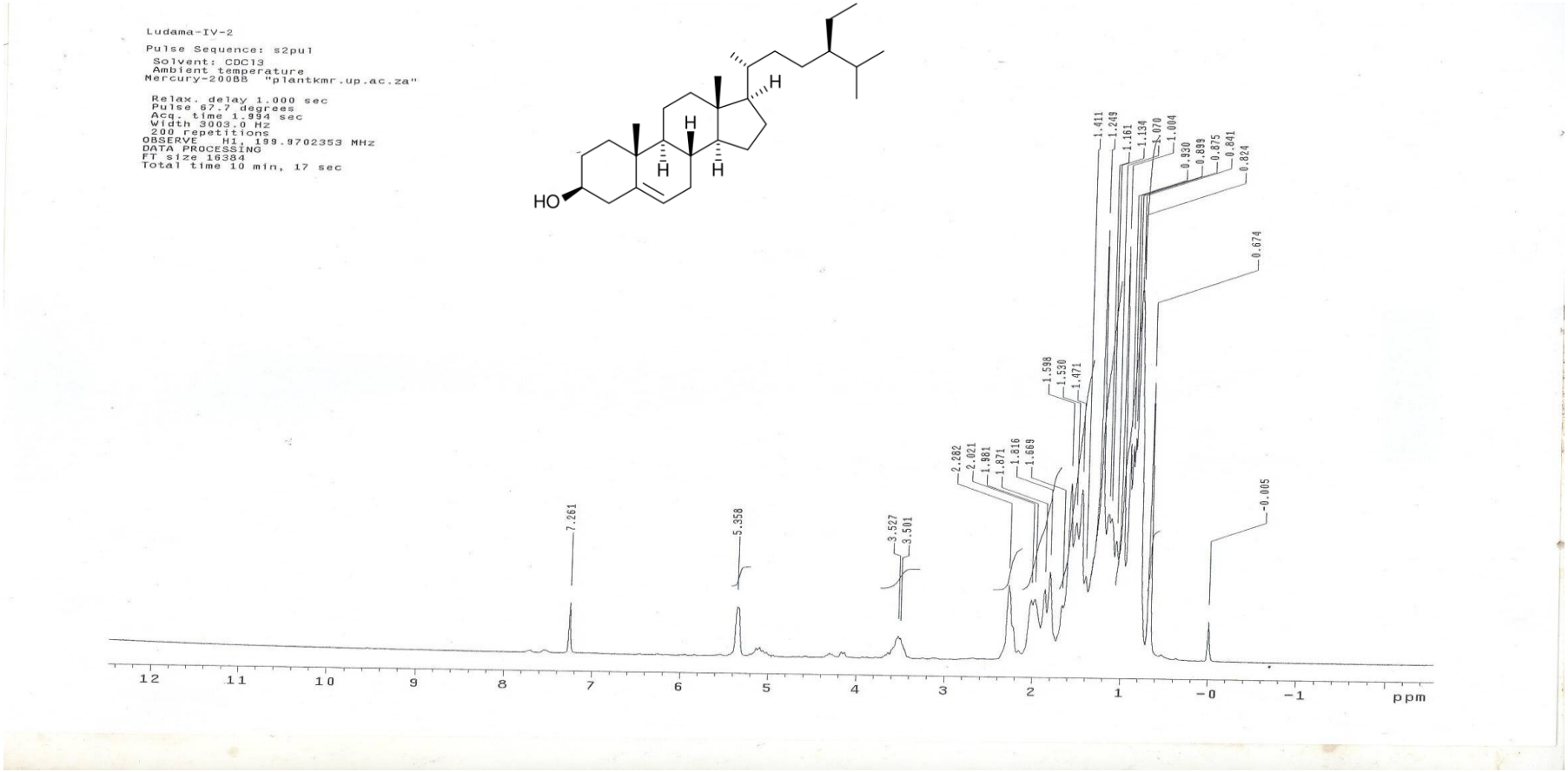

Figure 8. 1. ^{1}H NMR spectrum of compound 1 isolated from *P. capensis*

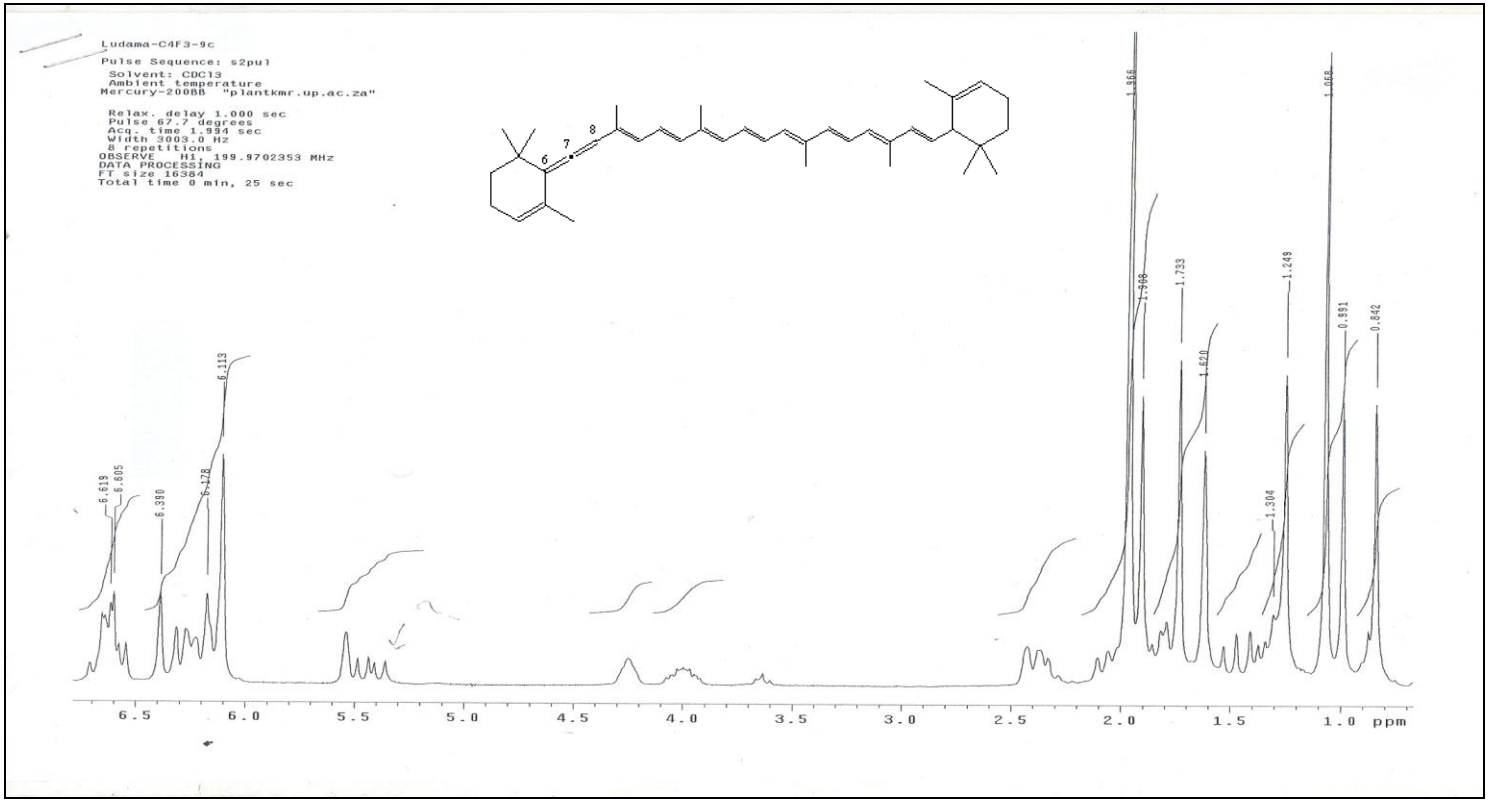

Figure 8. 2. ^{1}H-**NMR spectrum of compound 2 isolated from *P. capensis***

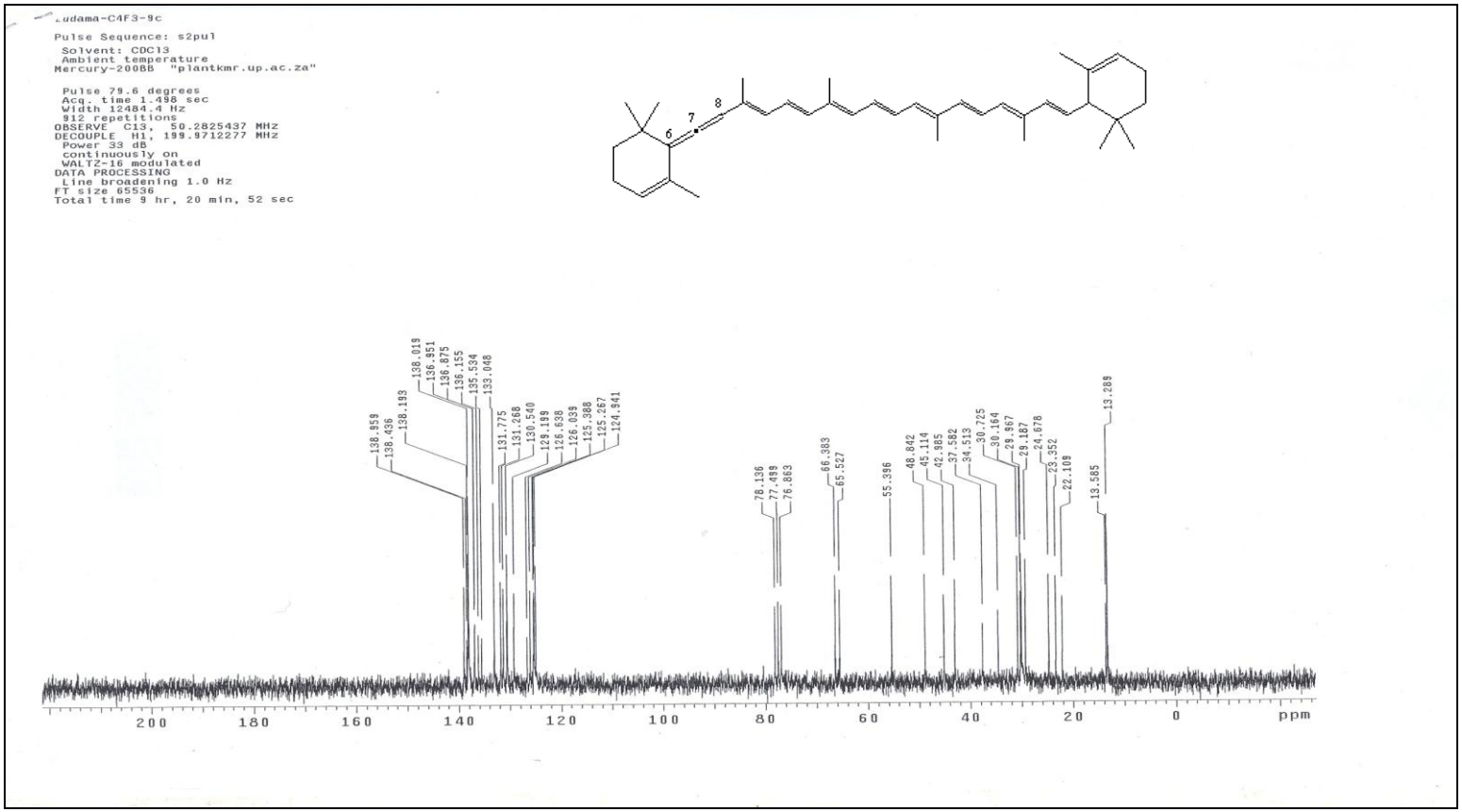

Figure 8. 3. ^{13}C NMR spectrum of compound 2 isolated from *P. capensis*

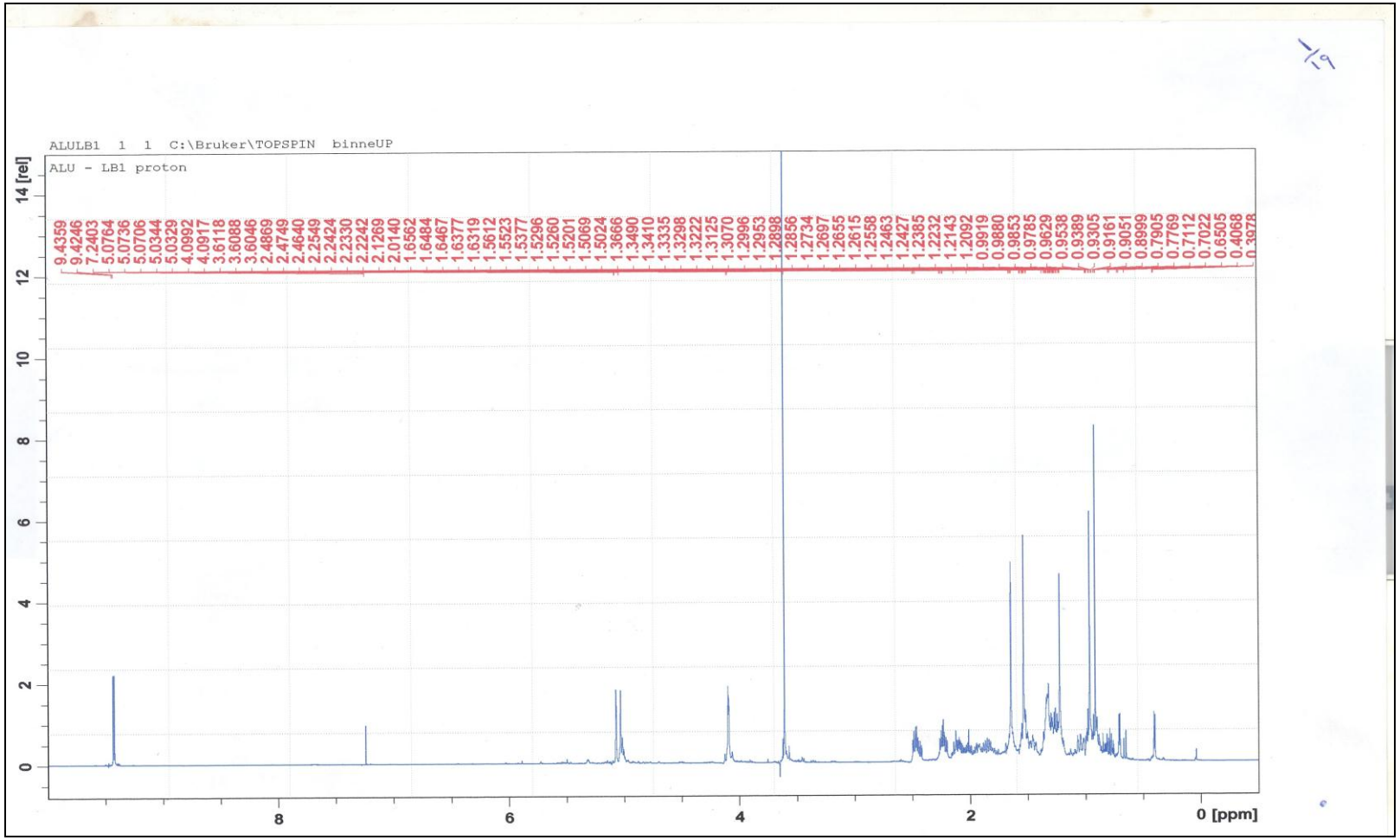

Figure 8. 4. ^{1}H-**NMR spectrum of compound isolated from *G. brighamii***

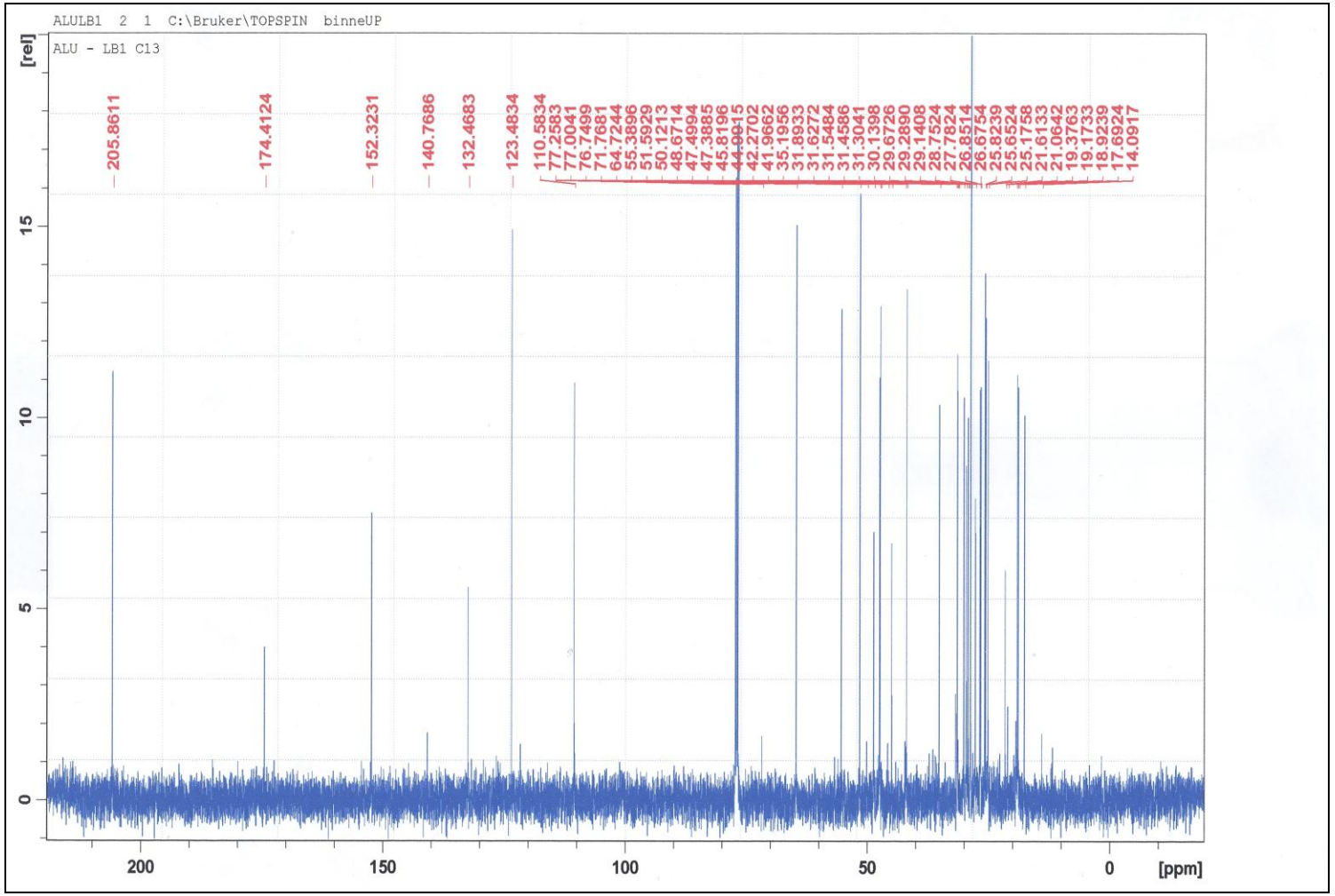

Figure 8. 5. ^{13}C NMR spectrum of compound isolated from *G. brighamii*