The antifungal activity of an aqueous *Tulbaghia violacea* plant extract against *Aspergillus flavus*

Xoliswa Vuyokazi Belewa

Submitted in fulfillment of the requirements for the degree of

Philosophiae Doctor

in the Faculty of Science at the

Nelson Mandela Metropolitan University

January 2015

Promoters: Dr. B.M. Somai

Prof. C. Frost

Table of Contents

Declaration

I, Xoliswa Vuyokazi Belewa, declare that this thesis represents my own unaided work and where necessary, due credit has been given. This thesis is in fulfilment of the requirements for the degree, *Philosophiae Doctor*, in the Faculty of Science at Nelson Mandela Metropolitan University, as per rule 304.5.3

Abstract

Fungal infections have been on the rise for many years. The emergence of opportunistic infections in immunocompromised patients has led to an increase in fungal infections, with *Aspergillus* being one of the causative agents. The emergence of resistance of fungal species towards antifungal agents and toxicity of these agents has led to a need for a search for alternative means of treatment. Medicinal plants have the potential for use as chemotherapeutic agents; however their mechanism of action is not fully understood.

Aqueous extracts of *Tulbaghia violacea* have been shown to have antifungal activity against a variety of microorganisms; however their mechanism of action has not been investigated. Analysis of the aqueous plant extract with TLC, using various solvent systems (EMW and HEA), showed that the plant extract had a mixture of polar and non-polar compounds. The active compounds of the plant extract was identified by an agar overlay technique and isolated from the other compounds by preparative TLC. Purification of the antifungal compounds isolated with EMW and HEA was done with RP-HPLC using three different columns. The RP-HPLC of EMW8 with a C18 column, showed a mixture of compounds within the fraction, which could be breakdown products of some of the compounds of the plant extract. The antifungal activity of the resolved peaks could not be established due to the low recovery of the peaks after fractionation. Analysis of fraction HEA1 with RP-HPLC, resulted in a major peak eluting at 3.7 min, and its antifungal activity was confirmed with the agar overlay assay. However, rechromatography of the antifungal peak showed a mixture of compounds within the peak, with more peaks being resolved. The lower yield of these peaks after fractionation did not make it possible to determine the antifungal activity of the peaks. PFP-HPLC analysis of HEA1 resulted in the identification of the antifungal peak with a similar retention time to that identified with C18 column. Spectrum analysis of this peak, confirmed its relative purity and rechromatography of the peak after fractionation showed that the peak contained a single compound. However, the identification and structure elucidation of HEA1 by NMR proved difficult as there were extremely low levels of the compound present. Comparison of HEA1 with known active compounds previously isolated from *T. violacea* and *T. alliacea* by predictive NMR analysis showed that HEA1 was different from the previously isolated ones, however a large amount of sample was still required to obtain a more conclusive result.

Phytochemical analysis of both HEA1 and the crude plant extract showed the presence of phenolics, tannins and saponins. Saponins were the predominant secondary metabolites and were mostly abundant in the plant extract and to a lesser extent in the active compound. Steroidal saponins, tannins and phenolics were also detected in the plant extract, but only the phenolics were detected in the active compound. The results of the phytochemical analysis showed that those compounds that were not present in the active compound could be removed from the crude extract during the TLC purification process. Investigation on the mechanism of action of the crude plant extract on the sterol production by *A. flavus* showed that the plant extract affected ergosterol biosynthesis by causing an accumulation of oxidosqualene in the ergosterol biosynthetic pathway resulting in a decline in ergosterol production. An oscillatory response in lanosterol production was observed in the presence of the plant extract, which may be an adaptation mechanism of *A. flavus* to unfavourable conditions and compensation for the loss of enzyme activity which may have occurred as a result of the accumulation of oxidosqualene. The antifungal activity of the plant extract on ergosterol production by *A. flavus* may also be due to saponins which target the cell membrane and ergosterol production in fungi.

The effect of the plant extract on the fungal cell wall of *A. flavus* also showed that the plant extract caused a decline in β-(1, 3) glucan production by inhibiting β-glucan synthase. The plant extract also affected the chitin synthesis pathway of *A. flavus*, by causing a decline in chitin production, which was due to the inhibition of chitin synthase. Investigation of chitinase production using 4MU substrates showed that the plant extract caused an accumulation of chitobioses, by activating chitobiosidases and endochitinases. A decline in N-acetylglucosaminidase activity in the presence of the plant extract was observed and this prevented the formation of N-acetylglucosamine. The accumulation of chitobiosidase and endochitinase may be as a result of autolysis that may be triggered by *A. flavus* as a survival mechanism in the presence of the plant extract and as a compensatory mechanism for the loss of β-glucans and chitin.

The antifungal effect of the plant extract on various components of the cell wall of *A. flavus*, makes *T. violacea* aqueous plant extract an ideal chemotherapeutic agent against both human and plant pathogens of *Aspergillus*. The broad spectrum of antifungal activity of *T. violacea* against

A. flavus also eliminates any chances of the fungus developing resistance towards it and would make it a candidate for use as a potential antifungal agent. Further identification and possible chemical synthesis is needed to shed light on the safety and efficacy of the active compound for further development as a chemotherapeutic agent.

Acknowledgements

I would like to express my sincere thanks and gratitude to the following without which, this study would have not been possible:

Dr. Somai and Prof. Frost for their guidance, patience, assistance and encouragement throughout the study and allowing me to develop my level of critical thinking.

My friends and family for always encouraging me and keeping me sane and grounded throughout this period

My beloved parents and brother for your continual love, support, encouragement and guidance. Thank you for your words of encouragement in times when I felt like giving up, keeping me grounded and being the wind beneath my wings.

My fellow lab members (CRH and Mitradev Pattoo) for the support and encouragement throughout the study

Technical staff of the department of Biochemistry and Microbiology for ensuring lab equipment and glassware are available and in good working condition

Prof P. Kaye (Rhodes University) for the NMR studies

NMMU, NRF and FoodBev SA for the financial support for the study

Most importantly, to the Lord Almighty for his undying and conditional love. Thank you for being a shoulder to cry on and being my refuge in those dark times, when there seem to be no light at the end of the tunnel.Without your love and support, this study would not have been possible.

List of Abbreviations

4MU	4-methylumbeliferone
4MU-$(GlcNAc)_2$	4-methylumbeliferyl-β-D-N-N-diacetylchitobioside
4MU-$(GlcNAc)_3$	4-methylumbeliferyl-β-D-N-N-N-triacetylchitotriose
4-MU-GlcNAc	4-methylumbeliferyl-N-Acetyl-β-D-glucosaminide
A. flavus	*Aspergillus flavus*
A. fumigatus	*Aspergillus fumigatus*
A. nidulans	*Aspergillus nidulans*
A. niger	*Aspergillus niger*
A. parasiticus	*Aspergillus parasiticus*
BSA	Bovine serum albumin
C. neoformans	*Cryptococcus neoformans*
C.albicans	*Candida albicans*
chi	Chitinase
chs	Chitin synthase
cms	Chitin synthase with a myosin motor-like domain
CF-FAB	Continuous flow fast atom bombardment
DTT	Dithiothreitol
EDTA	Ethylenediaminotetraacetic acid
EGTA	Ethyeleneglycol tetraacetic acid
ES	Electrospray
EMW	Ethyl acetate: methanol: water
$FeCl_3$	Ferric chloride
Folin C	Folin-Ciocalteu
FPP	Farnesyl pyrophosphate
GH	Glycoside hydrolase
GMM	Glucose minimal media
GPP	Geranyl pyrophosphate

GTP	Glucose Triphosphate
HCl	Hydrochloric acid
HEA	Hexane: ethyl acetate: acetone
INT	p-idionitrotetrazolium violet
IPP	Isopentyl diphosphate
$KHSO_4$	Potasium metabisulphite
KOH	Potassium hydroxide
MBTH	3-methyl-2-benzothiazolinone hydrozone hydrochloride hydrate
MEP	Methylerythritol phosphate
MS	Mass Spectrometry
MTT	3-[4,5–dimethylthiazol-2-yl]-2,5-diphenyltetrazolium chloride
$NaNO_2$	Sodium Nitrite
NaOH	Sodium Hydroxide
NMR	Nuclear Magnetic Resonance
PFP	Propyl flouro Pentyl
PMSF	Phenylmethylsulfonyl fluoride
Prep TLC	Preparative Thin layer chromatography
RP-HPLC	Reverse phase high performance liquid chromatography
S. cereviciae	*Saccharomyces Cereviciae*
T. violacea	*Tulbaghia violacea*
TLC	Thin layer chromatography
TMB	3, 3', 5, 5'-tetramethylbenzidine
TNZ	2, 3, 5-triphenyl-tetrazolium chloride
TSP	Thermospray
UDP	Uridine diphosphate
UDP-Glc-NAc	UDP-N-acetyl glucosamine
UV	Ultraviolet
UV-VIS	Ultra violet visible spectrum

WGA	Wheat germ agglutin
WHO	World Health Organisation
YES	Yeast extract sucrose
α	Alpha
β	Beta

List of figures

List of Tables

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Fungi are a diverse group of organisms, with over 25000 species distributed in the environment. These non-photosynthetic eukaryotes can exist as single cells (yeasts) or as multicellular forms (fungi) (Maheshwari, 2005). About 200 of these fungal species are pathogenic to humans, plants and animals, while many non-pathogenic species cause allergies in humans. In immune-compromised patients, exposure to most fungal species could be life threatening and could result in death (Krishnan *et al*., 2009).

The genus *Aspergillus* is widely distributed in the environment with at least 185 species being identified. Twenty of these species have been identified as human pathogens, which can result in invasive aspergillosis (Bennett, 2010). *Aspergillus flavus (A. flavus)* is the second most frequently isolated pathogenic species and is both a plant and a human pathogen. Its ability to tolerate high temperatures makes it one of the predominant fungal pathogens in dry, arid regions (Krishnan *et al.,* 2009). *Aspergillus flavus* and *A. parasiticus* result in major damage to crops and other food related products as they produce a mycotoxin, aflatoxin B1. Aflatoxin B1 is a highly toxic and potent hepatocarcinogenic compound and has been reported to cause human liver cancer in areas where food is often contaminated by this toxin (Bennett, 2010). The emergence of resistance to known antifungal agents and an increasing number of fungal infections, has led to a need to discover new antifungal agents and fungicides to combat the ever increasing fungal infections.

1.2 The fungal cell wall

The fungal cell wall is an important cellular structure that is crucial for the survival of the fungus. It makes up 30% of the cell's dry weight and acts as a barrier which allows the fungus to withstand and survive harsh environmental conditions (Diaz-Jiminez *et al*., 2012). The extracellular rigid layer provides shape and integrity and offers protection against osmotic pressure that is created during the formation of hyphae. The cell wall also acts as a filter for

larger molecules by allowing entry of nutrients and other macromolecules, which may be involved in cell-to-cell interaction (Diaz-Jiminez *et al.*, 2012; Perez and Ribaz, 2013). Approximately 90% of polysaccharides and 30% of glycoprotein make up the cell wall. The polysaccharides are made up of two fibrilar components, based on their ability to dissolve in an alkali solution. These are: alkali-insoluble and alkali soluble components. The alkali insoluble components consist of β-glucans, chitin and galactomannan and the alkali soluble component is made up of α-(1, 3) glucans and some galactomannan (Fig 1.1) (Latge, 2007; Latge 2010; Perez and Ribaz, 2013). The cell wall of *A. fumigatus* is made up of branched β-(1,3)/β-(1,6) glucan chains which are cross linked to chitin, galactomannan and β(1;3)/β(1;4) glucans (Gastebois *et al*., 2010).

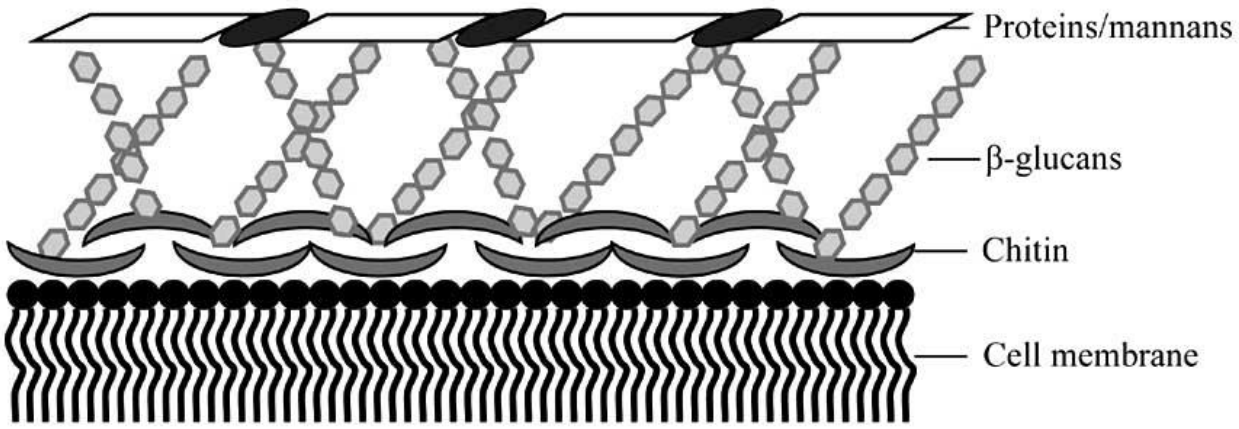


Figure 1.1: The fungal cell wall structure, showing the internal and the external layer of the cell wall with the cell membrane. The outer layer is made up of mannoproteins, while the internal layer is made up of chitin and glucans (Catalli and Kulka 2010)

The major components of the fungal cell wall (glucans, chitin, chitinases and glycoproteins) will be discussed further in subsequent sections.

1.2.1 Glucans

Glucan is the major polysaccharide of the fungal cell wall, making up 50-60% of the cell wall's dry weight. It is made up of repeating units of glucose residues which form chains by a variety of

linkages. Beta-(1, 3) glucan makes up 60-90% of the cell wall glucans, while other glucans such as β-(1, 6); β-(1, 3) (1, 4), β-(1, 6) glucan and a mixture of α glucans are also found in the cell wall (Fig 1.2). The major backbone of the cell wall is β-(1, 3) glucan linked with β (1, 6) branches. It plays a major role in cell wall structure, providing rigidity and fungal development processes (Abad *et al*., 2010; Bowman and Free, 2006). In *A. fumigatus*, β-(1, 3) glucan is synthesized by a plasma membrane bound enzyme, β-glucan synthase. This enzyme uses uridine diphosphate (UDP) and glucose as a substrate to catalyse the synthesis of glucose residues, which are linked to β-(1, 3) linkages (Bowman and Free, 2006; Gastebois *et al*., 2009). β-(1, 3) glucan is required for normal development of fungi and for the formation of the cell wall. It also serves as an attachment surface for other cell wall components and provides the cell wall with structural integrity (Cowen and Steinbach 2008).

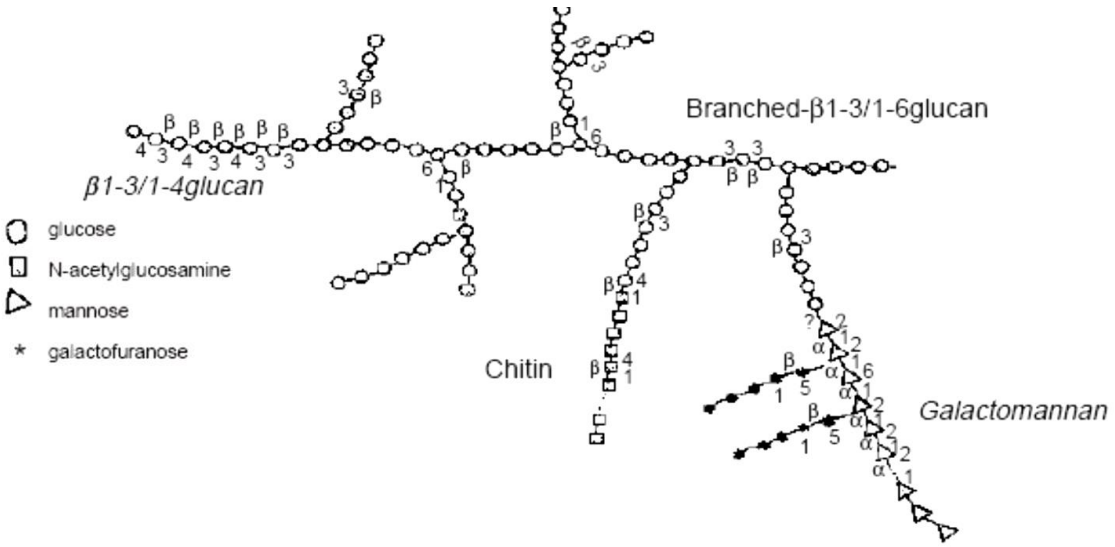


Figure 1.2: The carbohydrate components of the fungal cell wall (Bernard and Latge 2001).

1.2.2 Chitin

Chitin is long linked homopolymer of β-(1, 4)-N-acetyl glucosamine residues. It occurs as long thin microfibrils and short thick rods and has an important role in the shape and strength of the cell wall (Latge 2007; Catalli and Kulka, 2010; Jiminez-Ortigosa *et al*., 2012). In yeast, chitin

makes up 1-2% of the cell wall, while in filamentous fungi it is more than 40% (Perez and Ribaz, 2013; Rogg *et al*., 2012). Chitin synthesis occurs on the intracellular space adjacent to the plasma membrane and is regulated by chitin synthases (*Chs*). These enzymes catalyze the synthesis of N- acetyl glucosamine from UDP-N-acetyl glucosamine (UDP-GlcNAc) to a growing chain of chitin and release UDP (Fig 1.3) (Chaudhary *et al*., 2013). Chitin synthesis occurs at the sites of active growth such as the hyphal apex, the bud tip during polarized growth and the bud neck during cytokinesis (Bowman and Free 2006, Lenardon *et al*., 2010).

1.2.3 Chitin synthases

Chitin synthases consist of seven classes which are divided into two families. The first family is made up of classes I –III, while class IV-VII make up the second family of chitin synthases. The genes of class I, II and IV (*chsA, chsB* and *chsD*) are present in all fungi, while III, V, VI and VII (*chsC, chsE, csmA, csmB*) are only found in filamentous fungi, which indicates that they may regulate fungal growth (Jiminez-Ortigosa *et al*., 2012). The *chsA* gene has a small contribution in chitin formation of the cell wall, even though it can be detected in chitin synthase assays *in vitro*. A mutation of this gene in *A. fumigatus, A. nidulans* and *Cryptococcus neoformans* did not display any obvious phenotypic defects (Rogg *et al*., 2012), while in *Neurospora crassa* it resulted in inhibition of germination (Morcx *et al*., 2013). Class II genes play an important role in the regulation of *Saccharomyces cerevisiae* and *Candida albicans*. In *C. albicans*, *chs1*p regulates the formation of septum and cell wall chitin. The *chsB* class III synthase plays an important role in hyphal tip growth and conidiation of most filamentous fungi (Rogg *et al*., 2012). The *chsB* mutants are characterized by extremely small conidia and highly branched irregular slow-growing hyphae. This indicates the importance of the *chsB* gene for normal hyphal development, organization and in conidiation. The *chsD* class IV gene synthase functions together with *chsA* and *chsB* during conidiation as well as both hyphal growth and development (Fukuda *et al.,* 2009). A single mutation of *chsD* did not show any phenotypic changes when compared to the wild type strain. A double mutation of *chsA* and *chsD* resulted in reduced conidial formation and this indicates both the *chsA* and *chsD* genes have overlapping functions during conidiation (Horiuchi 2009).

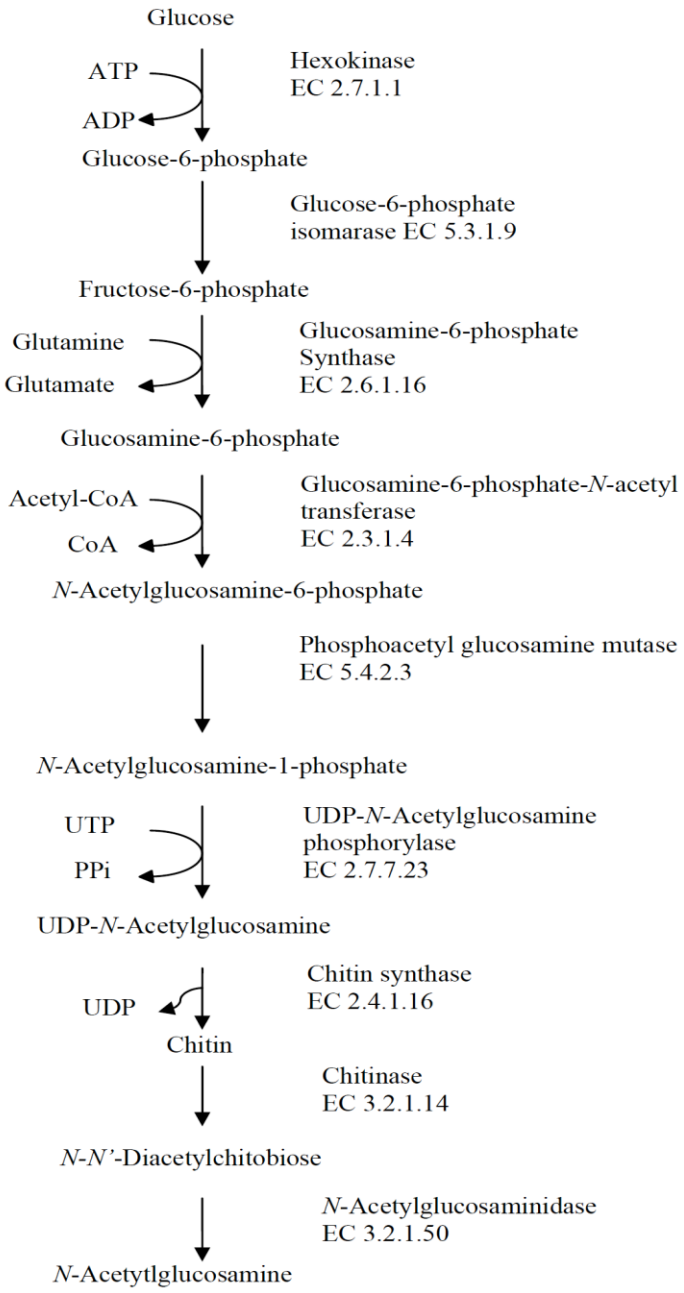


Figure 1.3: Chitin synthesis pathway (Chaudhary et al., 2013).

Chitin synthase with a myosin motor-like domain (*csmA)* and *csmB* are class VI and V gene products (Tsuizaki *et al*., 2013). Both of these gene products are found in the hyphal tips and form septa near the actin cytoskeleton, which is used to determine hyphal polarity and are thought to play a role in polarizing chitin synthesis at those sites (Tsuizaki *et al*., 2013). A deletion of either *csmA* or *csmB* resulted in abnormal hyphae that have a balloon appearance, formation of intracellular hyphae and lysis of hyphae under low osmotic conditions. This showed that both *csmA* and *csmB* play an important role in polarizing cell wall synthesis for hyphal tip growth, maintaining cell wall integrity and distribution of septa (Morcx *et al*., 2013).

1.2.4 Chitinases

Chitinases are enzymes that degrade chitin by breaking down the β-(1,4) glycosidic bonds between the glucosamine residues of chitin, releasing oligomeric, dimeric and trimer products in the process (Yamazaki *et al*., 2008, Alcazar-Fuoli *et al*., 2011; Tzelepis *et al*., 2012). They are widely distributed in fungi, bacteria, viruses and parasites and play an important role in cell nutrition, replication and division, morphogenesis and parasitism in bacteria. In fungi, chitinases are important for cell wall remodelling such as spore germination, hyphal formation and also spore differentiation (Brzezinska and Jankiewicz 2012, Tsirilakis *et al*., 2012).

1.2.4.1 Types and classification of chitinases

Chitinases belong to glycoside hydrolase (GH) family group 18 and 19. The GH family 18 is found in bacterial, plants, insects, mammals and viruses and have a common $(\alpha/\beta)_8$- barrel domain that is made up of eight α helices and β strands respectively (Matsumoto 2006; Seidel, 2008). The GH 19 family group is found mainly in plants and some *Streptomyces* species and have a high α content. Both families hydrolyse β-(1, 4) glycosidic bonds in chitin and will result in the release of short chain chitooligomers (Seidel 2008). The GH18 family is made up of two classes of chitinases: fungal/plant (class III) and fungal/bacterial (class V). Class V chitinases use fungal chitin during the digestion of exogenous chitin for energy, while Class III chitinases play a role in regulation of fungal morphogenesis (Alcazar-Fuoli *et al*., 2011).

Chitin is broken down by a chinolytic system of enzymes, namely, endochitinases, exochitinases and N-acetylglucominidases. Endochitinases hydrolyse chitin at the internal sites to form low molecular weight multiple forms of glucosamine dimmers such as diacylchitobiose, chitotrioses and chitotetraoses with diacetylchitobiose as the predominant form (Duo-Chang, 2006; Hamid *et al.*, 2013). Exochitinases release diacylchitobiosidases without the formation of monosaccharides or disaccharides. This process is regulated by chitobiosidases (Haran *et al*., 1995; Guthrie *et al*., 2005; Matsumoto 2006). The β-(1,4)-N-glucosaminidases also function as exochitinases and cleave the endochitinase products to release N-acetyl glucosamine monomers (Ren *et al*., 2000; Duo-Chang, 2006; Hamid *et al.*, 2013, Verwer *et al.*, 2013).

1.2.4.2 Function of chitinases

Chitinases have a variety of functions in fungal development and are divided into different subgroups. Chitobiosidases form part of subgroup A chitinases and are homologues of chitinase B (ChiB). They are expressed during unfavourable conditions when there are no nutrients available and will play a role in autolysis. During this process the older hyphae are digested and broken down to be used as a nutrient source together with other macromolecules within the cell (Hartl *et al*., 2012; Munster *et al*., 2013). This occurs during the later stages of fungal development and can be a form of programmed cell death for fungi (Shin *et al*., 2009). Chitinases belonging to subgroup B play a role in nutrition during fungal development. These enzymes are upregulated in nutrient limiting conditions and inhibited by glucose or other carbon sources that are easily broken down. In yeasts ChiB play an important role in cell division and remodelling. In filamentous fungi they are located on the hyphal tips and branching points and regulate cell wall remodelling during vegetative growth. Endo-N-acetyl-β-glucosaminidases also form part of subgroup ChiB and regulate protein deglycosylation (Munster *et al*., 2013). Group C chitinases are found in mycoparasites and play a role in cell wall penetration and degradation of chitin for nutrients (Hartl *et al*., 2012, Duo-Chang 2006).

1.2.5 β-glucanases

β-(1,3) glucanases are enzymes that breakdown β-(1,3) glucans during the cell wall remodelling process, where morphological changes occur. Changes in the linear glucan chains occur and this is followed by hyphal branching, elongation and ultimately degradation of the glucans (Mouyna

et al., 2013). The cell wall loses its rigidity to allow for entry of cell components for cell remodelling to take place. The β-(1,3) glucanases can be divided into exo- and endoglucanases. Endoglucanases cleave the internal long, fibril glucan chains at random sites resulting in a loss of cell wall rigidity and degradation. This allows for the hyphal branching and germination to take place (Mouyna *et al*., 2013; Onsori *et al*., 2005). Endoglucanases may therefore play an important role in cell wall remodelling. Exoglucanase brakes down cellulose by releasing glucose residues from the non-reducing ends of cellulose (Onsori, *et al*., 2005). Unlike endoglucanases, exoglucanases have a limited function in the cell wall.

1.2.6 Glycoproteins

Fungal cell wall proteins are glycoproteins made up of N- and O-linked oligosaccharides. The structure of these oligosaccharide chains vary amongst fungi (Perez and Ribaz, 2013). *Aspergillus fumigatus* has galactomannan structures made up of both mannose and galactose residues. Cell wall proteins maintain the cell shape, mediate adhesion for cell migration and fusion, offer protection against foreign substances, mediate absorption of molecules, transmit intracellular signals from external stimuli and synthesize and remodel cell wall components (Bowman and Free 2006; Free, 2013).

1.3 The fungal cell membrane

The cell membrane is made up of more than 500 naturally occurring proteins, which interact with the lipids found in the cell membrane. This leads to formation of pores, efflux of cellular components and changes in the membrane potential (Theis and Stahl, 2004). The lipid bilayer of the cell membrane consists of sphingolipids, phospholipids and sterols and these lipid components are found in most living organisms. The sterol component of higher eukaryotes such as mammals is cholesterol, whereas in fungi it is ergosterol. Fungal membranes play an important role in the cell they regulate nutrient uptake by the cell, transport membrane proteins and enzymes as well as regulate the process of signal transduction (Avis 2007). The fungal cell membrane also maintains cell shape and integrity. A disruption of the cell membrane in the cell can lead to changes in membrane protein function and enzymes, a loss of shape of the cell, leakage of cellular components and ultimately cell death (Avis 2007).

1.3.1 Ergosterol

Ergosterol is a major lipid component of the fungal cell membrane and is found in yeast and filamentous fungi. It regulates membrane fluidity, distributes integral proteins in the cell membrane, provides membrane asymmetry and integrity and controls the functioning of membrane-bound enzymes (Alcazar-Fuoli *et al*., 2013). Synthesis of ergosterol is a complex process that involves at least 10 enzymes in the conversion of squalene to ergosterol (Fig 1.4). This makes it an ideal target for antifungal agents used for treatment of fungal infections (Alcazar-Fuoli *et al*., 2006; Alcazar-Fuoli *et al*., 2008).

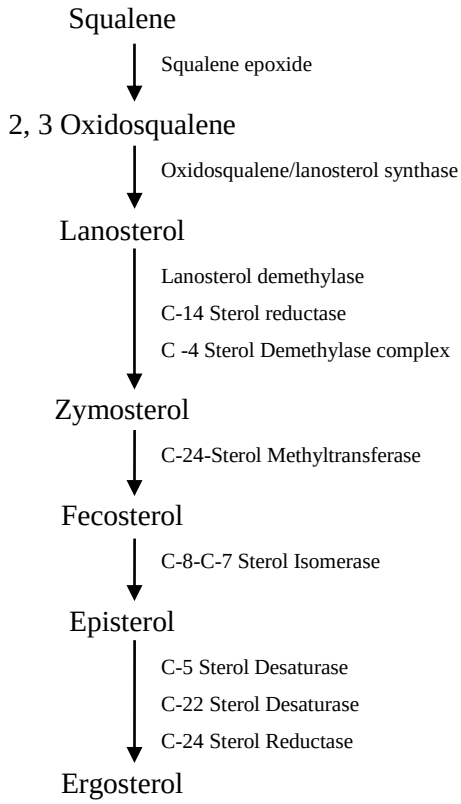


Figure 1.4: The ergosterol biosynthetic pathway with the enzymes involved in its formation from squalene to ergosterol (Gauwerky et al., 2009).

In *A. fumigatus*, the three sterol intermediates, lanosterol, fecosterol and episterol, branch into alternative pathways in their synthesis of ergosterol. The products formed in these alternative pathways may compensate for the loss of some of the sterol intermediates in the ergosterol biosynthesis pathway and allow for the synthesis of ergosterol (Alcazar-fuolli *et al*., 2008).

1.4 Antifungal agents

Different classes and various types of antifungal agents have been used for the treatment of fungal infections. Most of these agents target the cell wall and ergosterol biosynthesis. Those targeting the cell membrane are: polyenes, allylamines, azoles and morpholines. Echanocandins, nikkomycin and polyoxins target the cell wall (Denning and Hope, 2010; Johnson and Perfect, 2010).

1.4.1 Ergosterol biosynthesis inhibitors

1.4.1.1 Polyenes

Polyenes are antifungal agents with a broad spectrum of activity and are produced by *streptomyces*. Examples of these are amphotericin B, nystatin and natamycin (Denning and Hope, 2010; Vandeputte *et al*., 2011). Amphotericin B and nystatin target the cell membrane by binding irreversibly to ergosterol. This results in increased cell permeability, formation of pores causing leakage of cell membrane components and results in cell death (Borreli *et al*., 2008, Batista *et al*., 2013).

1.4.1.2 Allylamines

Allylamines act on the early steps of ergosterol biosynthesis by binding to squalene epoxidase. This prevents the conversion of squalene to 2,3-oxidosqualene (Fig 1.4), causing an accumulation of squalene and preventing the formation of lanosterol. Inhibition of lanosterol production will ultimately inhibit ergosterol biosynthesis. Terbinafine is an example of an allylamine that acts on the ergosterol biosynthetic pathway by inhibiting squalene epoxidase (Borrelli *et al*., 2008).

1.4.1.3 Morpholines

Morpholines also act on the ergosterol biosynthetic pathway by inhibiting sterol C_8-C_7 isomerase and C_{14} sterol reductase. These enzymes are involved in the conversion of lanosterol to zymosterol (Fig 1.4), which is converted to other sterol intermediates and ultimately ergosterol. Inhibition of these enzymes ultimately results in inhibition of ergosterol production (Gauwerky *et al*., 2009).

1.4.1.4 Azoles

Azoles block lanosterol (C-14) demethylase, which is involved in the initial stages of the conversion lanosterol to zymosterol. This will also result in the disruption of ergosterol biosynthesis and accumulation of toxic demethylated sterols that disrupt the membrane integrity of the cell and can result in death (Gauwerky *et al*., 2009; Vandeputte *et al.*, 2011; Cowen and Steinbach 2008).

1.4.2 Cell wall components inhibitors

1.4.2.1 Echanocandins

Echanocandins are regarded as a newer group of antifungal agents that target the cell wall. They are a group of semi synthetic lipopeptides which inhibit β-(1,3) glucan synthase and result in the inhibition of β-(1,3) glucan production. This will lead to the depletion of β-glucan production and can result in cell lysis. Examples of echanocandins include caspofungin, anidulafungin and micafungin and are fungicidal against *Candida* species and other yeasts and fungistatic against *Aspergillus* (Pierce *et al*., 2013).

1.4.2.2 Nikkomycin and polyoxin

Nikkomycin and polyoxin are peptide nucleoside antifungal agents, which are isolated from *Streptomyces* species (Vicente *et al*., 2003). They bind to the chitin synthase complex and inhibit chitin synthesis. This results in the disturbance of the fungal cell wall structure and fungal growth processes (Gauwerky *et al*., 2009).

1.4.3 Mannoprotein synthesis inhibitors

The various roles of mannoproteins in the fungal cell membrane, makes them ideal targets for antifungal agents. Pramidicin and benanomicin are inhibitors of mannoproteins and bind to mannoprotein and inhibit manan synthesis. This will cause a disruption of the fungal cell membrane, leakage of other cellular components and ultimately cell death (Vicente *et al*., 2003; Gauwerky *et al*., 2009).

1.4.4 Other antifungal agents

1.4.4.1 Pyrimidines

5-Fluorocytosine (5-FC) is a low molecular weight pyrimidine analog that acts on both DNA and RNA synthesis of the fungal cell. It enters the fungal cell through uptake by the fungal enzyme cytosine permease, deaminated to 5-fluorouracil and is then converted to a triphosphate. The triphosphate form gets incorporated into RNA, where it causes miscoding. 5-FU can also be converted to a deoxynucleoside which then inhibits thymidylate synthase and DNA synthesis (Vandeputte *et al*., 2011).

1.4.4.2 Sordarins

Sordarin was isolated from the fungus, *Sordaria araneosa* and inhibits protein synthesis by selectively binding to the elongation factor 2 /ribosome complex in fungi. It has been found to be highly effective against *C. albicans*, *S. cerevisiae* and *C. neoformans* (Liang 2008).

1.4.4.3 Sphingolipid inhibitors

Sphingolipids are essential for cell growth, development and therefore inhibition of their synthesis can result in cell death. Several enzymes that are involved in sphingolipid biosynthesis have been targeted by antifungal agents. These include serine palmitoyltransferase, ceramide synthase and inositol phosphoceramide (IPC) synthase. Antifungal agents involved in inhibiting sphingolipid biosynthesis include: Sphingofungin, lipoxamycin, viridiofungins, Fumonisin B1 and rustmucin (Vicente *et al*., 2003).

1.5 Mechanisms of resistance to antifungal agents

Fungal pathogens may have the ability to overcome the inhibitory action of antifungal agents by using various resistance mechanisms (Fig 1.5). These mechanisms are: (1) over expression of the target enzyme to prevent the antifungal agent from severely affecting the intended biochemical process within the cell; (2) changes to the antifungal agent to prevent binding to the target component; (3) activation of an efflux pump to pump the antifungal agent out of the cell; (4) formation of a barrier to prevent entry of the antifungal agent; (5) finding an alternative pathway to bypass the enzyme targeted by the antifungal agent; (6) inactivation and (7) degradation of the antifungal agent (Chelsea and White, 2009).

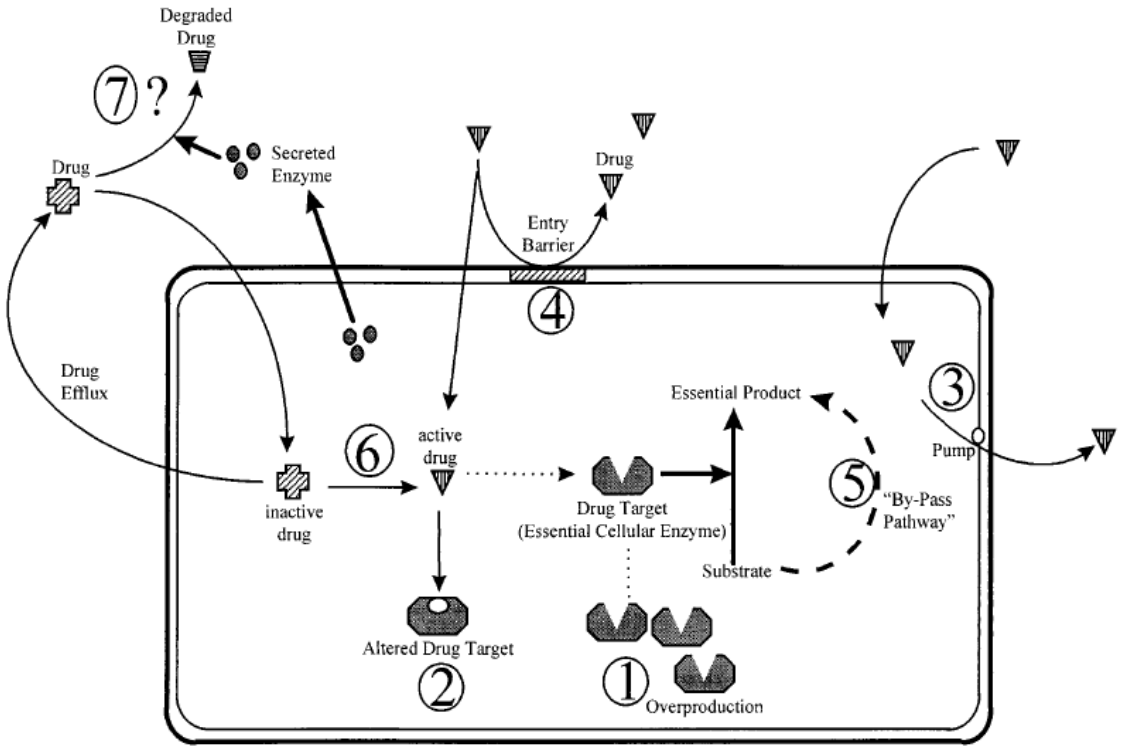


Figure 1.5: Mechanisms by which fungal pathogens develop resistance to antifungal agents (Ghannoum and Rice 1999).

1.6 Medicinal plants

The toxicity of some antifungal agents towards the host and the emergence of fungal resistant pathogens have led to a need for the development of alternative therapeutics. Plants have been an important source of medicine since the beginning of human kind (Mtiwari *et al*., 2013). The World Health Organization (WHO) estimates that 80% of the worlds population still relies on plant-derived medicines for their healthcare needs (Mabona and van Vuuren 2013). It is estimated that 25% of prescribed medication contains plant extracts and many active compounds of current medication has been isolated from plants. South Africa has a rich diversity of more than 300,000 plant species, with 300 of these species being used therapeutically. The WHO estimated that about 70% of South Africans use traditional medicines as their primary source of healthcare (Madhuri and Pandey 2009).

1.6.1 Bioassay directed isolation of compounds

The extraction process is an important step in the investigation of isolating active components from plants. It allows for the separation of the desired compounds and other compounds that may be present in the plant tissue and also the identification and characterization of these compounds. In this process a solvent diffuses into the plant tissue and will combine and solubilise compounds that have similar polarity (Ncube *et al.*, 2008). The quality of the extract obtained after the extraction process is influenced by a number of factors. These include: the type of plant material used (dried in oven, freeze dried or ground to obtain a homogenous mixture); the type of solvent used for the extraction process (aqueous or organic and polarity) and the extraction method. Other factors that have an effect of the quality of extract obtained after extraction are extraction temperature and extraction time (Ncube *et al.,* 2008; Sasidharan *et al*., 2011).

1.6.2 Choice of solvent

The type of solvent used plays an important role in successful extraction of bioactive compounds from plant material. The solvent used for extraction should have no or low toxicity, evaporate easily at low heat; have preservative properties and should not cause the extract to dissociate. Solvent choice is also influenced by the type of compounds that are being extracted (phenolics, flavonoids, etc.) and also what is intended for the extract (Tiwari *et al*., 2011). Water, methanol and ethanol are the most commonly used solvents in extracting compounds for detecting antimicrobial activity. Chloroform, acetone and dichloromethane have also been used for extraction processes. A combination of different solvents has been used to obtain the ideal solvent system for the extraction process. Ethanol and methanol have been shown to be ideal solvents for extracting polyphenols and hydrophilic compounds. A combination of water (30 %) and ethanol (70%) results in extraction of a higher concentration of flavonoids than 100% ethanol Dichloromethane is used for extraction of terpenoids and lipophilic compounds while a mixture of chloroform, hexane and methanol is used to extract terpenoid lactones (Sasidharan *et al.,* 2011,Tiwari *et al*., 2011). Water, acetone and methanol are used to extract saponins and a mixture of acetone and water has been shown to be a better solvent to extract tannins and other phenolics rather than aqueous methanol (Tiwari *et al*., 2011; Grabber *et al*., 2013).

1.6.3 Extraction methods

The type of extraction method used depends on a number of factors. These include the amount of time of the extraction period, the type of solvent used for the extraction procedure, pH, temperature, the particle size and solvent-to-sample ratio. Grinding of plant material to finer particles will result in an increase in the surface area for the extraction to take place. This will increase the rate at which extraction will take place, resulting in a shorter extraction period (Das *et al*., 2010; Ncube *et al*., 2008).

Homogenization in a solvent is the most commonly used method for extracting compounds from plant material. The plant material (wet or dried) is ground in a blender in the presence of a selected solvent into a homogeneous mixture. This is either left to stand for 24h or shaken at high speed for a short period of time (5-30 min) or at a gentle speed for 24h. The mixture is then filtered and fresh solvent may be added to the residue for another extraction for 24h. The filtrate is concentrated by drying under reduced pressure or freeze-dried to obtain a dried extract (Das *et al*., 2010; Liu 2011). Serial exhaustive extractions are methods used to extract a variety of compounds from plant tissue with the aim of obtaining a wide polarity range of compounds. This method uses successive extraction solvents with increasing polarity, starting from a non-polar to a more polar solvent. Other commonly used methods for extraction are, soxhlet extraction; steam distillation, volatile solvent extraction or supercritical fluid extraction (for extracting essential oils maceration (finely grounded plant material is mixed with a solvent in a stoppered container for a certain period of time until all of the material is dissolved in the solvent), percolation and infusion (plant material is mixed with cold or boiling water for a short period of time) (Tiwari *et al*., 2011; Das *et al*., 2010).

1.6.4. Chromatographic techniques for purification of plant extract compounds

The identification and isolation of the components of the plant extract is completed using a variety of chromatographic techniques. They are one of the simplest and affordable methods for identifying the different compounds in the plant extract and are also reproducible. These include column chromatography, thin layer chromatography (TLC), bioautography and high performance liquid chromatography (HPLC) (Patra *et al*., 2012).

1.6.4.1 Thin Layer Chromatography

This is the most commonly used chromatographic technique for separating plant extracts and identifying the active compounds. It allows for rapid analysis, provides qualitative and quantitative information on the separated compounds and allows for the purification of the active compound (Shobhen *et al*., 2011; Tistaert *et al*., 2011).

Thin layer chromatography is made up of two phases: a stationary and a mobile phase. Stationary phases include silica, alkyl-silica (C8 or C18), cellulose, dextran gels, polyamide and alumina. These are coated onto aluminium, plastic or glass plates. The mobile phase, which is used as the developing solvent, is an organic solvent that is made up of variety of liquids with different polarities, depending on the polarity or the type of compounds that are separated (Cheng, *et al*., 2011; Liu 2011).

Different methods are used for the detection of the separated compounds depending on their properties. Detection can be via ultraviolet (UV) light absorption, at a wavelength of 254 or 366 nm. Alternatively, compounds can be detected by spraying reagents, followed by heating until spots develop. Spraying reagents are used for detection of unknown compounds and examples of these include vanillin/sulphuric acid and iodine. In cases of known compounds, specific spraying reagents are used. These include ninhydrin Ferric chloride, anisaldehylde and Dragendorff's reagent (Liu, 2011; Sasidharan *et al*., 2011).

Once the active compounds have been localized, the plant extract is then separated on a preparative TLC plate to allow for the isolation of the active compounds. The same mobile phase used for the separation of the plant compounds is used and the band containing the active compound is scrapped from the TLC plate and eluted with a strong polar solvent. The active compound can then be subjected to other chromatographic techniques such as high performance liquid chromatography (HPLC) for further purification and fingerprinting, mass spectrometry (MS), nuclear magnetic resonance (NMR) for structure elucidation and characterization (Cheng *et al*., 2011; Patra *et al*., 2012).

1.6.4.2 High pressure Liquid Chromatography (HPLC)

This is one of the most commonly used chromatographic techniques for separating and analysing compounds of the plant extract and also developing a fingerprint of the sample. It has high resolution, selectivity and sensitivity and can be combined with different detectors. These include diode array detection (DAD), UV, MS and NMR amongst others (Springfield *et al*., 2005; Tistaert *et al*., 2011). The choice of the stationary phase is important in fingerprinting of plant extracts and the most commonly used method is reverse phase HPLC (RP-HPLC). In RP-HPLC, compounds are separated on their basis of their hydrophobicity, i.e. hydrophobic compounds elute before the hydrophilic ones. This type of stationary phase is also more suitable for separation of highly polar compounds (Liu, 2011).

The mobile phase is made up of an aqueous (water) and organic solvent (acetonitrile or methanol). The ratios of these solvents are adjusted to suit the elution ability of the sample, to ensure that there is interaction between the sample and the stationary phase. When a constant mobile phase is used to separate compounds it is known as an isocratic elution. Alternatively, the ratio of the organic solvent can also be increased in a step-wise manner or linear fashion and this is known as a gradient elution. This can be optimised until the best separation of the compounds is obtained (Liu 2011).

1.6.4.3 Liquid chromatography- Mass spectrometry (LC-MS)

Mass spectrometry is a highly sensitive detector which provides information on the molecular mass and structural features of the unknown compound (Hostettman; 1999; Cuyckens and Claeys 2004). The LC-MS coupling is made possible by the three interfaces: thermospray (TSP), continuous flow fast atom bombardment (CF-FAB) and electrospray (ES). The LC-TSP-MS is used for the ionization of moderately polar compounds in a range of 200- 800 MW, while LC-CF-FAB or LC-ES is used for the ionization of larger polar compounds with molecular weight greater than 800 (Hostettman 1999).

1.6.4.4 Nuclear magnetic resonance (NMR)

NMR provides structure elucidation and characterization of unknown compounds. It also provides structural content which includes chemical shifts,interaction between neighbouring

nuclei, intramolecular relationships, molecular motions in solution and ligand binding amongst others (Corcoran and Spraul, 2003; Simmler *et al*., 2014).

1.6.5 Bioautography techniques

Bioautography is a technique used to localize components of the plant extract that have antimicrobial activity against tested microorganisms. It uses a small amount of sample and can be used to determine the polarity of the active compounds, based on the polarity of the solvent used to develop the TLC plate. This technique is used to screen a large amount of samples for their bioactivity and also in bioactivity guided fractionation, where there is target-directed isolation of the active components (Choma and Grzelak, 2011).

1.6.5.1 Agar overlay technique

The agar overlay technique is a commonly used method for the detection of active compounds in the plant extract. It can be used for a broad spectrum of microorganisms and can produce well defined zones of clearing. Agar medium seeded with the microorganism is applied onto the developed TLC plate and incubated at the appropriate temperature to allow for growth of the microorganism. The plate is then sprayed with dehydrogenase-activity reagents such as tetrazolium salts and incubated at an appropriate temperature for 2-4 h. The tetrazolium salt is converted to a coloured formazan product by the dehydrogenases of actively growing microorganisms. A zone of clearing against a coloured background will indicate an active component of the plant extract. Examples of tetrazolium salts that are used in agar overlay include 3-[4, 5–dimethylthiazol-2-yl]-2, 5-diphenyltetrazoliumchloride (MTT); p-idionitrotetrazolium violet (INT) and 2, 3, 5-triphenyl-tetrazolium chloride (TZC) amongst others. These zones of clearing are then compared to the separated compounds of the plant extract corresponding parallel plate to localize active compounds (Choma and Grzelak, 2011; Marston 2011).

1.6.6 Antimicrobial activity of medicinal plants.

Medicinal plants produce a wide variety of secondary metabolites and other active compounds, which protect the plant against invaders and foreign material. Some metabolites exist as active

compounds in healthy plants, while others are activated and released as a defence mechanism when the plant is either damaged or invaded by foreign material (Ncube *et al*., 2008). Secondary metabolites include essential oils, terpenoids, phenolics and alkaloids (Yazdani *et al*., 2011).

1.6.6.1 Crude plant extracts

Crude plant extracts are used in initial stages of screening for antimicrobial activity. The antifungal activity of the aqueous and organic extract of *Acacia nilotica, Achras zapota* and *Eucalyptus globules* was tested against *Aspergillus* species. The aqueous and methanol extracts were highly active against *A. flavus* (Satish *et al*., 2007). Aqueous extracts of *T. violacea* and *Agapanthus africanus* were tested against *Puccinia triticina*, which causes leaf rust in wheat plants. The results showed that both extracts of *T. violacea* and *A. africanus* inhibited spore germination of *P. triticina* and prevented the germ tube formation (Cawood *et al*., 2010). The advantages stem from multiple compounds in the crude extract that may have greater efficacy as a mixture. These compounds might act synergistically, exhibit a broader range of antimicrobial activity and allow lower prevalence of resistance development (Yazdani *et al*., 2011).

1.6.6.2 Terpenes

Terpenes are one of the largest groups of plant secondary metabolites and form part of the essential oils. Plant fragrance of the plant is carried by the essential oil fraction consisting of isoprenoids. Additional oxygen in the terpene structure will give rise to terpenoids, (Das *et al*., 2010). Essential oils are hydrophilic compounds with volatile aroma compounds. They are extracted from plant material using steam. Most terpenes belong to the monoterpenoid group and have antibacterial, antifungal, antioxidant and anti-carcinogenic properties (Yazdani *et al*., 2011). Essential oils have a wide spectrum antimicrobial activity against bacteria; fungi (plant and human pathogens); yeasts and viruses and also anti-inflammatory properties (Ncube *et al*., 2008; Sati and Joshi 2011). It has been suggested that terpenes play a role in the disruption of the cell membrane, which can affect sterol production and the functioning of the cell (Paiva *et al*., 2010).

1.6.6.3 Alkaloids

Alkaloids are heterocyclic nitrogenous compounds with a ring structure that is composed of pyridine, pyrroles, indole, pyrrolidines, isoquinolines or piperidines (Das *et al*., 2010). They

possess antimicrobial activity against a variety of microorganisms. Barberine is an isoquinole alkaloid found in roots and stems of *Berbis* species and it has been found to be active against fungi, viruses and bacteria. Its mechanism of action is to accumulate in the cells causing DNA intercalation (Savioa 2012). Isoquinole alkaloids have also been found to possess anti-amoebic activity (Sati and Joshi 2012).

1.6.6.4 Phenolics and polyphenols

Phenolics are a large group of aromatic compounds made up of flavones, flavanoids, quinoles, polymeric phenolic compounds, coumarins and phenolics fused with benzene and pyrone groups (Hossain *et al*., 2011; Hossain *et al*., 2013; Karimi *et al*., 2011). They are found in plants and are released as a defence mechanism to pathogen attack. The antimicrobial activity of phenolic compounds depends on their structures. Flavanoids have been shown to inhibit cytoplasmic membrane and DNA gyrase and have anti-oxidant activity (Paiva *et al*., 2010; Hossain *et al*., 2011). Flavones disrupt cell morphology in filamentous fungi and inhibit DNA and RNA synthesis in *Vibrio harveyi* (Paiva *et al*., 2010). Other flavanoids are made up of two or more flavan-3-ol and these include catechin, epicatechin or gallocatechin (Hue *et al*., 2012). Catechin is an example of a flavanoid that is made up of two benzene rings and is considered to be the most powerful scavenger compared to other flavanoids. It is used as a standard to measure the amount of flavonoids in leaf samples and crude plant extracts (Hue *et al*., 2012).

1.6.6.5 Tannins

Tannins are polymeric phenolic compounds found in plants. They are divided into two groups : hydrolysable and condensed tannins. Hydrolysable tannins occur as multiple esters of glucose while the condensed ones are derived from flavanols and occur in oligomeric forms (Cowan, 1999; Savolia 2010). Condensed tannins or proanthocyaninids are made up of two or more benzene rings. Examples of theses tannins are epicatechin and gallocatechin (Hue *et al*., 2012). Tannins have antifungal and antibacterial properties and exert their action on microorganisms by binding to the cell membrane and inactivating enzymes and membrane bound proteins (Paiva *et al*., 2010).

1.6.6.6 Saponins

Saponins are a diverse group of secondary metabolites that are widely distributed in different plant species and accumulate in large quantities. They are made up of a hydrophobic aglycone backbone which is connected to hydrophilic sugar chains known as glycones (Sparg *et al*., 2004; Coleman *et al*., 2010; Karimi *et al*., 2011). Saponins are divided into two groups based on their aglycone backbone. These are known as steroidal and triterpenoid saponins. Steroidal saponins are regarded as one of the important secondary metabolites as they are used as starting material for the synthesis of steroidal hormones (Yang *et al.*, 2006). They are made up of a C-27 aglycone backbone that is made up of a six ring structure while the triterpenoid saponins are made up of C-30 aglycone backbone and have a pentacyclic structure (Sparg *et al*., 2004; Karimi *et al*., 2011). Both steroidal and triterpenoid saponins have antimicrobial activity against a variety of microorganisms. Antifungal activity has also been shown against a variety of fungal species which include *Candida*, *Aspergillus*, *Botryris ceneria* and *Trichoderma*, amongst others (Yang *et al*., 2006; Barile *et al*., 2007; Coleman *et al*., 2010, Lanzotti *et al*., 2012 (b)). Saponins are also used in household cleaning products. The combination of the hydrophobic aglycone and the hydrophilic sugar backbone allows the release of a foaming agent when mixed with water (Naoumkina *et al*., 2010). They are also used as emulsifying agents in food products and cosmetics (Sawai and Saito, 2011).

1.6.6.6.1 Biosynthesis of saponins

Triterpenoids are synthesized by the condensation of C5 units isopentyl diphosphate (IPP), which is derived from the mevalonic acid pathway and methylerythritol phosphate (MEP) (Augustin *et al*., 2011; Sawai and Saito 2011). IPP is condensed to geranyl pyrophosphate (GPP) followed by the addition of the second unit of IPP to form farnesyl pyrophosphate (FPP). Two FPP units are linked together to form squalene, which is converted to 2,3-oxidosqualene by squalene synthase (Kim *et al*., 2011; Augustin *et al*., 2011). The initial stages of the biosynthesis of triterpenoids leading to the formation of 2,3-oxidosqualene are the same for phytosterol and ergosterol biosynthesis in plants and fungi respectively (Fig 1.6) (Alcazar-Fuoli *et al*., 2006, 2008 and Alcazar-Fuoli and Mellado 2013; Augustin *et al*., 2011; Kim *et al*., 2011).

After the formation of 2,3-oxidosqualene, steroidal saponins and other sterol biosynthesis diverge for the formation of their respective end products (phytosterol and ergosterols). The cyclization of 2,3-oxidosqualene by 2,3 oxidosqualene synthase is considered as the first committed step in the biosynthesis of saponins and sterols (Meesapyodsuk *et al*., 2007). The cyclization of sapogenins can occur in two ways: the chair-chair-chair or the chair-boat-chair conformation. The chair-boat-chair conformation also gives rise to sterols (Augustin *et al*., 2011). In plants the cyclization of oxidosqualene is carried out by β-amyrin synthase (β-AS). This enzyme has been characterized in several plant species and will result in the formation of the triterpenoid skeleton known as β-amyrin (Naoumkina *et al*., 2010). The β-amyrin is then modified to a hydrophobic aglycone, known as the sapogenin. This process is a result of the oxidation of triterpenoid skeleton by cytochrome P_{450} monooxygenase. The sapogenin then undergoes a glycosylation process which is carried out by UDP-dependent glycosyltransferases (UGT). Glycosylation is important for saponin biosynthesis as it increases the water solubility of the terpenoid and also confers biological activity (Naomkina *et al*., 2010; Sawai and Saito, 2011).

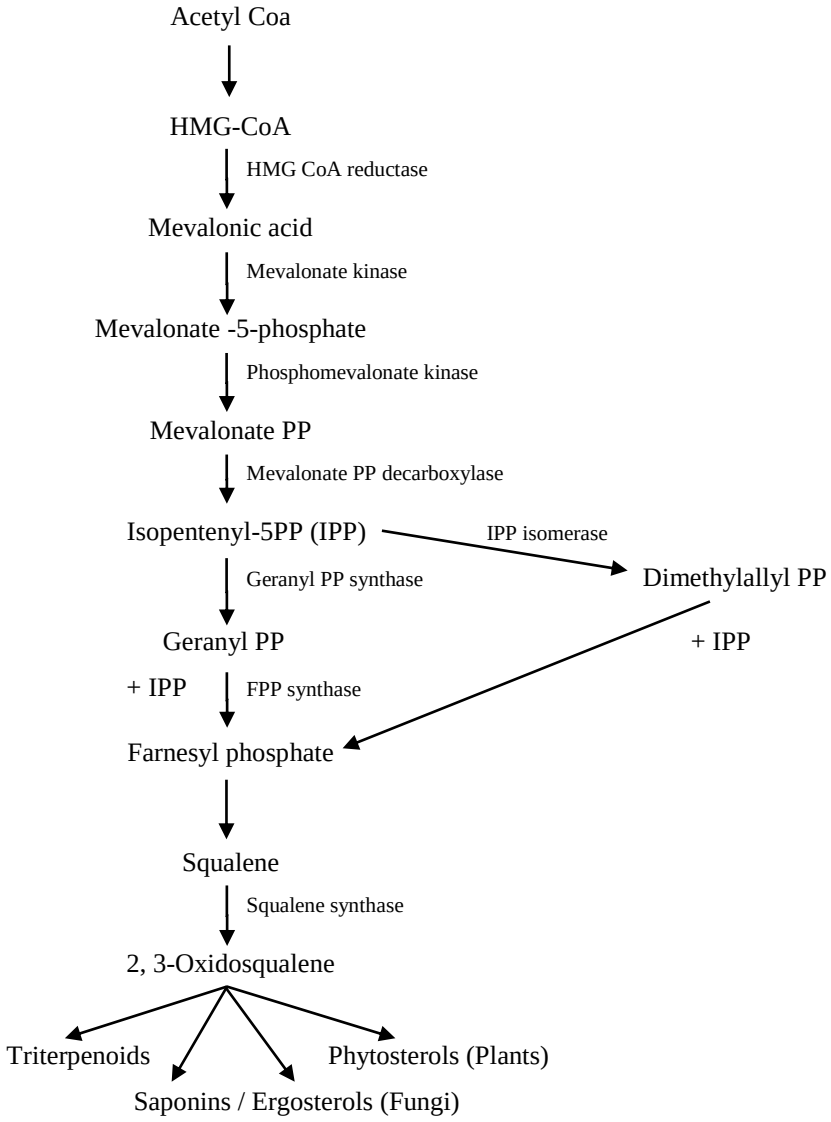


Figure 1.6: Initial steps of triterpenoid, phytosterol and sterol biosynthesis, leading to the formation of the common precursor (2,3-oxidosqualene). IPP = Isopentenyl pyrophosphate; DMPP = Dimethylallyl pyrophosphate; GPP = Genaryl pyrophosphate; FPP = Farnesyl Pyrophosphate (Adapted from Meesapyodsuk *et al*., 2007; Buhaescu and Izzedine, 2007).

1.6.6.6.2 Antimicrobial activity of saponins

Saponins have antimicrobial activity against a variety of microorganisms (Simons *et al*., 2006). In fungi, the mechanism of action of saponins is thought to be the formation of the sterol/saponin complex by binding to the cell membrane. This will result in the formation of pores in the cell membrane, which will cause leakage of cellular components, disruption of the cell membrane integrity and can cause cell death (Mert-Turk 2006; Simons *et al*., 2006; Avis 2007; Ahmed *et al*., 2012; Teshima *et al*., 2013). Various *Allium* species produce saponins. The bulb sections of these plants have been shown to contain a high content of saponins which have antifungal activity against various microorganisms (Barile *et al*., 2007; Lanzotti 2012(a and b), Teshima *et al*., 2013). Steroidal saponins in garlic have also displayed cholesterol lowering effects in hypercholesteremic rat models (Matsuura 2001). The antimicrobial activity of saponins makes them ideal candidates as possible therapeutic agents in treatment of fungal infections.

1.7 *Tulbaghia* genus

The genus *Tulbaghia* belongs to the *Alliacea* family and consists of 30 species which are indigenous to the Southern Africa region (Lyantagaye, 2011). The majority of the species are found in the Eastern Cape and Kwazulu-Natal, in South Africa (Jager and Stafford 2012). Plants of *Tulbaghia* have a prominent corona, are crown shaped, produce a garlic-like odour when the leaves are crushed or damaged and most species produce steroidal saponins (Lyantagaye, 2011).

1.7.1 *Tulbaghia violacea (T. violacea)*

Tulbaghia violacea is one of the most widely used medicinal plants in Southern Africa. It is a bulbous plant with long strap-like leaves. The bulbs are used for medicinal purposes to treat a variety of ailments such as asthma, fever, colds and flu, tuberculosis, etc. while the leaves are used as a condiment (Soyingobe *et al*., 2013) (Fig 1.7). *Tulbaghia violacea* and other *Tulbaghia* species are produce sulphur-containing compounds, which give them the characteristic garlic odour **(**Lyantagaye, 2011). Various extraction techniques and solvents yield various compounds (Table 1.1) .

Figure 1.7: A typical example of a *Tulbaghia violacea* plant (van Wyk and Gericke 2000).

Table 1.1: Chemical composition of *Tulbaghia violacea* extracted from different plant parts with different solvents

Plant part	Extractant	Compounds	References
Leaves	Water	2,4,5,7-tetrathiaoctane-2,2-dioxide 2,4,5,7-tetrathiaoctane Sugars	Burton and Kaye (1992)
Rhizomes	Methanol	2,4,5,7-tetrathiaoctane-4-oxide Marasmicin Methiin Ethiin	Kubec *et al*., (2002)
Aerial (leaves,stem,flower)	Diethyl ether (Steam distillation)	Volatile compounds	Pino *et al*., (2008)
Rhizomes	Methanol:water (1:1)	Methyl-α-D-glucopyranoside	Lyantagaye 2013

2,4,5,7-tetrathiaoctane-2,2-dioxide, 2,4,5,7-tetrathiaoctane, 2,4,5,7-tetrathiaoctane-2,27,7-tetraoxide and 2,4,5,7-tetrathiaoctane-4,4-dioxide are breakdown products of marasmicin which are produced due to the unstable nature of this compound (Kubec *et al*., 2002). *Tulbaghia violacea* also produces flavanols and saponins (Lyantagaye 2011).

1.7.2 Antimicrobial activity of *T. violacea* extracts

Tetrathiaoctane compounds have antibacterial activity (Burton and Kaye 1992), while the aqueous plant extract showed antifungal activity against plant pathogens in both *in vitro* and *in vivo* studies with no toxicity studies reported for either (Lindsey and van Staden, 2004; Nteso and Pretorius 2006a and b). Marasmicin has been shown to have fungistatic activity against three *Candida* species (Thamburan *et al*., 2006). Antifungal activity of *T. violacea* aqueous plant extract has also been reported for *C. albicans* (Motsei *et al*., 2003) and *A. flavus* (Belewa *et al*., 2011). This makes the plant extract a potential therapeutic agent for the development of newantifungal agents.

1.8 References

Abad, A., Fernandez-Molina, J.V., Bikandi, J., Ramirez, A., Margareto, J., Sendino, J., Hernando, F.L., Ponton, J., Garaizar, J. and Rementeria, A. 2010. What Makes *Aspergillus fumigatus* a Successful Pathogen? Genes and Molecules involved in Invasive Aspergillosis. *Revista Iberomericana de Micologia* **27**(4): 155-182.

Ahmed, D.B., Chaieb, I., Salah, K.B., Boukamcha, H., Jannet, H.B., Mighri, Z. and Daami-Remadi, M. 2012. Antibacterial and Antifungal Activities of *Cestrum parqui* Saponins: Possible Interaction with Membrane Sterols. *International Research Journal of Plant Science* **3**(1): 001-007.

Alcazar-Fuoli, L., Mellado, E., Garcia-Effron, G., Buitrago, M.J., Lopez, J.F., Grimalt, J.O., Cuenca-Estrella, J.M. and Rodriguez-Tudela, J.L. 2006. *Aspergillus fumigatus* C-5 Sterol Desaturases Erg3A and Erg3B: Role in Sterol Biosynthesis and Antifungal Drug Susceptibility. *Antimicrobial Agents and Chemotherapy* **50**(2): 453-460.

Alcazar-Fuoli., L., Mellado, E., Garcia-Efron, G., Lopez J.F., Grimalt, J.O., Cuensa-Estrella, J.M. and Rodriguez-Tudela, J.L. 2008. Ergosterol Biosynthesis Pathway in *Aspergillus fumigatus*. *Steroids* **73**: 339-347.

Alcazar-Fuoli, L., Clauvad, C., Lamarre, C., Aimanianda, V., Seidl-Seiboth, V, Mellado, E. and Latge, J-P. 2011. Functional Analysis of the Fungal/Plant Class Chitinase Family in *Aspergillus fumigatus*. *Fungal Genetics and Biology* **48**: 418-429.

Alcazar-Fuoli, L. and Mellado, E. 2013. Ergosterol Biosynthesis in Aspergillus fumigatus: Its Relevance as an Antifungal Target and Role in Antifungal Drug Resistance. *Frontiers in Microbiology* **3**(439): 1-6.

Augustin, J.M., Kuzina, V., Andersen, S.V. and Bak, S. 2011. Molecular Activities, Biosynthesis and Evolution of Triterpenoid Saponins. *Phytochemistry* **72**: 435-457.

Avis, T.J. 2007. Antifungal Compounds that Target Fungal Membranes: Application and Disease Control. *Canadian Journal of Plant Pathology* **29**: 323-329.

Barile, E., Bonanomi, G., Antignani, V., Zolfaghari, B., Sajjadi, S.E., Scala, F. and Lanzotti, V. 2007. Saponins from *Allium minutiflorum* with Antifungal Activity. *Phytochemistry* **68**: 596-603.

Batista, M.V., Costa, S.F., Shikanai-Yasuda, M.A. and Moss, R.B. 2013. Current Treatment Options for Invasive Aspergillosis. *Drugs of Today* **49**(3): 213-226.

Belewa, V., Baijnath, H. and Somai, B.M. 2011. Aqueous Extracts from the Bulbs of *Tulbaghia violacea* are Antifungal against *Aspergillus flavus*. *Journal of Food Safety*. **31**: 176-184.

Bennett, J.W. 2010. An Overview of the Genus *Aspergillus Aspergillus: Molecular Biology and Genomics*. M. A. G. Machida, K. Sendai**:** 1-17.

Bernard, M and. Latge, J-P. **2001.** *Aspergillus fumigatus* Cell Wall: Composition and Biosynthesis. *Medical Mycology* **39**(1): 9-17.

Borelli, C., Schaller, M., Niewerth, M., Nocker, K., Baasner, B., Berg, D., Tiemann, R., Tietjen, K., Fugmann, B., Lang-Fugmann, S. and Korting, H.C. 2008. Modes of Action of the new *Arrylguanidine Abafungin* beyond Interference with Ergosterol Biosynthesis and *in vitro* Activity against Medically Important Fungi. *Chemotherapy* **54**: 245-259.

Bowman, S.M. and Free, S.J. 2006. The Structure and Synthesis of the Fungal Cell Wall. *Bioassays* **28**(8): 799-808.

Brzezinska, M.S. and Jankiewicz, U. 2012. Production of Antifungal Chitinase by *Aspergillus niger* LOCK 62 and Its Potential Role in The Biological Control. *Current Microbiology* **65**: 666-672.

Buhaescu, I. and Izzedine, H. 2007. Mevalonate Pathway: A Review of Clinical and Therapeutical Implications. *Clinical Biochemistry* **40**:575-584.

Burton, S.G. and Kaye, PT. 1992. Isolation and Characterization of Sulphur Compounds from *Tulbhaghia violacea*. *Planta Medica* **58**: 295-296.

Catalli, A. and Kulka, M. 2010. Chitin and β-Glucan Polysaccharides as Immunomodulators of Airway Inflammation and Atopic Disease. *Recent Patents on Endocrine, Metabolic and Immune Drug Discovery* **4**: 175-189.

Cawood, M.E., Pretorius, J.C., Westhuizen, A.J. and Pretorius, Z.A. 2010. Disease Development and PR-protein activity in Wheat (*Triticum aesitivum*) Seedlings Treated with Plant Extracts Prior to Leaf Rust (*Puccinia triticina*) Infection. *Crop Protection* **29**: 1311-1319.

Chaudhary, P.M., Tupe, S.G. and Deshpande, M.V. 2013. Chitin Synthase Inhibitors as Antifungal Agents. *Mini-Reviews in Medicinal Chemistry* **13**(2): 222-236.

Cheng, S-C. Huang, M-Z. and Shie, **J**. 2011. Thin layer chromatography/mass spectrometry. *Journal of Chromatography A* **1218** (19): 2700-2711

Chelsea, M., and White, T.C. 2009. Genetic Basis of Antifungal Drug Resistance. *Current Fungal Infection Reports* **3**: 163-169.

Choma, I.M. and Grezelak, E.M. 2011. Bioautography Detection in Thin Layer Chromatography. *Journal of Chromatography A* **218**(19): 2684-2691.

Coleman, J.J., Okoli, I., Tegos, G.P., Holson, E.B., Wagner, F.F., Hamblin, M.R. and Mylonakis, E. 2010. Characterization of Plant-Derived Saponin Natural Products against *Candida albicans*. *American Chemical Society* **5**(3): 321-332.

Corcoran, O. and Sparaul, M. 2003**.** LC–NMR–MS in Drug Discovery. *Drug Discovery Today* **8**(14): 624-631.

Cowan, M.M. 1999. Plants as Antimicrobial Agents. *Clinical Microbiology Reviews* **12**(4): 564-582.

Cowen, L.E. and Steinbach, W.J. 2008. Stress, Drugs and Evolution: The Role of Cellular Signaling in Fungal Drug Resistance. *Eukaryotic Cell.* **7**: 747-764.

Cuyckens, F. and Claeys, M. 2004. Mass Spectrometry in the Structural Analysis of Flavonoids. *Journal of Mass Spectrometry* **39**: 1-15.

Das, K., Tiwari, R.S.K. and Shrivastava, D.K. 2010. Techniques for Evaluation of Medicinal Plant Products as Antimicrobial Agents: Current Methods and Future Trends. *Journal of Medicinal Plant Research* **4**(2): 104-111.

Denning, D.W. and Hope, W.W. 2010. Therapy for Fungal Diseases: Opportunities and Priorities. *Trends in Microbiology* **18**(5): 195-204.

Di Liberto, M., Svetaz, L., Furlan, R.L.E., Zacchino, S.A., Delporte, C., Novoa, M.A., Asencio, M. and Cassels, B.K. 2010. Antifungal Activity of Saponin-Rich Extracts of *Phytolacca dioica* and of the Sapogenins Obtained through Hydrolosis. *Natural Products Communication* **5**(7): 1013-1018.

Diaz-Jiminez, D., Perez-Garcia, L.A., Martinez-Alvarez, J.A. and Mora-Montes, H.M. 2012. Role of the Antifungal Cell Wall in Pathogenesis and Antifungal Resistance. *Current Fungal Infection and Respiration* **6**: 275-282.

Duo-Chang, L. 2006. Review of Fungal Chitinases. *Mycopathologia* **161**: 345-360.

Free, S.J. 2013. Fungal Cell Wall Organization and Biosynthesis. *Advances in Genetics* **81**: 1-43.

Fukuda, K., Yamada, K., Deoka, K., Yamashita, S., Ohta, A. and Horiuchi, H. 2009. Class III Chitin Synthase ChsB of *Aspergillus nidulans* Localizes at the Sites of Polirized Cell Wall Synthesis and is required for Conidial Development. *Eukaryotic Cell* **8**(7): 945-956.

Gastebois, A., Clavaud, C., Aimanianda, V. and Latge, J-P. 2009. Aspergillus fumigatus: Cell Wall Polysaccharides, Their Biosynthesis and Organization. *Future Microbiology* **4**(5): 583-595.

Gastebois, A., Mouyna, I., Simenel, C., Clavaud, C., Coddeville, B., Delepierre, M., Latge, J-P. and Fontaine, T. 2010. Characterization of a new β (1-3) -Glucan Branching Activity of *Aspergillus fumigatus*. *Journal of Biological Chemistry* **285**(4): 2386-2396.

Gauwerky, K., Borelli, C. and Korting, H.C. 2009. Targeting Virulence: A New Paradigm for Antifungals. *Drug Discovery Today* **14**(3/4): 214-222.

Ghannoum, M.A and Rice, L.B. 1999. Antifungal Agents: Mode of Action, Mechanism of Resistance, and Correlation of these Mechanisms with Bacterial Resistance. *Clinical Microbiology Reviews* **12**: 501-517.

Guthrie, J.L., Khalif, S. and Castle, A.J. 2005. An Improved Method for Detection and Quantification of Chitinase Activities. *Canadian Journal of Microbiology* **51**: 491-495.

Grabber, J.H., Zeller, W.E. and Mueller-Harvey, I 2013. Acetone Enhances the Direct Analysis of Procyanidin-and Prodelphinidin-Based Condensed Tannins in Lotus Species by the Butanol-HCl-Iron Assay. *Agriculture and Food Chemistry* **61**: 2669-2678.

Haran, S., Schickler, H., Oppenheim, A. and Chet, I. 1995. New Components of the Chitinolytic System of *Trichoderma harzianum*. *Mycology Research* **99**(4): 441-446.

Hamid, R., Khan, M.A., Ahmad, M., Ahmad, M.M., Abdin, M.Z., Musarrat, J. and Javed, S. 2013. Chitinases: An Update. *Journal of Pharmacy and Bioallied Science* **5**(1): 21-29.

Hartl, L., Zach, S. and Seidl-Seiboth, V. 2012. Fungal Chitinases: Diversity, Mechanistic Properties and Biotechnological Potential. *Applied Microbiology and Biotechnology* **93**(2):533-543.

Horiuchi, H. 2009. Functional Diversity in Chitin Synthases of *Aspergillus nidulans* in Hyphal Growth, Conidiophores Development and Septum Formation. *Medical Mycology* **47**:547-552.

Hossain, M.A., Shah, M.D., Gnanaraj, C. and Iqbal, M. 2011. *In vitro* Total Phenolics, Flavanoids Contents and Antioxidant Activity of Essential Oil, Various Organic Extracts from the Leaves of Tropical Medicinal Plant *Tetrastigma* from Sabah. *Asian Pacific Journal of Tropical Biomedicine*:717-721.

Hossain, M.A., Al-Raqmi, K.A.S., Al-Mijizy, Z.H., Weli, A.M. and Al-Riyami, Q. 2013. Study of Total Phenol, Flavanoids Contents and Phytochemical Screening of Various Leaves Crude Extracts of Locally Grown *Thymus vulgaris*. *Asian Pacific Journal of Tropical Biomedicine* **3**(9):705-710.

Hostettmann, K. 1999. Strategy for the Biological and Chemical Evaluation of Plant Extracts. *IUPAC*. (http://www.iupac.org/symposia/proceedings/phuket97/hostettmann.html)

Hue, S.-M., Boyce, A.M. and Somasundrum, C. 2012. Antioxidant Activity, Phenolic and Flavanoid Contents in the Leaves of Different Varieties of Sweet Potato (*Ipomoea batatas)*. *Australian Journal of Crop Science* **6**(3):375-380.

Jiminez-Ortigosa, C., Aimanianda, L.M., Mouyna, I., Alsteens, D., Pire, S., Krappmann, S., Beauvais, A., Dufrene, Y.F., Roncero, C. and Latge, J-P. 2012. Chitin Synthase with a Myosin Motor-Like Domain Control the Resistance of Aspergillus fumigatus to Echinochandins. *Antimicrobial Agents and Chemotherapy* **56**(12):6121-6131.

Jager, A.K. and Stafford, G.I. 2012. Quality Assessment of Tulbaghia Rhizomes. *South African Journal of Botany* **82**:92-98.

Johnson, M.D. and Perfect, J.R. 2010. Use of Antifungal Combination Therapy: Agents, Order, and Timing. *Current Fungal Infection and Respiration* **4**:87-95.

Karimi, E., Jaafar, H.Z.E. and Ahmad, S. 2011. Phytochemical Analysis and Antimicrobial Activities of Methanolic Extracts of Leaf, Stem, Root from Different Varieties of *Labsia pumila* Benth. *Molecules* **16**: 4438-4450.

Kim, T-D., Han, J-Y., Hun, G.H. and Choi, Y-E. 2011. Expression and Functional Characterization of Three Squalene Synthase Genes Associated with Saponin Biosynthesis in Panax ginseng. *Plant and Cell Physiology* **52**(1):125-137.

Krishnan, S., Manavathu, E.K. and Chandrasekar, P.H. 2009. *Aspergillus flavus:* An Emerging non-*fumigatus Aspergillus* Species of Significance. *Mycoses* **52**: 206-222.

Kubec, R., Velisek, J. and Musah, R.A. 2002. The Amino Acid Precursors and Odour Formation in Society Garlic (*T. violacea Harv*). *Phytochemistry* **60**: 21-25.

Lanzotti, V., Romano, A., Lanzuise. S., Bonanomi, G. and Scala, F. 2012(a). Antifungal Saponins from Bulbs of White Onion, *Allium cepa L. Phytochemistry* **74**: 133-139.

Lanzotti, V., Barile, E., Antignani, V., Bonanomi, G. and Scala, F. 2012(b). Antifungal Saponins from Bulbs of Garlic, *Allium sativum* L. var. *Voghiera*. *Phytochemistry* **78**: 126-134.

Latge, J-P. 2007. The Cell Wall: A Carbohydrate Armour for the Fungal Cell. *Molecular Microbiology* **66**(2): 279-290.

Latge, J-P. 2010. Tasting the Fungal Cell Wall. *Cellular Microbiology* **12**(7): 863-872.

Lenardon, M.D., Munro, C.A. and Gow, N.A.R. 2010. Chitin Synthesis and Fungal Pathogenesis. *Current Opinion in Microbiology* **13**: 416-423.

Liang, H. 2008. Sordarin, an Antifungal Agent with a Unique Mode of Action. *Beilstein Journal of Organic Chemistry* **31**: 1-14.

Lindsey, K.L. and van. Staden, J. 2004. Growth Inhibition of Plant Pathogenic Fungi by Extracts of *Allium sativum* and *Tulbaghia violacea*. (Abstract). *South African Journal of Botany* **70**: 671-673.

Liu, W.J.H. 2011. *Traditional Herbal Medicine Research Methods*. Singapore, John Wiley & Sons Incorporated.

Lyantagaye, S.L. 2011. Ethnopharmacological and Phytochemical Review of Allium Species (Sweet Garlic) and Tulbaghia Species (Wild Garlic) from Southern Africa. *Tanzanian Journal of Science* **37**: 59-72.

Lyantagaye, S.L. 2013. Methyl-α-D-Glucopyranoside from *Tulbaghia violacea* Extract Induces Apoptosis *in vitro* in Cancer Cells. *Bangladesh Journal of Pharmacology* **8**: 93-101.

Mabona, U. and van. Vuuren, S.F. 2013. Southern African Medicinal Plants Used to Treat Skin Diseases. *South African Journal of Botany* **87**: 175-193.

Madhuri, S. and Pandey, G. 2009. Some Anticancer Medicinal Plants of Foreign Origin. *Current Science* **96**: 779-783.

Maheshwari, R. 2005. Fungal Biology in the 21st Century. *Current Science* **88**(9): 1406-1418.

Matsuura, H. 2001. Saponins in Garlic as Modifiers of the Risk of Cardiovascular Disease. *Journal of Nutrition* **131**: 1000-1005.

Marston, A. 2011.Thin-Layer Chromatography with Biological Detection in Phytochemistry. *Journal of Chromatography A* **1218**: 2676-2683.

Matsumoto, K. S. 2006. Fungal Chitinases. *Advances in Agricultural and Food Biotechnology*. R. G. A. T.-P. Guevara Gonzalez, I. Kerela, India, Research Signpost: 289-304.

Meesapyodsuk, D., Balsevich, J., Reed, D.W. and Covello, P.S. 2007. Saponin Biosynthesis in *Saponaria vaccaria*. cDNAs Encoding β-Amyrin Synthase and Triterpene Carboxylic Acid Glucosyltransferase. *Plant Physiology* **143**: 959-969.

Mert-Turk, F. 2006. Saponin versus Plant Fungal Pathogens. *Journal of Cell and Molecular Biology* **5**: 13-17.

Morcx, S., Kunz, C., Choquer, M., Assie, S., Blondet, E., Simond-Cote, E., Gajek, K., Chapeland-Leclerc, F., Expert, D. and Soulie, M-S. 2013. Disruption of Bcchs4, Bcchs6 or Bcchs7 Chitin Synthase Gene in *Botryis cinerea* and the Essential Role of Class VI Chitin Synthase (*Bcchs6*). *Fungal Genetics and Biology* **52**: 1-8.

Motsei, M.L., Lindsey, K.L., van Staden, J. and Jager, A.K 2003. Screening of Traditionally Used South African Plants for Antifungal Activity against *Candida albicans*. *Journal of Ethnopharmacology* **86**: 235-241.

Mouyna, I., Hartl, L.and Latge, J-P. 2013. β-1,3-Glucan Modifying Enzymes in *Aspergillus fumigatus*. *Frontiers in Microbiology* **4**(81): 1-9.

Mtiwari, P.G., Ayeka, P.A., Ondicho, J., Matu, E.N. and Bii, C.C. 2013. Antimicrobial Activity and Probable Mechanisms of Action of Medicinal Plants of Kenya: *Withania somnifera, Warbugla ugandensis, Prunus, africana and Plectrunthus*. *PLOS ONE* **8**(6): 1-9.

Munster, J.M., Nitsche, B.M., Krijgsheld, P., van Wyk, A., Dijkhuizen, L., Wosten, H.A., Ram, A.F. and van der Maarel, M.J.E.C. 2013. Chitinases CtcB and Cflcl Modify the Cell Wall in Sporulating Aerial Mycelim of *Aspergillus niger*. *Microbiology* **159**: 1853-1867.

Naoumkina, M.A., Modolo, L.V., Huhman, D.V., Urbanczyk-Wochniak, E., Tang, Y., Sumner, L.W. and. Dixon, R.A 2010. Genomic and Co-expression Analyses Predict Multiple Genes Involved in Triterpene Saponin Biosynthesis in *Medicago truncatula*. *The Plant Cell* **22**: 855-866.

Ncube, N.S., Afolayan, A.J. and Okoh, A.I. 2008. Assessment of Techniques of Antimicrobial Properties of Natural Compounds of Plant Origin: Current Methods and Future Trends. *African Journal of Biotechnology* **7**(12): 1797-1806.

Nteso, L. and Pretorius, J.C. 2006a. *Tulbaghia violacea* L.I: *In Vitro* Antimicrobial Properties towards Plant Pathogens. *Australian Journal of Agricultural Research* **57**: 511-516.

Nteso, L. and Pretorius, J.C. 2006b. *Tulbaghia violacea* L.II: *In Vivo* Antifungal Properties towards Plant Pathogens. *Australian Journal of Agricultural Research* **57**: 517-523.

Onsori, H., Zamani, M.H., Motallebi, M. and Zarghami, N. 2005. Identification of Over Producer Strain of Endo-β-1,4-Glucanase in *Aspergillus* Species: Characterization of Crude Carboxymethyl Cellulase. *African Journal of Biotechnology* **4**(1):26-30.

Paiva, P.M.G., Gomez, F.S., Napoleao, T.H., Sa, R.A., Correia, M.T.S. and Coelho, L.C.B.B. 2010. Antimicrobial Activity of Secondary Metabolites and Lectins from Plants *Current Research Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*. A.Mendez-Vilas. Formatex 396-405.

Patra, J.K., Gouda, S., Sahoo, S.K. and Thatoi, H.N 2012. Chromatographic Separation, ^{1}H NMR Analysis and Bioautography Screening of Methanol Extract of *Excoecaria agallocha* L. from Bhitarkanika, Orissa, India. *Asian Pacific Journal of Tropical Biomedicine*:50-56.

Perez, P. and Ribas, J.C. 2013. Fungal Cell Wall Analysis. *Laboratory Protocols in Fungal Biology: Current Methods in Fungal Biology*. V. K. Gupta, Springer Science and Business Media.

Pierce, C.G., Srinivasan, A., Uppuluri, P., Ramasubramanian, A.K. and Lopez-Ribot, J.L. 2013. Antifungal Therapy with Emphasis on Biofilms. *Current Opinion in Pharmacology* **13**: 726-730.

Pino, J.A., Quijano-Celís, C.E. and Fuentes, V. 2008. Volatile Compounds of *Tulbaghia violacea* Harv. *Journal of Essential Oil Bearing Plants* **11**(2): 203-207.

Ren, Y., Wee, K.E. and Chang, F.N. 2000. Deficiency of Current Methods in Assaying Endochitinase Activity. *Biochemical and Biophysical Communications* **268**: 302-305

Rogg, L.E., Fortwendel, J.R., Juvvadi, P. and Steinbach, W. 2012. Regulation of Expression, Activity and Localization of Fungal Chitin Synthase. *Medical Mycology* **50**: 2-17.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K.M. and Latha, L.Y. 2011. Extraction, Isolation and Characterization of Bioactive Compounds from plant extracts. *African Journal of Traditional and Complementary Alternative Medicine* **8**(1): 1-10.

Sati, S.C. and Joshi, S. 2011. Aspects of Antifungal Potential of Ethnobotanically Known Medicinal Plants. *Research Journal of Medicinal Plants* **5**(4): 377-391.

Satish, S., Mohana, D.C., Raghavendra, M.P. and Raveesha, K.A. 2007. Antifungal Activity of Some Plant Extracts against Important Seed Borne Pathogens of *Aspergillus sp*. *Journal of Agriculture and Technology* **3**: 109-119.

Savoia, D. 2012. Plant-Derived Antimicrobial Compounds: Alternatives to Antibiotics. *Future Microbiology* **7**(8): 979-990.

Sawai, S. and Saito, K. 2011. Triterpenoid Biosynthesis and Engineering in Plants. *Frontiers in Plant Science* **2**(25): 1-6.

Seidl, V. 2008. Chitinases of Filamentous Fungi: A large Group of Diverse Proteins with Multiple Physiological Functions. *Fungal Biology Reviews* **22**: 36-42.

Simmler, C., Napolitano, J.G., McAlpine, J.B., Chen S-N. and Pauli, G.F. 2014. Universal Quantitative NMR Analysis of Complex Natural Samples. *Current Opinion in Biotechnology*. **25**:51-59

Simons, V., Morrissey, J.P., Latijnhouwers, M., Csukai, M., Cleaver, A., Yarrow, C. and Osbourn, A. 2006. Dual Effects of Plant Steroidal Alkaloids on *Saccharomyces cerevisiae*. *Antimicrobial Agents and Chemotherapy* **50**(8): 2732-2740.

Shin, K-W., Kwong, K-J., Kim, Y.H., Park, H-S., Kwong, G-S. and Yu, J-H. 2009. Differential Roles of the ChiB Chitinase in Autolysis and Cell Death of *Aspergillus nidulans*. *Eukaryotic Cell* **8**(5):738-746.

Shobhen, R., Patel, N.M. and Patel, P.M. 2011. A Review on Modification of Analytical Techniques in Herbal Research. *International Journal of Research in Ayurveda and Pharamcy* **2**(5):1483-1485

Singh, S., Pandey, J.M.B., Singh, U.P. and Pandey, V.B. 2009. Antifungal Activity of the Alkaloids from *Eschescholtzia californica*. *Folia Microbiology* **54**(3):204-206.

Soyingbe, O.S., Oyedeji, A.O., Basson, A.K., Singh, M. and Opoku, A.R. 2013. Chemical Composition, Antimicrobial and Antioxidant Properties of the Essential Oils of *Tulbaghia violacea* Harv L.F. *African Journal of Microbiology Research* **7**(18):1787-1793.

Sparg, S.G., Light, M.E. and van Staden, J. 2004. Biological Activities and Distribution of Plant Saponins. *Journal of Ethnopharmacology* **94**:219-234.

Springfield, E.P., Eagles, P.F.K. and Scott, G. 2005. Quality Assessment of South African Herbal Medicines by Means of HPLC Fingerprinting. *Journal of Ethnopharmacology* **101**:75-83.

Teshima, Y., Ikeda, T., Imada, K., Sasaki, K., El-Sayed, M.A., Shygo, M., Tanaka, S. and Ito, S. 2013. Identification and Biological Activity of Antifungal Saponins from Shallot (*Allium cepa* l. Aggregatum Group). *Journal of Agriculture and Food Chemistry* **61**:7440-7445.

Thamburan, S., Klaasen, J., Mabusela, W.T., Cannon, J.F., Folk, W. and Johnson, Q. 2006. *Tulbaghia alliacea* Phytotherapy: A Potential Anti-Infective Remedy for Candidiasis. *Phytotherapy Research* **20**:844-850.

Theis, T. and Stahl U. 2004. Antifungal Proteins: Targets, Mechanisms and Prospective Applications. *Cellular and Molecular Life Sciences* **61**:437-455.

Tistaert, C., Dejaegher, B. and Vander Heyden, Y. 2011. Chromatographic Separation Techniques and Data Handling Methods for Herbal Fingerprints: A Review. *Analytica Chimica Acta* **690**:148-161

Tiwari, P., Kumar, B., Kaur, M., Kaur,G and Kaur, H. 2011. Phytochemical Screening and Extraction: A Review.*International Pharmaceutica Sciencia* **1**(1):98-106.

Tsuizaki, M., Ohta, A. and Horiuchi, H. 2013. Myosin-Like Domain of Class VI Chitin Snthase CsmB of *Aspergillus nidulans* is not Functionally Equivalent to that of Class V Chitin Synthase CsmA. *Bioscience, Biotechnology and Biochemistry*.**77**(2):369-374.

Tzelepis, G.D., Melin, P., Jensen, D.F., Stenlid, J. and Karlsson, M. 2012. Functional Analysis of Glycoside Hydrolase Family 18 and 20 genes in *Neurospora crassa*. *Fungal Genetics and Biology* **49**:717-730.

Tsirilakis, K., Kim, C., Vicencio, A.G., Andrade, C., Casadevall, A. and Goldman, D.L. 2012.Methylxanthine Inhibit Fungal Chitinases and Exhibit Antifungal Activity. *Mycopathologia* **173**:83-91.

Van Wyk, B-E. and Gericke, N 2000. *People's Plants: a Guide to Useful Plants of Southern Africa*. *Pretoria, Briza Publications*.

Vandeputte, P., Ferrari, S. and Coste, A.T. 2011. Antifungal Resistance and New Strategies to Control Fungal Infections. *International Journal of Microbiology* **20**:1-26.

Verwer, P.E.B., ten Kate, M.T., Falcone, F.H., Morroll, S., Verbrugh, H.A., Bakker-Woudenberg, I.A.J.M. and van de Sande, W.W.J. 2013. Evidence Supporting a Role for Mammalian Chitinases in Efficacy of Caspofungin against Experimental Aspergillosis in Immunocompromised Rats. *PLOS ONE* **8**(10): 1-9.

Vicente, M.F., Basilio, A., Cabello, A. and Pelàez, F. 2003. Microbial Natural Products as a Source of Antifungals. *Clinical Microbiology and Infectious Diseases* **9**: 15-32.

Yamazaki, H., Tanaka, A., Kaneko, J., Ohta, A. and Horiuchi, H. 2008. *Aspergillus nidulans* ChiA is a Glycosylphosphatidylinositol (GPI) - Anchored Chitinase Specifically Localized at Polarized Growth Sites. *Fungal Genetics and Biology* **45**: 963-972.

Yang, C.-R., Zhang, Y., Jacob, M.R., Khan, S.I., Zhang, Y.-J. and Li, X.-C. 2006 Antifungal Activity of C-27 Steroidal Saponins. *Antimicrobial Agents and Chemotheraphy* 50: 1710-1714.

Yazdani, D., Tan, Y.H., Zainal Abidin, M.A. and Jaganath, I.B. 2011. A Review on Bioactive Compounds Isolated From Plants Against Plant Pathogenic Fungi. *Journal of Medicinal Plant Research* **5**(30): 6584-6589.

CHAPTER 2

INTRODUCTION TO THE STUDY

2.1 Introduction

Fungal infections have steadily been on the rise over the past decade and opportunistic pathogens causing many of these infections have become a major problem world-wide (Krishnan *et al*., 2009). *Aspergillus* species cause severe infections in humans, plant and animals and amongst these, *A. flavus* is second only to *A. fumigatus* as the leading cause of invasive aspergillosis (Tian *et al*., 2012). *Aspergillus flavus* is also the leading cause of fungal contamination of food products such as mealies (maize) and nuts and produces the toxic secondary metabolite aflatoxin B1. In many developing countries, maize is the staple food of the majority of the population and infection of these food products with aflatoxin B1 poses a major health risk (Williams *et al*., 2004).

Currently, although antifungal agents are available for the treatment of fungal infections, most of these agents are very old and some fungi have to developed resistance towards them and multi drug resistance strains have begun to emerge (Morschhauser 2010). Some antifungal agents have been used although they have been shown to exhibit toxicity towards the host. Synthetic fungicides (pesticides) have been used for the treatment and control of plant pathogens however many are expensive and are toxic to the environment. With continued use and an increase in resistance, higher doses of these fungicides may be required for effective control of fungal pathogens. This may therefore increase the toxicity of the product to the environment and may pose a health risk to humans (Castillo *et al*., 2012). There is therefore an urgent need to develop alternative antifungal agents that are affordable, non-toxic and safe to use for antifungal therapy.

Medicinal plants have been used worldwide as an alternative treatment for various ailments formulated either as a crude extract or after purification of their active compounds with minimal to no side-effects (Arif *et al*., 2011). The chemical diversity of plant compounds within the different species has provided the opportunity to identify and isolate many active components and for the exploration of their antimicrobial nature against a variety of microorganisms (Singh

et al., 2009; Di Liberto *et al*., 2010). Some plant extracts also target a variety of cellular components within a microorganism and this decreases the chances of resistance development. Extracts of *T. violacea* have been shown to exhibit antifungal activity towards a variety of both human and plant pathogens with no reported toxicity or side effects (Motsei *et al*., 2003; Nteso and Pretorius 2006a and b). This makes the compounds derived from plant extracts ideal candidates for use as therapeutic agents for the treatment of fungal infections. Previous studies have shown that an aqueous extract of *T. violacea* has antifungal activity against *A. flavus* and *A. parasiticus* and inhibited fungal germination which is crucial to the development of infection (Belewa *et al*., 2011; Somai and Belewa 2011). This extract affected lipid formation in *A. flavus* and *A. parasiticus* (Belewa 2009). Based on these findings, the plant extract presented as a potential antifungal agent for treatment of *Aspergillus* infections and this study further investigates the mechanism of inhibition of *A. flavus*.

2.2 Objectives of the study

i. To identify, isolate and characterize the antifungal compound(s) within the *T. violacea* aqueous plant extract using TLC and RP-HPLC. With TLC, compounds of the plant extract will be separated with various solvents and the active compounds will be identified using agar overlay technique. The active compounds will be purified from other compounds with preparative TLC followed by RP-HPLC. The structure of the active compounds will be characterized and elucidated by NMR.

ii. To determine secondary metabolites present in the plant extract and the active compound(s) using various biochemical assays.

iii. To investigate the effect of the plant extract on the morphology and structure of *A.flavus* using light and fluorescence microscopy

iv. To determine the effect of the plant extract on ergosterol and other sterol intermediates produced by *A. flavus* using a biochemical assay and RP-HPLC.

v. To investigate the effect of the plant extract on *A. flavus* cell wall components (β-(1, 3) glucan, chitin and chitinases). The effect on β-(1, 3) glucan production and β-glucan synthase activity will be investigated using the aniline blue fluorescence assay. Total chitin content and chitin

synthase activity will be determined with chitin biochemical assay and chitin synthase assay respectively. The effect of the plant extract on chitinase production will be investigated with flourimetric enzyme activity assay using fluorescent substrates of 4-methylumbeliferone glycosides of N- acetyl glucosamine oligosaccharides (4MU-$(GlcNAc)_{1-3)}$.

2.3 References

Arif, T., Mandal, T.K. and Dabur, R. 2011. Natural Products: Antifungal Agents Derived from Plants. *Opportunity, Challenge and Scope of Natural Products in Medicinal Chemistry*. 283-311. Reasearch Signpost, India

Belewa, X.V. 2009. The Effect of *Tulbaghia violacea* Aqueous Plant Extract on the Growth of *Aspergillus* Species. MSc Dissertation., Nelson Mandela Metropolitan University

Belewa, V., Baijnath, H. and Somai, B.M. 2011. Aqueous Extracts from the Bulbs of *Tulbaghia violacea* are Antifungal against *Aspergillus flavus*. *Journal of Food Safety*. **31**:176-184.

Castillo, F., Hernandez, D., Gallegos, G., Rodriguez, R. and Aguilar, C.N. 2012. Antifungal Properties of Bioactive Compounds from Plants. *Fungicides for Plants and Animal Diseases.* Dr. Dharumadurai Dhanasekaran (Ed.) In Tech, China and Europe.

Di Liberto, M., Svetaz, L., Furlan, R.L.E., Zacchino, S.A., Delporte, C., Novoa, M.A., Asencio, M. and Cassels, B.K. 2010. Antifungal Activity of Saponin-Rich Extracts of *Phytolacca dioica* and of the Sapogenins Obtained through Hydrolosis. *Natural Products Communication* **5**(7):1013-1018

Krishnan, S., Manavathu, E.K. and Chandrasekar, P.H. 2009. *Aspergillus flavus:* An Emerging non-*fumigatus Aspergillus* Species of Significance. *Mycoses* **52**: 206-222

Morschhäuser, J. 2010. Regulation of Multidrug Resistance in Pathogenic Fungi. *Fungal Genetics and Biology*.**47**:94-106

Motsei, M.L., Lindsey, K.L., van Staden, J. and Jager, A.K 2003. Screening of Traditionally Used South African Plants for Antifungal Activity against *Candida albicans*. *Journal of Ethnopharmacology* **86**: 235-241.

Nteso, L. and Pretorius, J.C. 2006a. *Tulbaghia violacea* L.I: *In Vitro* Antimicrobial Properties towards Plant Pathogens. *Australian journal of Agricultural Research* **57**:511-516.

Nteso, L. and Pretorius, J.C. 2006b. *Tulbaghia violacea* L.II: *In Vivo* Antifungal Properties towards Plant Pathogens. *Australian journal of Agricultural Research*.**57**:517-523.

Singh, S., Pandey, J.M.B., Singh, U.P. and Pandey, V.B. 2009. Antifungal Activity of the Alkaloids from *Eschescholtzia californica*. *Folia Microbiology* **54**(3):204-206.

Somai, B.M. and Belewa, V. 2011. Aqueous Extracts of *Tulbaghia violacea* Inhibit Germination of *Aspergillus flavus* and *Aspergillus parasiticus* Conidia. *Journal of Food Protection* **74**(6):1007-1011

Tian, J., Ban, X., Zeng, H., He, J., Chen, Y. and Wang, Y. 2012. The Mechanism of Antifungal Action of Essential Oil from Dill (*Anethum graveolens* L.) on *Aspergillus flavus*. *PLoS One*. **7**(1):1-10

Williams, J.H., Phillips, T.D., Jolly, P.R., Stilles, J.K., Jolly, C.M. and Aggarwal, D. 2004. Human Aflatoxicosis in developing countries: A Review of Toxicology, Exposure, Potential Health Consequences and Intervention. *American Journal of Clinical Nutrition*. **80**: 1106-1122

CHAPTER 3

ISOLATION AND PARTIAL PURIFICATION OF THE ANTIFUNGAL COMPONENT OF *Tulbaghia violacea* AQUEOUS PLANT EXTRACT

3.1 Introduction

Plants, their products and /or plant extracts have been used as a source of medicine for many years throughout the world. The WHO estimates that about 80% of the world's population rely solely on medicinal plants as a source of medicine (Scorzoni *et al*., 2007). The advent of modern medicine has led to investigations into the safety, efficacy and mechanism of action of many prepared plant extracts (Eloff, 1998; Sasidharan *et al*., 2011). Amongst others, this involves the extraction, isolation and identification of the active compound(s) within the plant, followed by its chemical and biochemical characterization.

Crude plant extracts are traditionally used as herbal medicine for the treatment of a variety of ailments. The extracts are taken as infusions, decoction or as herbal tea (Wendakoon *et al*., 2012). The variety of active chemical components within a single plant extract can provide an indication of its efficacy and potential antimicrobial activity. There is therefore a need for the isolation and characterization of these chemical components for their development as potential chemotherapeutic agents (Mir *et al*., 2013). Most of the active chemical components in plant extracts are present in small quantities and their chemical composition is influenced by factors such as seasonal variation, extraction method and environmental growth conditions (Hoai *et al*., 2009). Chromatographic techniques have been used as a fingerprinting method for the identification and isolation of the active components within crude plant extracts and provides information on the chemical composition of the extract. They are also used as a tool for assessment of the quality of plant extracts and traditional medicines (Sirikatitham *et al*., 2007; Tang *et al*., 2010).

Extracts of *T. violacea* have been used to treat a variety of ailments which include asthma, tuberculosis, colds and flu (Soyingobe *et al*., 2013). The rhizomes of *T. violacea* are known to possess sulphur-containing compounds which have previously been extracted either with

methanol or a mixture of methanol and water (Kubec *et al*., 2002; Lyantagaye *et al*., 2013). Saponins and flavanols are some of the phytochemicals that have also been found in *T. violacea* (Lyantagaye 2011) and these may contribute to the antimicrobial activity of this plant and its extracts.

The aim was to use TLC and RP-HPLC techniques to isolate and purify the active antifungal component/s within *T. violacea* aqueous plant extracts and also perfom phytochemical analysis of plant extract and the antifungal fraction.

.

.

3.2 Materials and Methods

3.2.1 Reagents and equipment used

The reagents used in this study were of analytical grade and were purchased from Sigma Aldrich and Merck Millipore South Africa. The following reagents were bought form Merck Millipore: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium chloride (MTT), acetone, acetonitrile, analytical thin layer chromatography plates (20 x 20cm, $F_{254;}$ Aluminium backed); ethyl acetate, ethanol, hexane, hydrochloric acid, methanol, sucrose, sulphuric acid, vanillin and yeast extract sucrose (YES). The following were purchased from Sigma: Folin-Ciocalteu reagent, ammonium ferric chloride, anisaldehyde catechin, diosgenin, ferric chloride, gallic acid, pyrogallol and preparative TLC plates. Phenomenex C18 (150mm x 4.6mm, 5µm) and PFP (150mm x 4.6mm, 5µm) columns were purchased from Separations and a Scalar Prep Column (100mm x 4.6mm; 5µm) was purchased from Agilent Technologies. An HPLC system from Agilent (Agilent1100 series), consisting of a degasser, binary pump, autosampler, fraction collector and a diode array detector was used for the separation of the plant extract and for obtaining the UVspectra. All experiments were done three times.

3.2.2 Collection and extraction of plant material

The *T. violacea* plants were obtained from Prof Baijnath at the University of Kwazulu Natal (UKZN) and the voucher specimen deposited at the herbarium at UKZN. The leaves, bulbs (rhizomes) and roots were separated and the bulbs were washed under running water to remove soil debris. Whole bulbs were then used for the extraction process. The bulbs (1000g) were mixed with sterile deionised water (2l) and ground with a blender to obtain a slurry. The slurry was allowed to stand for 24hr at room temperature with intermittent mixing to allow the extraction to take place. The mixture was filtered through Whatmann No. 24 filter paper and the extraction and filtration process was repeated at least once. The filtrates were pooled, filter sterilized by sequentially passaging through a 0.45µm and 0.22µm filters, frozen at -80°C and freeze-dried (Virtis, SP Scientific) overnight to obtain a dry powder, which gave a total yield of 50% (weight of bulbs/weight of freeze-dried sample X 100). This was kept in the dark at 4°C until further use.

3.2.3 Separation and partial purification of the antifungal compound within the aqueous plant extract

3.2.3.1 Thin layer chromatography (TLC)

For TLC separation, initially the freeze-dried plant extract was resuspended in water at a concentration of 100 mg/ml and 15µl of the plant extract (1.5 mg) was applied onto the surface of several analytical silica gel 60 F_{254} TLC plates. To optimize the maximum separation of most of the compounds, various solvent mixtures were used as developing agents. These included different ratios of ethyl acetate: hexane (EH, 95:5; 60:40; 55:45), hexane: ethyl acetate: acetone (HEA, 80:10:10) and ethyl acetate: methanol: water (EMW, 40:5.4:5). After development with the solvent mixture, plates were allowed to dry thoroughly and the components of the plant extract were visualised by spraying each plate with a mixture of vanillin-sulphuric acid (0.1 g vanillin; 28 ml ethanol; 1 ml concentrated sulphuric acid). Plates were then heated at 110°C for 5 min or until there was colour development of the compounds (Masoko and Eloff, 2006).

3.2.3.2 Identification of the antifungal compound: Agar overlay assay

Once the optimal mobile phase combinations were identified (EMW 80:10:10; HEA1 40:5.4:5) for the separation of the plant extract, duplicate plates were spotted as mentioned above (section 3.2.3.1) and developed using the optimized solvent system. The plates were dried, one plate was stained to visualize the bands as described above (section 3.2.3.1) and an unstained duplicate plate was then overlaid with YES agar medium (1g Yeast extract, 10g Sucrose, 15g agar and 1L double distilled water) seeded with 1 x 10^6 *A. flavus* spores. Once the agar had solidified, overlaid plates were incubated for 48hr to allow for germination of the spores. After 48hr, plates were sprayed with a 5mg/ml MTT solution and incubated for a further 4hrs to allow for the conversion of the tetrazolium salt (yellow in colour) in MTT into a purple formazan derivative by living fungal cultures. Plates were observed for any zone(s) of clearing originating over any of the separated compounds/bands on the TLC plate which would indicate the position of the active (antifungal) compound (Masoko and Eloff, 2006).

3.2.3.3 Isolation of the antifungal compound: Preparative TLC

After identification of the antifungal band by TLC separation and agar overlay above (section 3.2.3.2.1), the plant extract was loaded onto preparative TLC plates and separated using the optimized protocols as described above (section 3.2.3.1). The antifungal compound was then visualized by UV, scraped off the plate and eluted from the silica gel with 100% methanol. The elution process was repeated at least once to maximize removal of the compound from the silica.The methanol extracts were combined and left to evapourate. The remaining residue was freeze-dried to obtain a white powder. This was kept at 4°C in the dark until further use (Sharma *et al*., 2009).

3.2.4 Partial purification of the antifungal compound

3.2.4.1 High pressure liquid chromatography (HPLC)

The antifungal compound from several isolations prepared on preparative TLC extractions were combined and further purified with RP-HPLC separation. Three different columns; the Phenomenex C18 column (150 mm x 4.6 mm, 5µm), the Phenomenex pentafluorophenyl (PFP) column (150 mm x 4.6 mm; 5µm) and the Agilent Scalar preparative C18 column (100 mm x 4.6mm; 5µm) were used. A 10µl solution containing 0.5 mg of the antifungal compound was injected onto an HPLC column at a flow rate of 0.5 ml/min, using various ratios of acetonitrile-water as the isocratic mobile system. A wave scan was completed to determine the ideal wavelength at which the components(s) present within the antifungal band absorbed maximally and this wavelength was subsequently used for all further RP- HPLC analyses. Individual peaks were collected, dried and resuspended in water before being tested for antifungal activity using the TLC agar overlay assay as described above (section 3.2.3.2.1). Once the antifungal fraction (HEA1) was positively identified, additional preparative TLC purified plant extract material was repetitively subjected to RP-HPLC separation and identified antifungal fractions were collected for further analysis.

3.2.5 Phytochemical analysis of the crude plant extract and the active HEA1 fraction

3.2.5.1 Quantification of total phenolic compounds

The total phenolic content in the *T. violacea* aqueous plant extract and in HEA1 was determined using the Folin-Ciocalteu (Folin C) assay. Fifty microliters of the crude plant extract (resuspended in water) or HEA1 (resuspended in water) were each separately mixed with 950 µl of sterile double distilled water. This was followed by the addition of 500 µl of Folin C reagent and 2.5 ml of 2% sodium carbonate to each tube. Each solution was vortexed and incubated at room temperature for 40 min. A blank consisting of water (instead of the plant extract or active compound) was also prepared. The tubes were read at 725nm (Biochrom Libra S12) against the blank. A standard curve (Appendix 1, Fig A1) using gallic acid ranging from 0 -250 µg/ml was generated and used to determine the total phenolic content of the crude extract and HEA1 respectively. These were expressed as gallic acid equivalents. The experiment was done at least three times (Ncube *et al*., 2011).

3.2.5.2 Quantification of tannins

A colorimetric butanol-HCl assay was used to quantify condensed tannins in the plant extract and in HEA1. Five hundred microliters of the crude plant extract (resuspended in water) or HEA1 (resuspended in water) were separately mixed with 3ml butanol-HCl (95:5) and 100µl of ferric reagent. These were vortexed and placed in a water bath at 95°C for 60 min. After the incubation period, the samples were allowed to cool and absorbance was determined at 550nm (Biochrom Libra S12). A blank consisting of only water instead of the plant extract or HEA1 was also prepared. Pyrogallol ranging from 0 -70 mg/ml was used to generate a standard curve (Appendix 1, Fig A2) to quantify the amount of condensed tannins present within the plant extract and HEA1 and these values were expressed as pyrogallol equivalents (Ncube *et al*., 2011).

3.2.5.3 Vanillin-HCl assay for detection of flavanols

Flavanols were detected using a modified procedure of Sun *et al*., (1998). Fifty microliters of crude plant extract (resuspended in water) or HEA1 (resuspended in water) was mixed with 950 µl of sterile double distilled water. An equal volume of 1% vanillin in methanol (Appendix 1) was added to the plant extract or HEA1, followed by the addition of 2.5 ml of methanol-HCl

(95:5). The tubes were incubated at room temperature for 20min and after colour development the absorbance was read at 500 nm (Biochrom Libra S12). A blank consisting of only water instead of the plant extract or the active compound was also prepared. Catechin was used as a reference and a standard curve ranging from 0 -100 mg/ml was generated to quantify the amount of flavanols present in the plant extract and in HEA1 (Appendix 1, Fig A3).

3.2.5.4 Quantification of saponins

3.2.5.4.1 Total Saponin content

For the quantification of the total saponin content, 125 µl of the crude plant extract (resuspended in water) or HEA1 (resuspended in water) was mixed with an equal volume of 8% vanillin (in ethanol) respectively. This was followed by the addition of 2.5 ml of 72% sulphuric acid followed by incubation at 65°C for 10min. After the incubation period tubes were placed on ice for 5min. After the tubes had cooled, the absorbance was measured at 535 nm (Biochrom Libra S12). Diosgenin was used as the standard and a concentration ranging from 0 – 4 mg/ml was used to generate a standard curve for the quantification of saponins within the crude plant extract and HEA1. A suitable blank containing vanillin and sulphuric acid was prepared. The amount of saponin detected was expressed as diosgenin equivalents (Appendix 1, Fig A4) (Ncube *et al*., 2011).

3.2.5.4.2 Steroidal saponin content

Steroidal saponins were quantified by a modified anisaldehyde-sulphuric acid procedure as described by Ncube *et al*., (2011). Three hundred microliters of plant extract (resuspended in water) or HEA1 (resuspended in water) was mixed with 2ml ethyl acetate in a test tube. This was followed by the addition of 1ml anisaldehylde-ethyl acetate reagent (0.5:95.5 v/v) and 1ml of sulphuric acid-ethyl acetate reagent (50:50). The mixture was vortexed for 2min, followed by incubation at 60°C for 20min. The tubes were then allowed to cool at room temperature for 10min and the absorbance measured at 430nm (Biochrom Libra S12). A blank containing only ethyl acetate instead of the sample was also prepared. A standard curve was prepared with diosgenin standard at a concentration ranging between 0 – 2 mg/ml. Plant extract and active compound steroidal saponin content were extrapolated from the standard curve (Appendix 1, Fig A5) and expressed as diosgenin equivalents.

3.3 Results

3.3.1 TLC separation and screening of crude plant extract

TLC was used to separate the different types of compounds present within *T. violacea* crude plant extract, using solvents of various polarities. Initially, a combination of ethyl acetate: hexane was used. With a solvent of 95:5 ethyl acetate: hexane, one major band close to the solvent front (R_f = 0.99) was observed and minor bands (R_f = 0.15 and 0.31 respectively) were found near the point of origin (Fig. 3.1). With an increase in the percentage of hexane, the R_f value of the major compound decreased. Progressively fewer minor bands were obtained and at the highest concentration of hexane (45%), the minor bands failed to migrate from the origin. Based on differential staining, the brighter staining products were considered to be major elution products since they were at higher concentrations while the lighter staining bands were considered minor elution products since their concentrations were low. In these solvents most of the detectable material remained at the origin, and a small amount seemed to have migrated. This indicates that there may be a need for a higher percentage of the nonpolar solvent and a third polar solvent may be needed to improve the separation of the plant extract.

Subsequently, a combination of hexane: ethyl acetate: acetone (HEA; 80:10:10) was tested. With this solvent, more bands were eluted from the origin than with the ethyl acetate: hexane (HE) solvent. A major band (R_f = 0.29) close to the origin was detected and several minor bands (R_F = 0.21, 0.41, 0.69 and 0.87) eluting higher than the major band were also found (Fig 3.2).

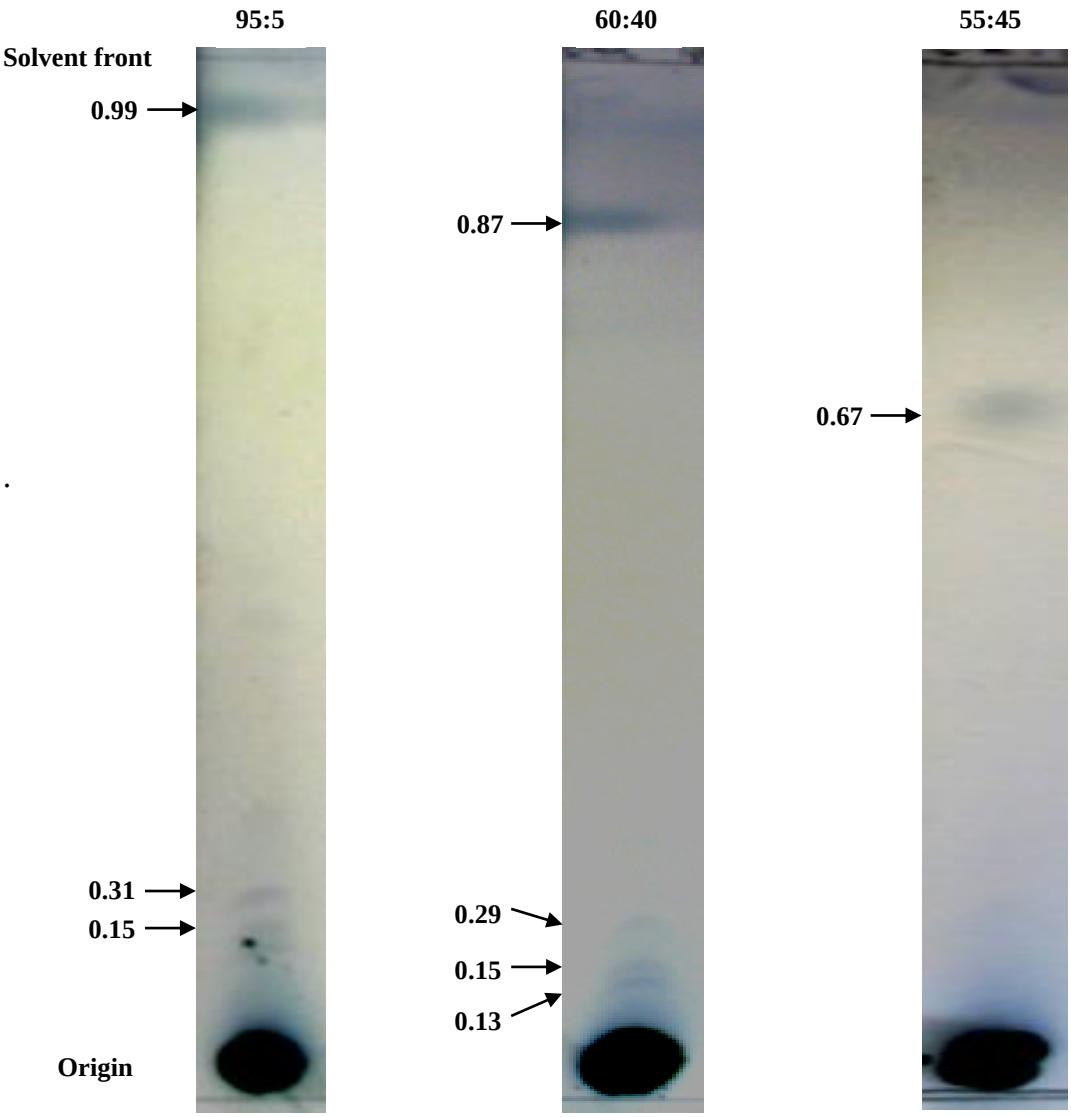


Figure 3.1: **TLC analysis of the *Tulbaghia violacea* crude plant extract with various ratios of ethyl acetate and hexane. The plates were stained with vanillin sulphuric acid and after heating at 100°C for 5 min, stained bands (indicated with arrows) were observed and their respective R_f values were calculated.**

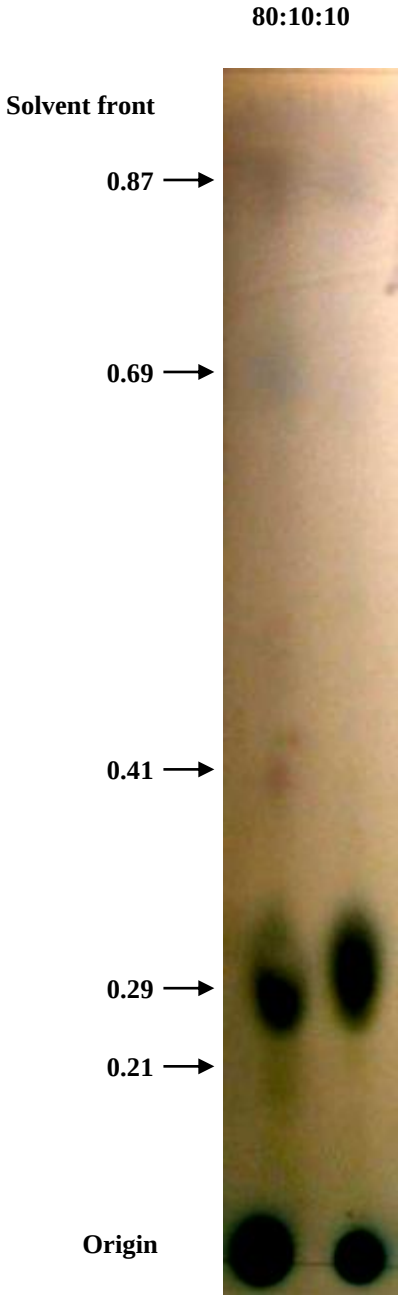


Figure 3.2: TLC analysis of *Tulbaghia violacea* crude plant extract with HEA (80:10:10) as the mobile phase. The plate was stained with vanillin sulphuric acid and after heating at 100°C for 5 min, various bands were obtained and their respective R_f values were calculated.

A combination of ethyl actetate: methanol: water (EMW; 40:5.4:5, Eloff *et al*., 2011) was also tested. Again, similar to the HEA solvent, one major band (R_f = 0.33) close to the origin was obtained and several minor bands (R_f = 0.64; 0.79; 0.81; 0.87 and 0.94) close to the solvent front was observed (Fig 3.3).

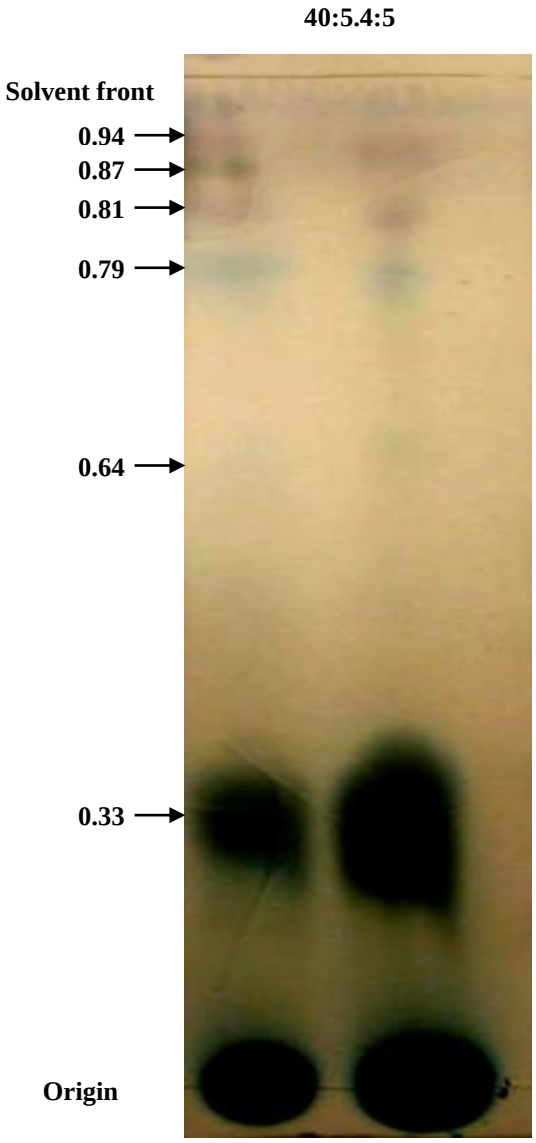


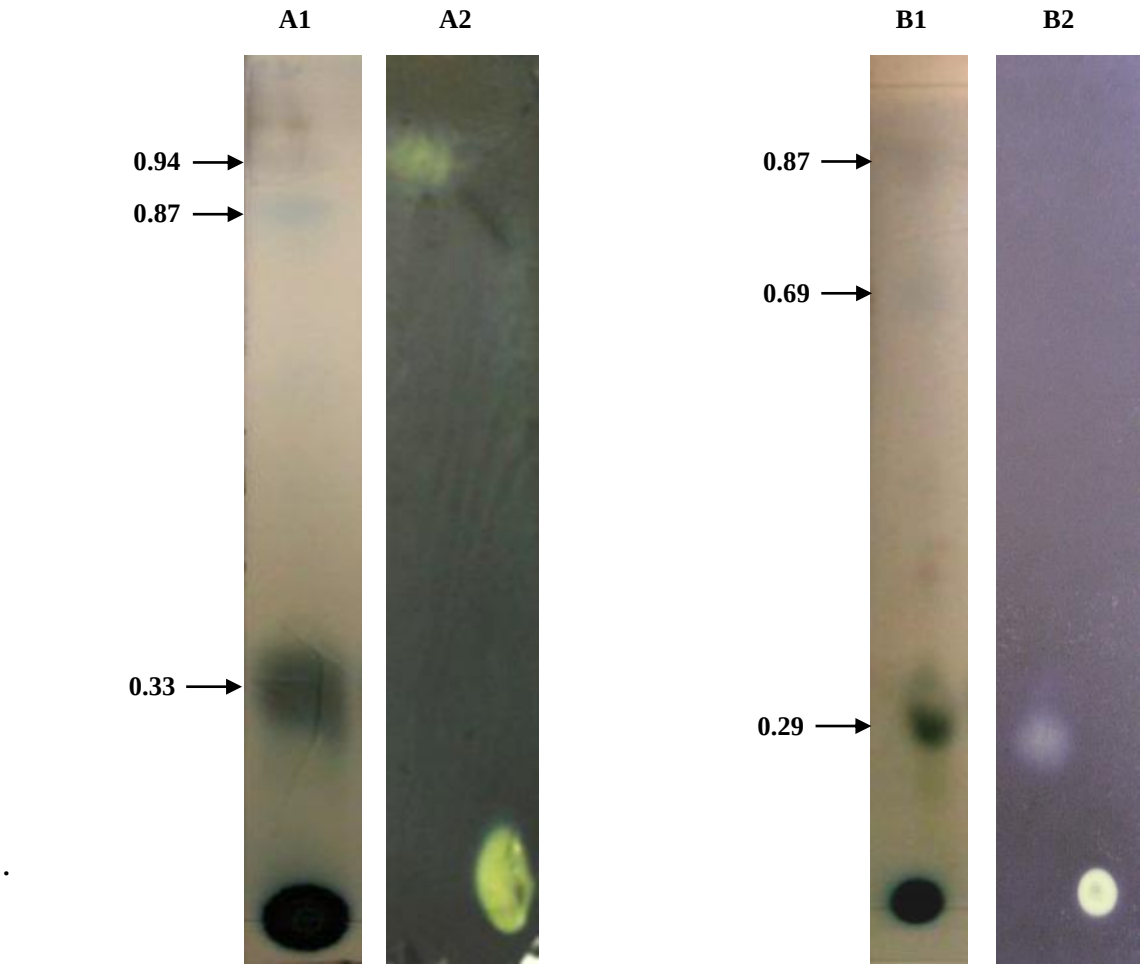
Figure 3.3: TLC of the aqueous plant extract using EMW (40:5.4:5) as the mobile phase. The plate was stained with vanillin sulphuric acid and after heating at 100°C for 5 min to visualize the various bands, their respective R_f values were determined.

The use of the different solvents indicated that various types of compounds were present in the crude plant extract as shown by the different coloured compounds obtained after development of the TLC plates with vanillin sulphuric acid. The TLC separation with different ratios of HEA indicated that the crude extract had polar and nonpolar compounds present. Better resolution of the plant extract components was obtained with the EMW solvent system as this solvent mixture generated more compounds that were distinctly separated from one other. For all solvent

systems, a large amount of sample remained bound at the origin indicating that a large amount of polar compounds present in the sample could not be separated from the silica with either solvent system. With all solvent systems tested, the bluish-black band with R_f values ranging from 0.29 to 0.33 could indicate the presence of saponins in the plant extract (Wettasinghe *et al*., 2001; Sindhu 2011; Taganna *et al*., 2011; Jayashree 2013). The purple bands with $R_f = 0.94$ and $R_f = 0.87$ possibly indicates the presence of terpenoids, while the light blue compounds with $R_f = 0.69$, $R_f = 0.71$ and $R_f = 0.84$ could indicate the presence of putative flavanoids (Taganna et *al*., 2011) because these are the staining colours of these compounds

3.3.2 Agar overlay assay

Since EMW and HEA gave the best separation of the plant extract compounds, these solvents were subsequently used to separate the components of the plant extract using TLC. The TLC separation was completed in duplicate, one plate was stained to visualize the bands and the second unstained plate was used to determine which band/component exhibited antifungal activity using an agar overlay assay. After 48 hr of incubation and after spraying the plates with MTT, a zone of clearing/inhibition around a single distinct band against the purple stained background was observed for each solvent indicating the presence of an antifungal agent. The EMW solvent system showed the presence of the bioactive compound at an $R_f = 0.94$. From the stained duplicate plate and calculation of the R_f value, the band was identified as the one closest to the solvent front and appeared to be co-migrating with the solvent front. With the HEA solvent, the antifungal agent was located at an $R_f = 0.29$ (Fig 3.4). Nystatin, a known antifungal agent was used as a positive control and exhibited a zone of inhibition/clearing on the same agar overlay plate indicating that the agar overlay system was functional.



.

Figure 3.4: **Agar overlay(A) of *T violacea* aqueous crude plant extract, separated with EMW (40:5.4:5) and (B) HEA (80:10:10) solvent systems. A1 = plate was stained with vanillin-suplhuric acid; A2 = agar overlay assay plate stained with MTT after 48hr of incubation; B1 = plate stained with vanillin-sulphuric acid; B2 = agar overlay assay plate stained with MTT after 48hr of incubation. The zones of clearing against the purple background represent the antifungal band**

3.3.3 Isolation of the bioactive compound by preparative TLC

In order to test individual bands for their antifungal activity, the plant extract was then separated on preparative TLC plates using either the EMW or HEA solvent systems. Individual bands scraped off the TLC plates and extracted from the silica were then concentrated, resuspended in water and separated on analytical TLC plates in duplicate. Again an agar overlay was completed to confirm the presence of the antifungal band. Analysis of the preparative TLC plates under UV

light indicated the presence of many more bands than obtained with vanillin-sulphuric acid staining. Eight distinct bands were obtained from the plant extract using the EMW solvent system (Fig 3.5A). This indicated that additional compounds had been eluted from the plant extract and were only detectable by UV and were not stained by vanillin. From the agar overlay assay of the isolated bands (Fig 3.5B) only the 8th band (EMW8) with R_f = 0.94 tested positive for antifungal activity.

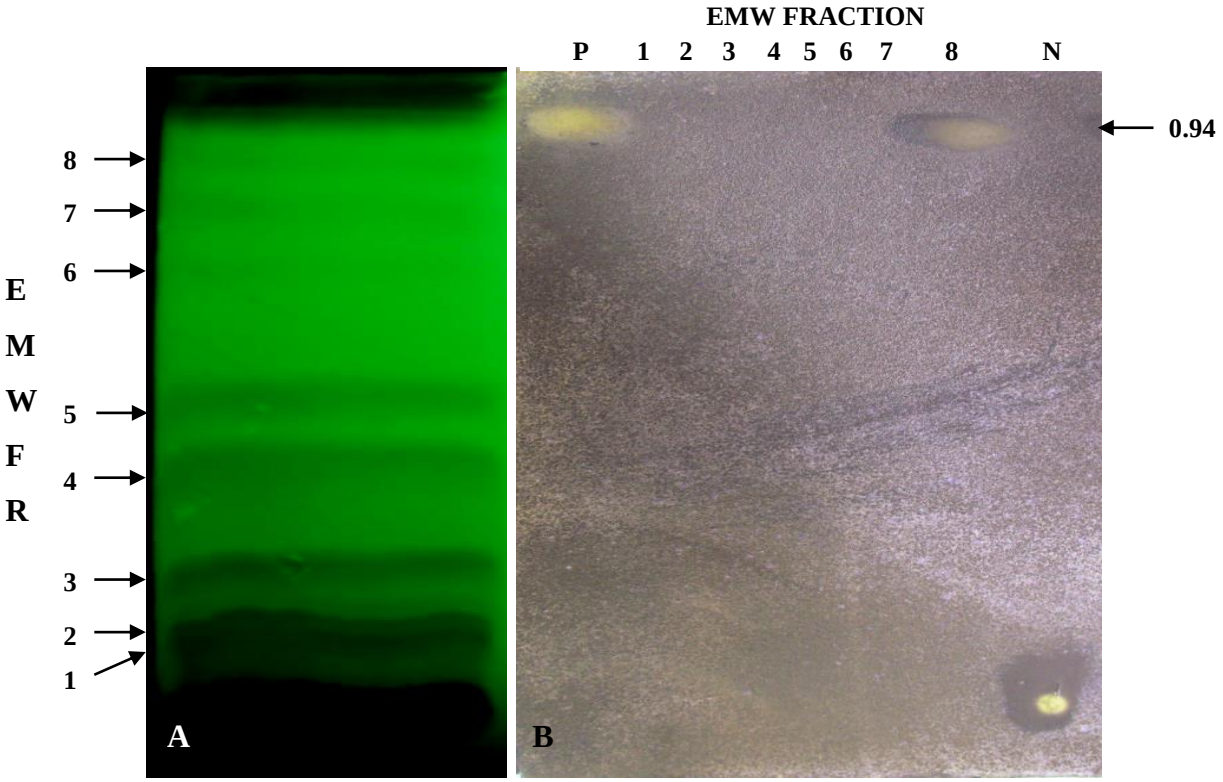


Figure 3.5: Preparative TLC (A) of the *T. violacea* aqueous plant extract with EMW (40:5.4:5) as the mobile phase and visualization under UV light at 320nm. (B) Agar overlay assay on an analytical TLC plate after isolation of the bands obtained from A. P = crude plant extract; 1-8 = compounds isolated from the preparative TLC in A; N = Nystatin.

Analysis of the plant extract after development with the HEA solvent similarly displayed more fractions when visualized with UV light than with vanillin staining. The individual fractions were scraped, isolated from the silica, concentrated, separated on analytical TLC plates and used

for an agar overlay assay. The agar overlay showed that band no 1 (HEA1) at R_f = 0.29 exhibited the antifungal activity (Fig 3.6).

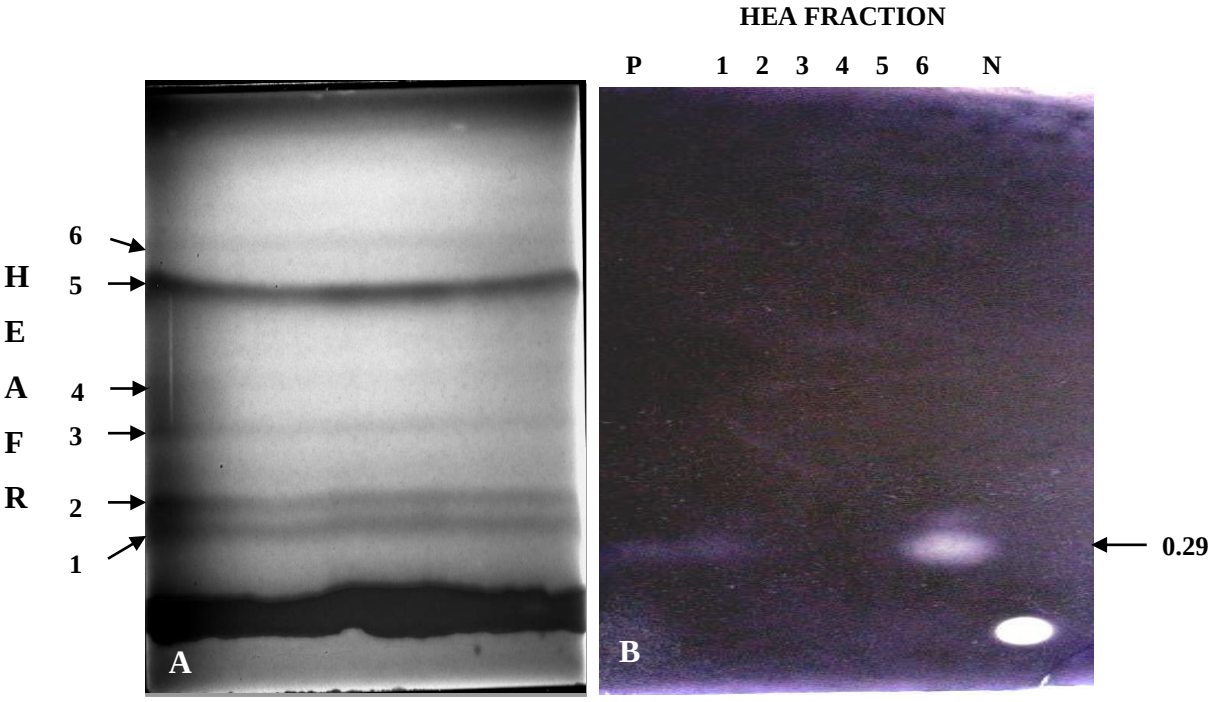


Figure 3.6: Preparative TLC (A) and agar overlay (B) of *T. violacea* plant extract after developing with HEA, (80:10:10) as the mobile phase. Numerous compounds that separated were visualized under UV light at 320nm (A). An agar overlay was done on the isolated compounds (B) to confirm the location of the antifungal compound. P = plant extract; 1-6 represent the compounds isolated from preparative TLC plate in A; N = Nystatin. Band no.1 tested positive for antifungal activity as indicated by the zone of clearing.

3.3.4 Chemical fingerprinting and purification by RP-HPLC

Three columns were used for fingerprinting the plant extract: a Phenomenex C18 Agilent Scalar prep C18 and a Phenomenex PFP. A RP-HPLC analysis of the plant extract was done to obtain fingerprints of the various compounds present within the extract for each column. The optimal wavelength for detection of HEA1 was found to be 210 nm. A ratio of 60:40 water: acetonitrile mixture with an optimal flow rate of 0.5 ml/min gave optimal separation with the lowest retention times for the peaks. The Phenomenex C18 column separated 12 component peaks from the plant extract with major peaks detected at 2.32.4 and 3.7 min respectively (Fig 3.7A). There

were 7 peaks resolved with the Scalar prep column with major peaks eluting at 1, 1.4, 1.6 and 1.8 min (Fig 3.7B). The resolution of these peaks was poor and resulted in the column not being used for further analyses. Only 6 peaks were resolved using the PFP column with the major peak detected at 2.7 min (Fig 3.8). These crude plant extract-derived fingerprints were then compared to the fingerprints derived from the partially purified antifungal compounds (EMW8 and HEA1) using the same columns and HPLC conditions.

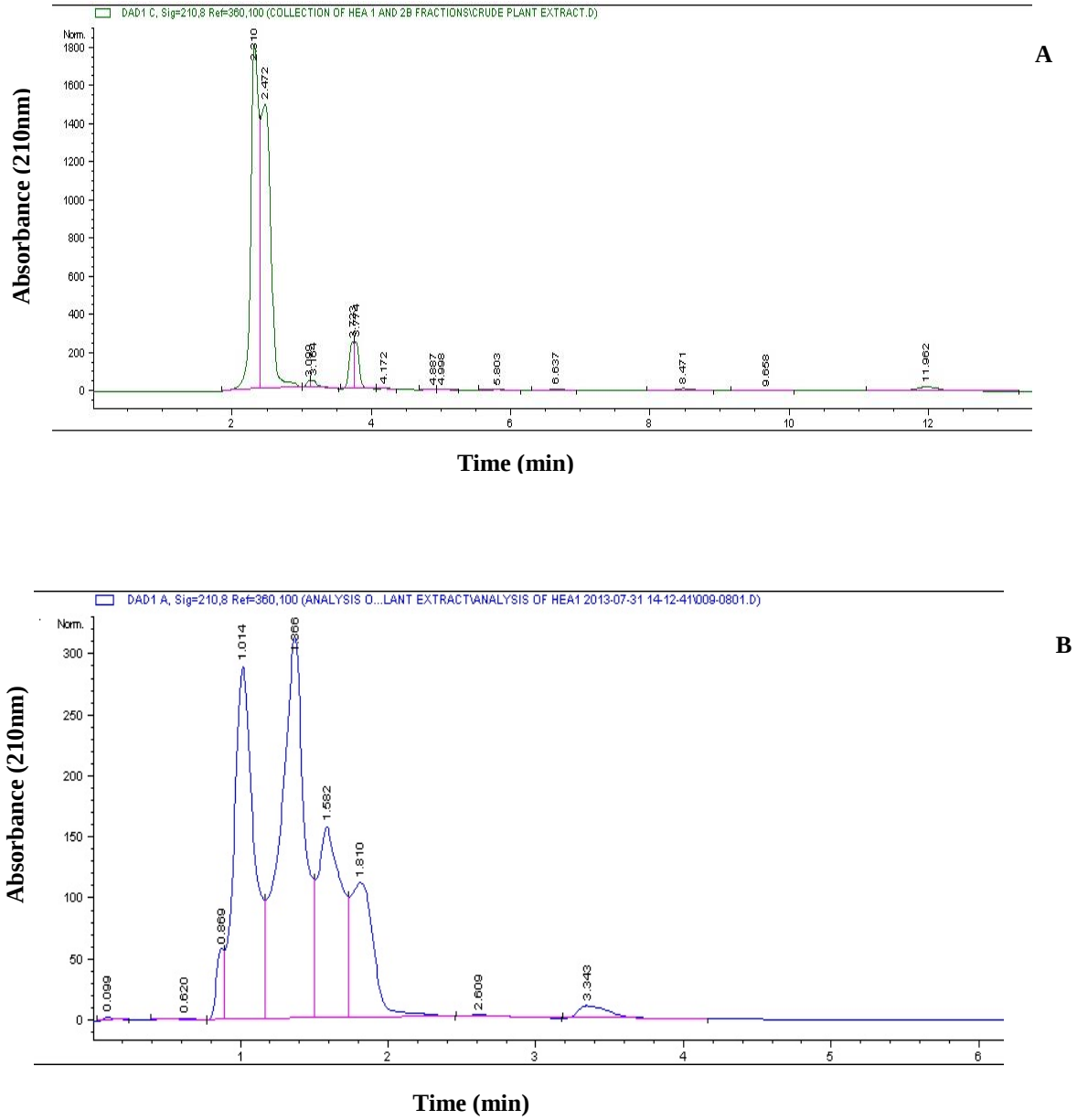


Figure 3.7: The RP- HPLC chromatograms of *T. violacea* plant extract using the (A) Phenomenex C18 and (B) Scalar prep C18 columns at a wavelength of 210 nm and flow rate of 0.5 ml/min.

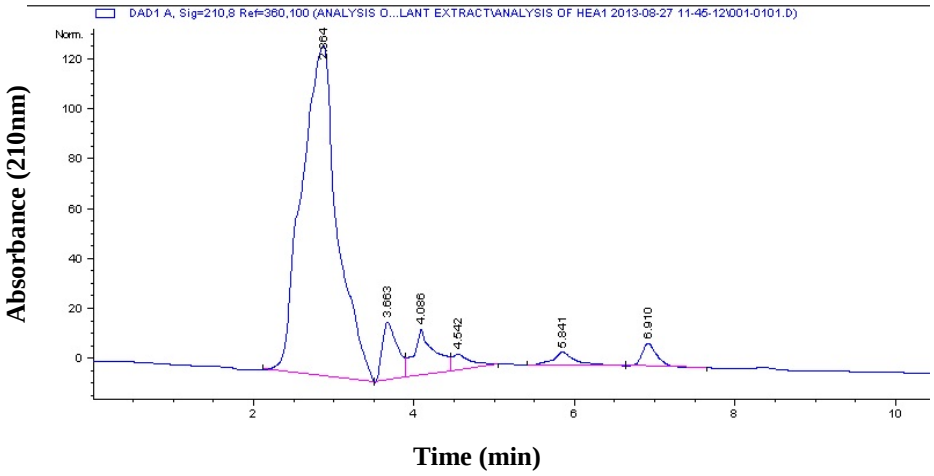


Figure 3.8: A typical RP-HPLC chromatogram of *T violacea* plant extract using the PFP column at a wavelength of 210nm and flow rate of 0.5 ml/min.

The RP-HPLC profile of EMW8 using the Phenomenex C18 column resolved 16 peaks with various retention times (Fig 3.9). Major peaks were detected at 2.5, 2.6 and 3.9 min. Overall the profile of EMW8 was similar to that of the plant extract with all 16 peaks having eluted within 7 min. This indicates that there was probably a mixture of compounds present in EMW8 implying that components within the crude plant extract had not adequately separated by TLC. As a result a switch was made to a more non-polar solvent in an attempt to obtain better separation of these compounds. An identification of the antifungal peak after fractionation of the peaks from the Phenomenex C18 column proved difficult due to the extremely low yield of compounds that were obtained after fractionation.

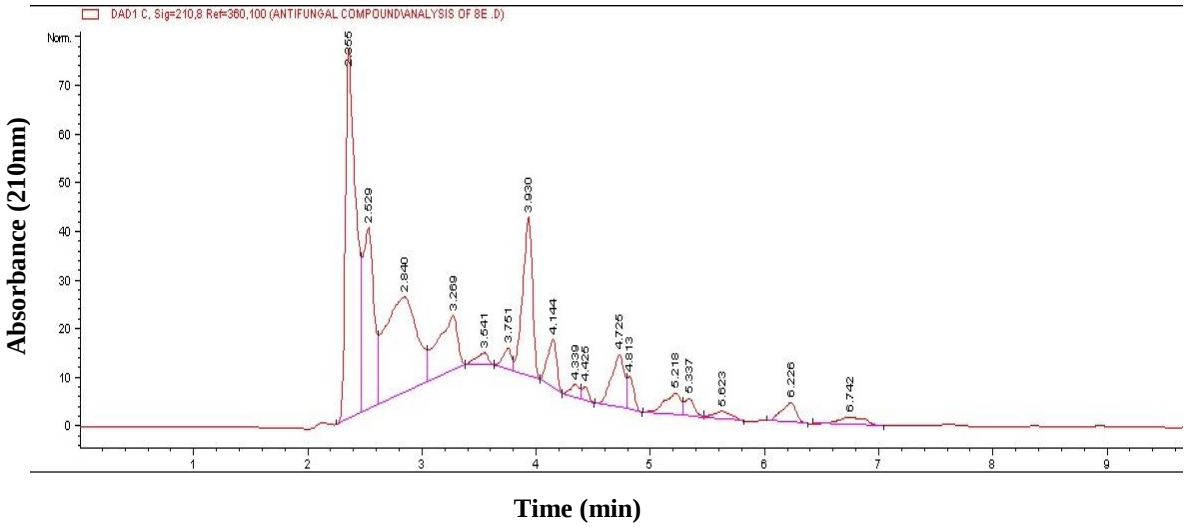


Figure 3.9: A typical HPLC chromatogram of EMW8 with the Phenomenex C18 column at a detection wavelength of 210nm and flow rate of 0.5ml/min.

The HEA1 fraction was then analysed with three different HPLC columns, fractions collected and individually tested for antifungal activity. Again, various peaks were resolved with the Phenomenex C18 column. Six minor peaks were obtained together with a single major peak which eluted at 3.9 min. (Fig 3.10A). The major peak was then fraction collected and tested for antifungal activity using the TLC agar overlay assay. This major peak tested positive for antifungal activity (data not shown). Re-chromatography of this antifungal peak using RP-HPLC showed the presence of a mixture of compounds with 3 major peaks being resolved at 2.4, 3.9 and 4.7 min (Fig 3.10B). Owing to the low yield of material that was obtained after re-chromatography, it was not possible to re-test the antifungal activity of these new peaks. Reverse phase-HPLC analysis of HEA1 with the Scalar prep C18 column resulted in 5 major peaks at 1, 1.3, 1.6, 1.8 and 3.6 min (Fig 3.10C). However, the fraction yields as determined by the absorbance units were lower than that with the Phenomenex column and antifungal testing of the peaks was therefore not done.

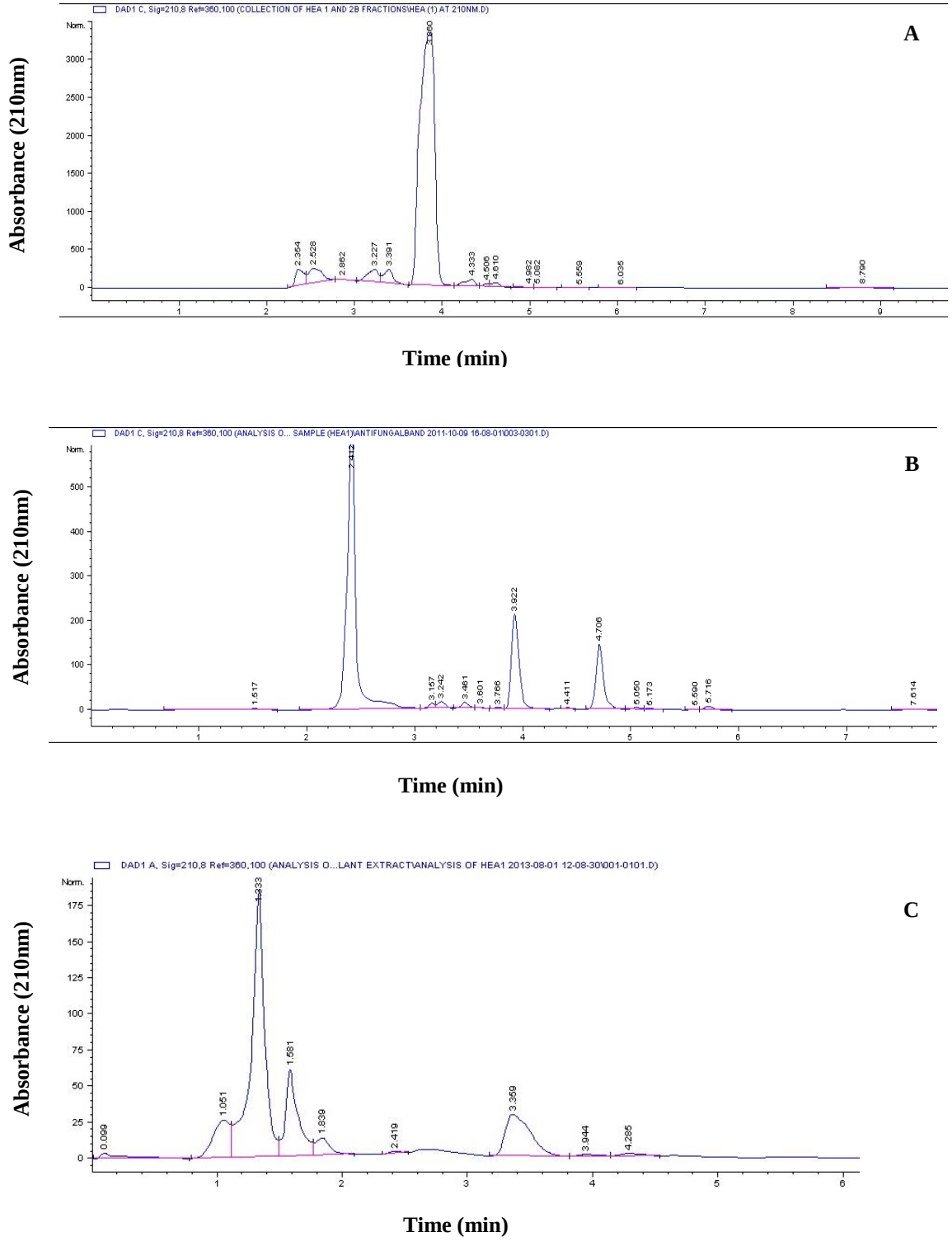


Figure 3.10: The RP-HPLC chromatograms of HEA1 (A) and re-chromatography of the major peak collected at an elution time of 3.7min (B) on the Phenomenex C18 column. (C) Scalar prep C18 column chromatography of HEA1. Detection was done at a wavelength of 210nm at a flow rate of 0.5ml/min.

The PFP-HPLC of HEA1 resulted in a number of peaks, with 8 peaks resolved from HEA1 (Fig 3.11A). The major peak at 3.9 min was collected and its antifungal activity was confirmed with the agar overlay assay (Fig 3.11B). Rechromatography of the antifungal peak using PFP-HPLC chromatography indicated that the peak was primarily a single compound (Fig 3.12A) and spectrum analysis showed that it was relatively pure (Fig 3.12B). The peak was then fractionated for further analysis.

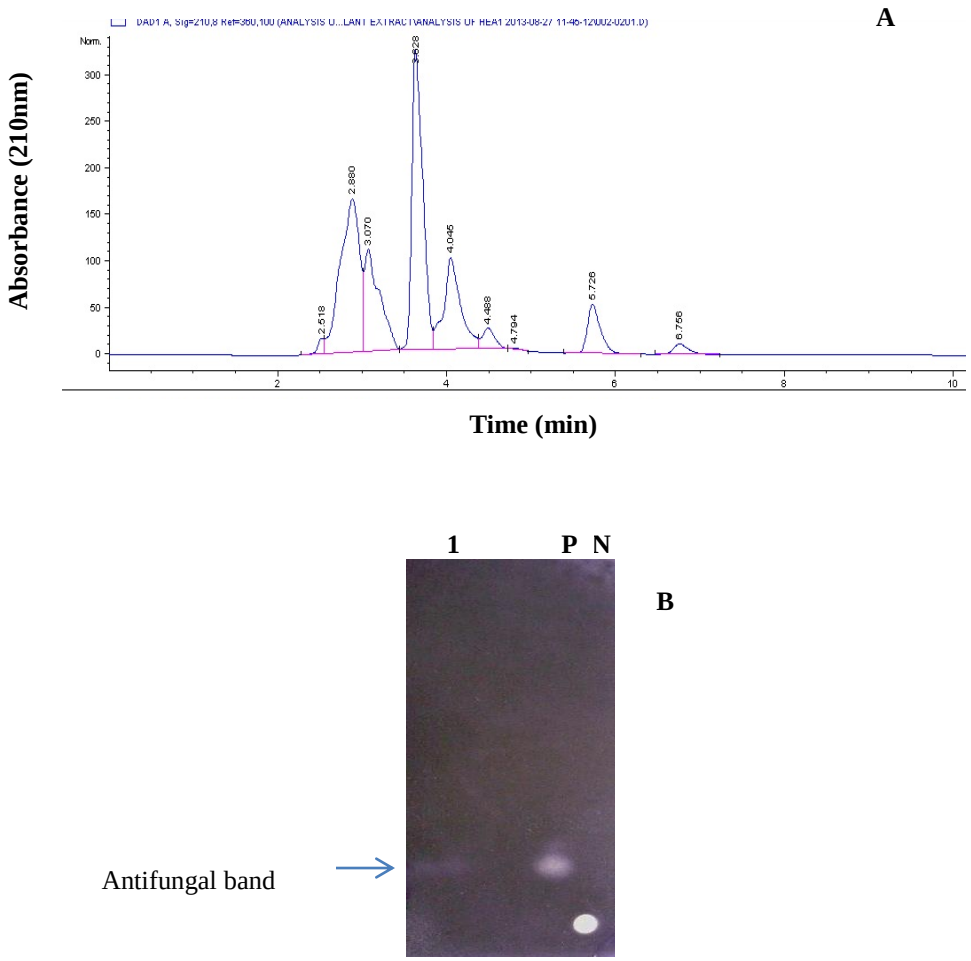


Figure 3.11: PFP-HPLC chromatogram of HEA1 (A) and an agar overlay (B) of the major peak collected after PFP-HPLC separation. The TLC plate for the agar overlay assay was developed with HEA1 solvent system. 1 = peak collected after PFP-HPLC separation showing antifungal activity; P = plant extract; N = Nystatin

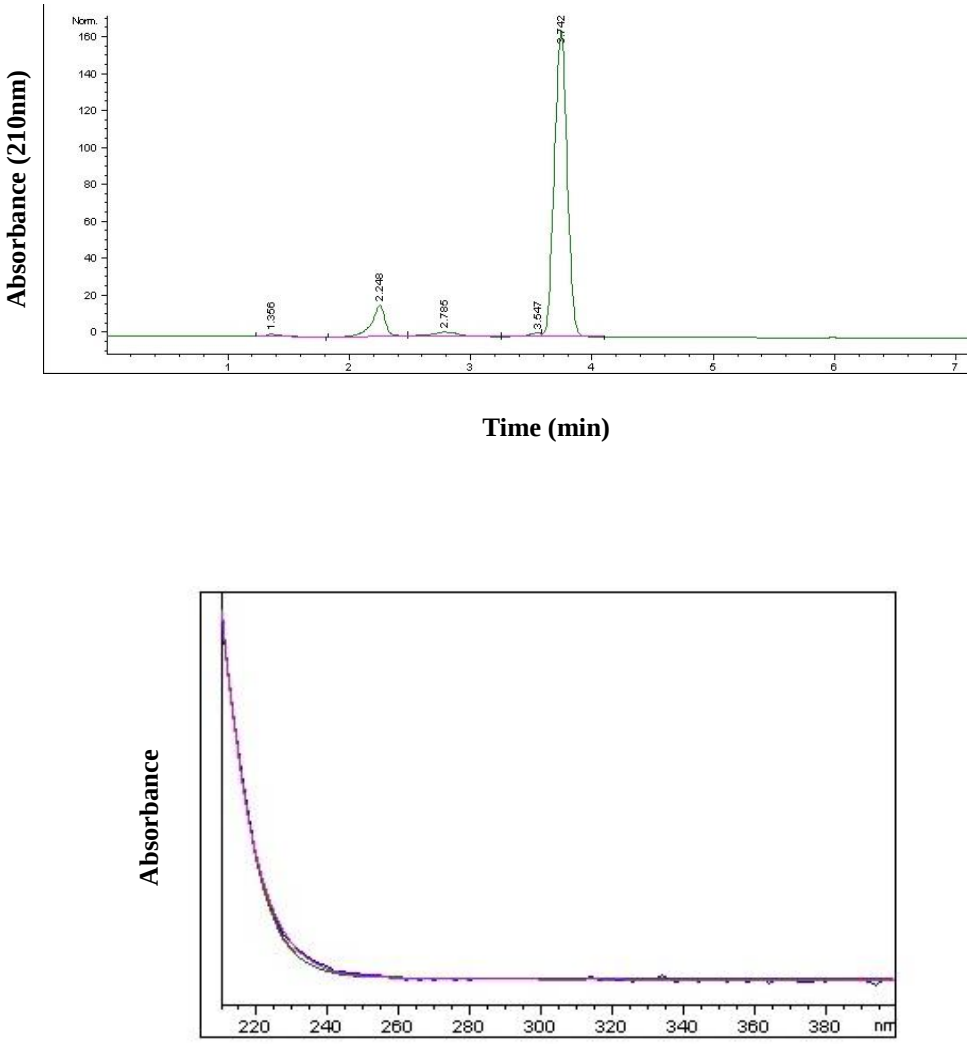


Figure 3.12: PFP-HPLC chromatogram (A) of the antifungal peak collected at 3.9min (Fig 3.11) eluting a major peak at 3.7min and (B) spectral analysis of the fraction collected at 3.7 min

3.3.5 Phytochemical analysis of the crude plant extract and HEA1

Phytochemical analysis of the plant extract and the partially purified HEA1 compound was done to examine for the presence of phenolics, tannins, flavanols and saponins. The concentration of each of the above compounds present in both the crude plant extract and HEA1 are shown in Table 3.1.

Table 3.1: Secondary metabolites present within the crude plant extract and HEA1

PHYTOCHEMICAL	CRUDE EXTRACT[¥]	HEA1[¥]
Total saponins (in *diosgenin equivalents*)	2040 ± 0.07µg/ml	220 ± 9µg/ml
Steroidal saponins (in *diosgenin equivalents*)	137 ± 0.030µg/ml	Not detected
Phenolics (in *gallic acid equivalents*)	69.33 ± 2.31µg/ml	89.33 ± 2.31µg/ml
Tannins (in *pyrogallol equivalents*)	2070 ± 0.06µg/ml	Not detected
Flavonols (in *catechin equivalents*)	Not detected	Not detected

[¥]Values represent mean ± standard deviation (n=3)

The presence of phenolic compounds in the crude plant extract and HEA1 were investigated by a colorimetric Folin C method. The assay is based on the reduction of tungsten and molybdenum oxides of the yellow Folin-Ciocalteu reagent to a blue phosphotungstic-phosphomolybdic complex and this is measured at 725nm (Appendix 1, Fig A1; Hue *et al*., 2012; Blainski *et al* 2013; Khoddami *et al*., 2013). Both the crude plant extract and the HEA1 samples turned light blue and after extrapolation from the standard curve (Fig 3.13), a concentration of 69.33 µg/ml and 89.33 µg/ml of gallic acid equivalents was obtained (Table 3.1).

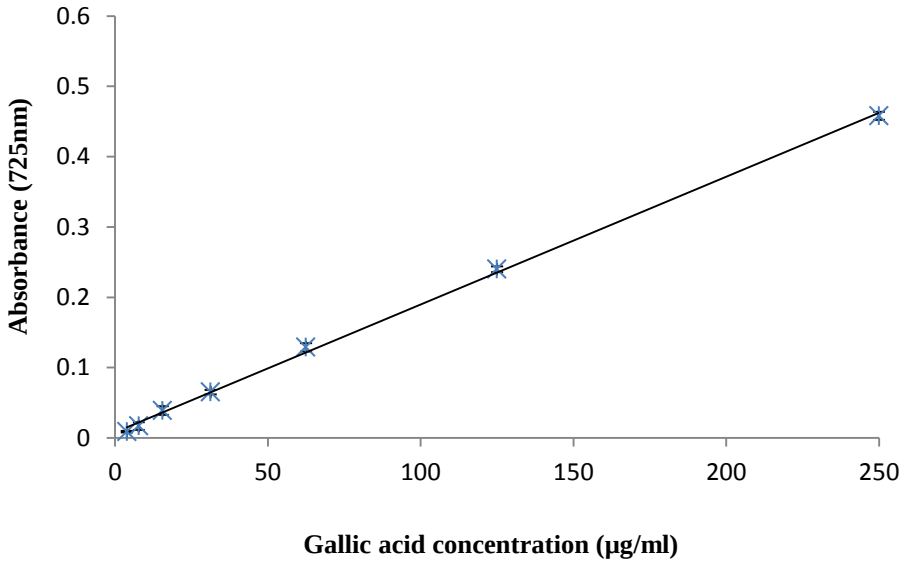


Figure 3.13: A gallic acid standard curve for the quantification of total phenolic content within the crude plant extract and in HEA1. Gallic acid was dissolved in water and a range of 0µg/ml to 250µg/ml was used to construct the standard curve. These were expressed as gallic acid equivalents. y = 0.0018x + 0.0063 R^2 = 0.9987. Error bars represent standard deviation (n = 3)

The assay for condensed tannins is based on the oxidative depolymerisation of the condensed tannins in hot acid to form a heat stable anthocyanidin (Schofield *et al*., 2001; Khoddami *et al*., 2013). The ferric reagent which is used in the assay will give a violet colour which is converted to a yellowish brown colour when the anthocyanidin is released giving a maximum absorbance at 550nm (Fig 3.14; Appendix 1, Fig A2). Tannins were only present in the crude plant extract (2070µg/ml pyrogallol equivalents) and none were detected in HEA1.

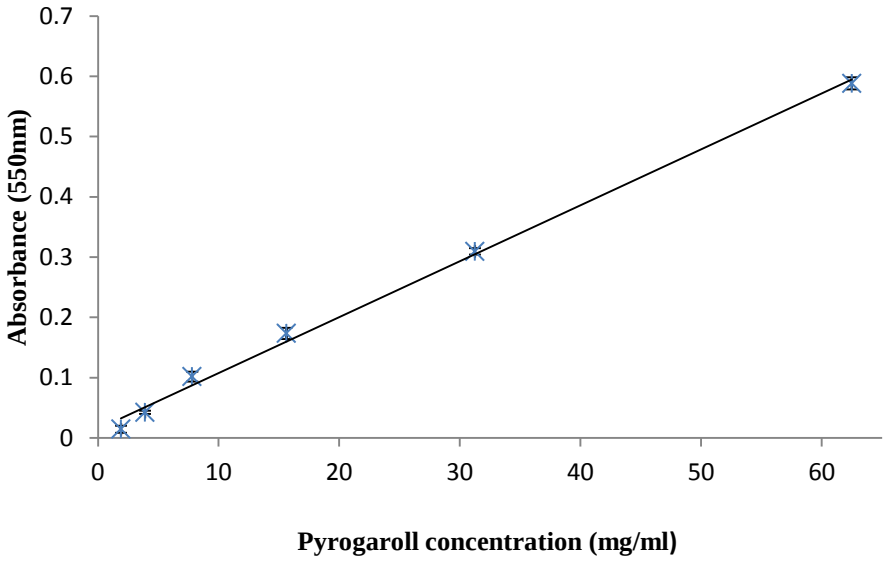


Figure 3.14: A pyrogallol standard curve for the detection of condensed tannins using the butanol-HCl assay. Pyrogallol was dissolved in water and a concentration range of 0mg/ml to 70mg/ml was used to generate the standard curve. Values are expressed as pyrogallol equivalents. y = 0.0093x + 0.00148 R^2 = 0.9963. Error bars represent the standard deviation (n = 3).

The flavanol assay for both the crude extract and HEA1 resulted in no colour change of the samples (Fig 3.15). Based on the method used, there was no detectable flavanoids in the samples tested. Determination of the flavanol content is based on the protonation of vanillin in an acidic solution to give an electrophilic radical and this will react with the flavanoid ring at position 6 or 8. The intermediate compound is dehydrated and will release a red coloured compound (Appendix 1 Fig A3; Sun *et al*., 1998; Nakamura *et al*., 2003).

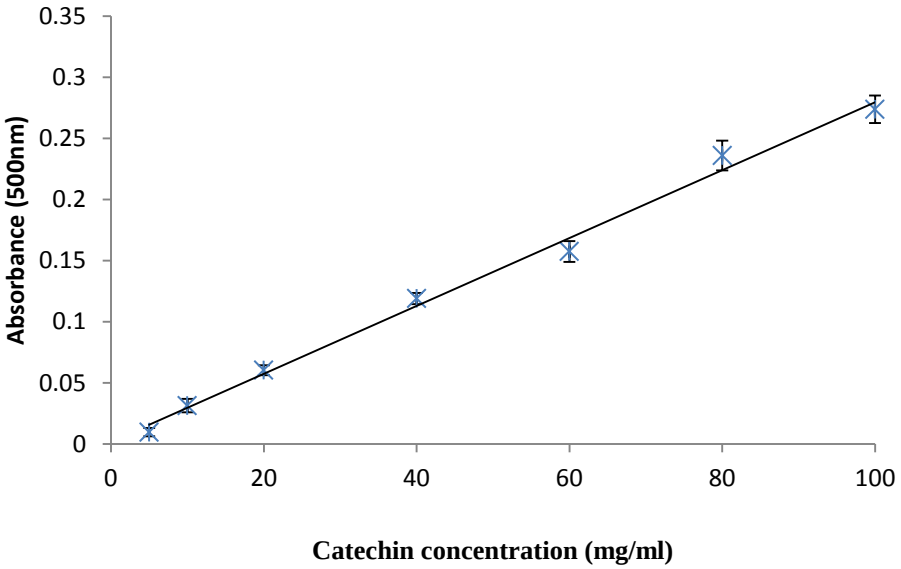


Figure 15 : Catechin standard curve for the detection of flavanols using the vanillin assay. Catechin was dissolved in methanol and a concentrations ranging from 0mg/ml to100mg/ml was used to generate a standard curve. The standard curve was used to measure the flavanol concentration of the plant extract and HEA1 expressed as catechin equivalents. y = 0.0028x + 0.0018; R^2 = 0.9937. Error bars represent the standard deviation (n = 3).

The total saponin assay is based on vanillin-sulphuric acid reaction. Vanillin reacts with the aldehydes in the presence of sulphuric acid to produce an aglycone. The colour of the aglycone depends on the type of saponin detected and the intensity is measured at 535nm (Fig 3.16; Appendix 1, Fig A4). The plant extract and HEA1 turned brownish-black indicating the presence of saponins within both samples and concentrations of 2400µg/ml and 222µg/ml for the plant extract and HEA1 respectively were found.

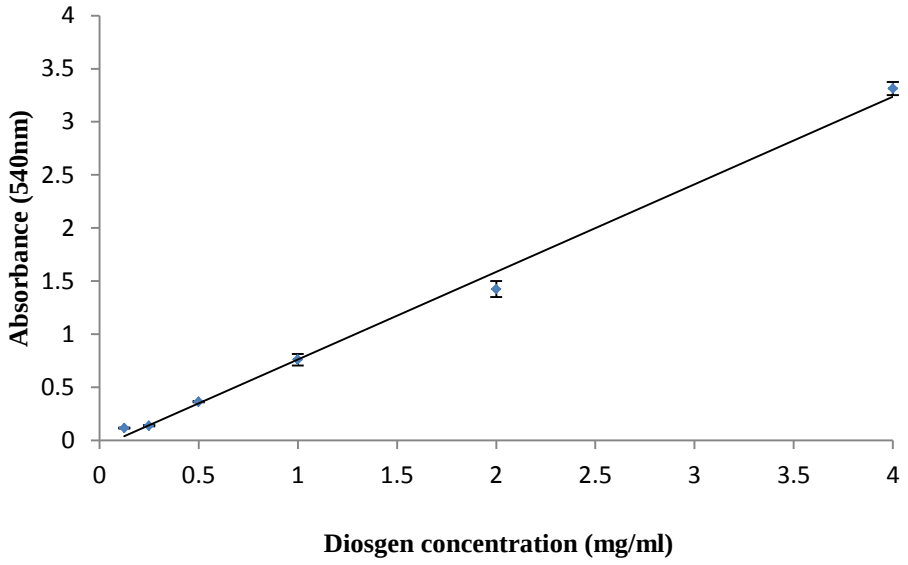


Figure 3.16: Diosgenin standard curve for quantification of total saponin content using the vanillin-sulphuric acid assay. Diosgenin was dissolved in water and a concentration range of 0mg/ml to 4mg/ml was used for the construction of the standard curve. Total saponin content was expressed as diosgenin equivalents. y = 0.8245x – 0.062, R^2 = 0.9949. Error bars represent the standard deviation (n = 3)

Steroidal saponins are assayed with anisaldehylde-sulphuric acid. Anisaldehyde reacts with the aromatic aldehyldes in the presence of sulphuric acid to form steroidal saponin producing a deep yellow coloured compound which is detected at 440nm (Fig 3.17; Appendix 1, Fig A5) (www.vidverto.info). The steroidal saponins were only detected in the crude plant extract at a concentration of 137µg/ml but none were detectable in the HEA1 fraction.

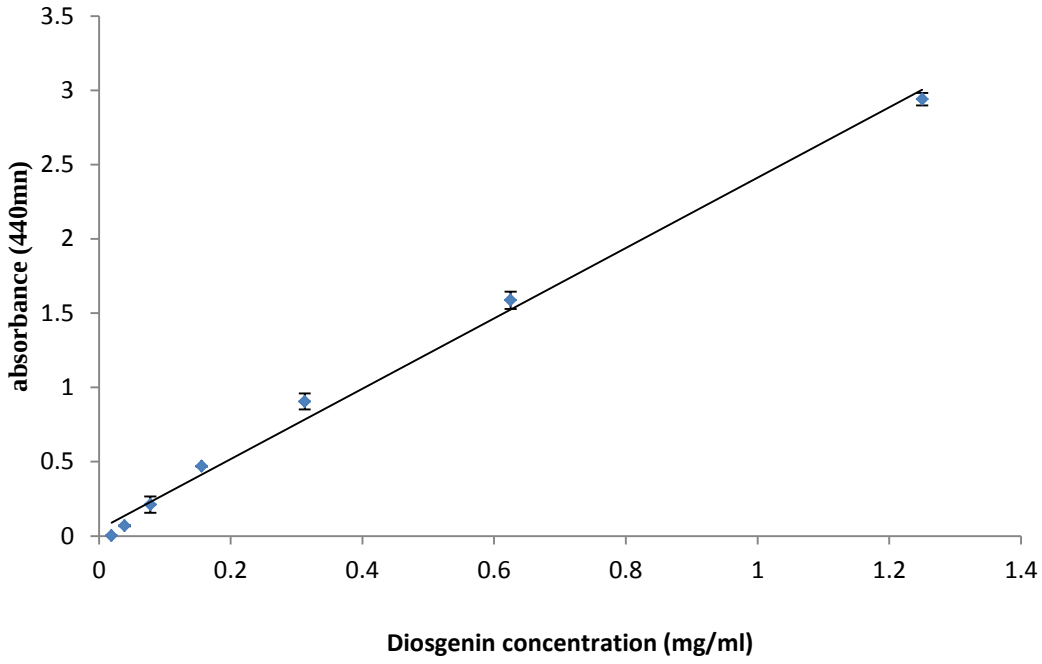


Figure 3.17: Diosgenin standard curve for quantification of steroidal saponin content using the anisaldehyde- sulphuric acid assay. Diosgenin was dissolved in water and a concentration ranging between 0mg/ml to 2mg/ml was used for the construction of the standard curve. Steroidal saponin content was expressed as diosgenin equivalents. y = 2.3667x + 0.0451, R^2 = 0.9944. Error bars represent standard deviation (n = 3).

3.4 Discussion

Plant extracts contain a mixture of active and non-active compounds, most of which are present in low quantities. The chemical components of these compounds are dependent on a number of factors such as growth season, part of the plant used, solvent used for the extraction and the extraction process (Webster *et al*., 2008; Hoai *et al*., 2009). Products of medicinal plants contain a wide variety of biologically active phytochemical compounds known as secondary metabolites. They contain bioactive compounds which contribute to the antimicrobial activity of plant extracts (Lim *et al*., 2006). These secondary metabolites are produced in specific pathways and their site of production varies from species to species and this is dependent on the type of compound produced (Ribera and Zuniga 2012). Examples of secondary metabolites present in plants include phenolics, alkaloids, flavonoids, terpenes, tannins and isoprenoids (Bourgaud *et al*., 2001).

Chromatographic fingerprinting has been regarded by the WHO as a good method for the quality control of plant extracts. It is also regarded as an effective technique for the assessment of traditional medicine since it can be used to separate the different compounds within the plant extracts (Gunalan *et al*., 2012; Ciesla 2012; Jiang *et al*., 2007, Deo *et al*., 2011). In this study, a decision was taken to use a non-toxic solvent and therefore water was chosen for the preparation of the plant extract and TLC and RP-HPLC were used as the chromatographic techniques for the identification and partial purification of the antifungal compound from the crude extract.

Analysis of *T. violacea* bulb and rhizome extracts by TLC with solvents of varying polarities has shown a presence of a variety of compounds (Motsei *et al*., 2003; Jäger and Stafford 2012). Two of these active compounds, allicin and marasmicin (Ankri and Mirelman 1999; Kubec *et al*., 2002; Thamburan *et al*., 2006), have been shown to have antifungal activity against *C. albicans* (Ankri and Mirelman 1999; Thamburan *et al*., 2006). It has been shown that some antifungal compounds isolated from *T. violacea* rhizome extracts were sulphur-containing compounds with marasmicin being the predominant compound (Jäger and Stafford 2012). In the present study the EMW solvent separated a variety of compounds and TLC-bioautography (agar overlay) localised the active compound at R_f = 0.94. The HEA solvent system separated a number of compounds from the plant extract, with the predominant compound located at an R_f = 0.29. This compound was identified as the active compound using the agar overlay assay. A large amount of sample still remained at the origin after separation with both solvent systems. This could be due to the overloading of the TLC plate with the crude plant extract. It was not possible to avoid this, since some compounds within the extract were present in small quantities and required higher concentrations of starting material to enable their visualisation after separation and staining. Sulphur compounds of *T. violacea* species have been found to be generally unstable after crushing the rhizomes due to the enzymatic conversion and degradation of chemical constituents (Ankri and Mirelman 1999; Kubec *et al*., 2002). However, it has been previously determined that the aqueous extracts used here were stable at 4^{o}C for at least a week (Belewa 2009).

High performance liquid chromatography fingerprinting is the most commonly used technique for the assessment of and quality control of plant extracts. It is used for separating a mixture of compounds in the plant extract and also for the identification of the active compound within the

plant extract (Deo *et al*., 2011; Ciesla 2012,). This is done by collecting the separated fractions of the plant extract and testing them for antimicrobial activity to identify the fraction with the active compound. Method development and optimization play an important role in creating a fingerprint of the plant extract and its compounds (Jiang *et al*., 2007). The detection wavelength plays a crucial role in producing a reliable and reproducible fingerprint of the plant extract and its compounds (Zhou *et al*., 2007). The RP-HPLC fingerprinting of the plant extract with all three columns showed that the C18 column separated more peaks than the other two columns. Davison *et al*., (2012) reported that PFP-HPLC separated more peaks of the *T. violacea* organic extract when compared to the C18 RP-HPLC. The PFP column has ionic interactions and has a pentaflourophenyl phase which incorporates fluorine atoms on the phenyl ring to provide aromatic and polar selectivity (www.phenomenex.com/Kinetex) to separate both polar and non-polar compounds. The differences observed in these two studies are due to the type of extract that was used (aqueous vs. organic) and the polarity of the compounds present in the extract. The organic extract had a mixture of both polar and non-polar compounds, while the aqueous extract used in the present study was found to be highly polar and the C18 column seemed to have retained most of these polar compounds.

The active compounds identified with the two different solvent systems were each isolated from the plant extract and analysed by RP-HPLC. Fraction EMW8 was separated with the C18 column and several peaks were resolved. The numbers of peaks were more than those obtained for the plant extract fingerprint. This fraction could therefore be a combination of some of the compounds that had lower R_f values or could be breakdown products of some of the other compounds present in the extract. It migrated together with the solvent front and was not used for further analysis since it proved difficult to test for antifungal activity of each of the many peaks that were collected after fractionation. Analysis of fraction HEA1 with RP-HPLC identified numerous peaks as well; however, there were fewer peaks than with EMW8, with the major peak at R_f = 3.7min. The antifungal activity of this peak was confirmed with the agar overlay assay. However, rechromatography of this peak with RP-HPLC showed the presence of numerous other peaks, which indicated the presence of a mixture of compounds that co-eluted because they did not properly resolve during HPLC. The scalar prep-C18 column is an analytical version of the C18-preparative column and is used for method development for preparative

applications that will involve the use of a preparative column and also to check for the purity of the sample (www.agilent.com). Analysis of HEA1 with this column resulted in separation of additional compounds (numerous peaks); however the low yield of the various fractions obtained after collection of each peak made it impossible to test each fraction for antifungal activity. Pentaflourophenyl-HPLC was found to be more suitable for the separation of the different compounds within HEA1. A better yield of the collected fractions was obtained and the antifungal peak was identified at R_f = 3.7min using the agar overlay assay. Rechromatography of the antifungal peak with PFP-HPLC showed that the peak did not contain any other compounds and that ion-pairing chromatography was more suitable for the analysis of HEA1.

Attempts to characterize the structure of the active compound proved difficult. NMR analysis of the active compound showed that there was still a mixture of compounds present in the sample and a higher yield of sample, than the 1mg obtained, was required to obtain a conclusive result (Appendix 2). Predictive NMR analysis was also completed by comparing active compounds isolated from *T. alliacea* (Maoela 2005) and known *T. violacea* sulphur containing compounds, with that of HEA1. None of the structures matched that of HEA1, which indicated that HEA1 was different from the previously identified *T. violacea* compounds. In a study conducted by Jäger and Stafford (2012), it was found that aqueous extracts of *T. violacea* rhizomes, had extremely low levels of sulphur-containing compounds below the levels required for other analyses such as NMR. Further purification of this compound is therefore necessary and may involve the use of other extraction techniques such as solid phase extraction to obtain better purification and higher yields. These could include freeze-drying of the bulbs before the extraction to minimize degradation of compounds. An introduction of an organic solvent such as ethanol during extraction could increase the amount of sulphur compounds extracted (Jäger and Stafford 2012).

Phytochemical screening of the crude plant extract and HEA1 indicated the presence of a high level of phenolics and saponins (Table 3.1). The majority of saponins were detected in the crude plant extract (2400µg/ml diosgenin equivalents), with HEA1 having a smaller proportion (137µg/ml diosgenin equivalent). Saponins are produced by plants in response to pathogen attack and have a variety of antimicrobial effects including antifungal, antibacterial and insecticidal

activities (Augustin *et al*., 2011). In fungi, saponins are known to interact with the cell membrane (Ahmed *et al*., 2012). They bind to sterols in the cell membrane and aggregate to form saponin/sterol complexes. This results in pore formation in the cell membrane, leading to increased cell permeability, leakage of cell components from the cell and loss of cell integrity (Ahmed *et al*., 2012; Ribera and Zuniga 2012). Saponins cause disruption in sterol production leading ultimately to cell death.

Bulbs of Allium plants have been shown to produce saponins that possess antifungal activity against *Botrytis cinerea* and *Trichoderma artoviridae* (Barile *et al*., 2007; Lanzotti *et al*., 2012a and b Teshima *et al*., 2013). Most of the isolated saponins were showed to be steroidal and have synergistic interaction with saponins to exert their antifungal activity. *Tulbaghia violacea* has also previously been shown to produce high levels of saponins which exhibited antifungal activity against *C. albicans* (Ncube *et al*., 2011). In the current study only low levels of steroidal saponins (220µg/ml diosgen equivalents) were detected in the crude plant extract and none were detectable in HEA1. Since HEA1 exhibited antifungal activity, it can be concluded that steroidal saponins did not contribute to this effect. Based on this and previous studies (Belewa *et al* 2011; Somai and Belewa 2011) saponins may contribute to the antifungal nature of the crude extract and HEA1.

Other metabolites which are produced by *T. violacea* include phenolics, tannins and flavanoids. Tannins were detected in the crude extract only but not in HEA1. They possess antimicrobial activity by inhibiting and inactivating membrane-bound enzymes (Schofield *et al*., 2001). It has been reported that tannins have antimicrobial activity against bacteria and yeast but no activity against fungi, including *A. flavus* and *A. niger* (Lim *et al*., 2006; Sulaiman *et al*., 2011). The thick fungal cell wall and the high chitin content act as a barrier against many foreign substances and this makes it difficult for compounds such as tannins to penetrate their cell wall and exert their antimicrobial effect (Lim *et al*., 2006). Owing to this and the fact that no tannins were detected in HEA1, tannins were discarded from contributing towards the antifungal activity reported here. Flavanoids were not detected in either the crude extract or HEA1. The vanillin assay is highly sensitive to quenching of the colour reaction by water (Hagermann, 2002). In this study both the plant extract and HEA1 were prepared in water and quenching could have

occurred in both cases, resulting in the inability to detect flavanols. It was later determined that methanol can be used for preparation of samples for flavanol detection. Phenolics were detected for both the crude extract and HEA1 (69.33µg/ml and 89.33µg//ml gallic acid equivalents, respectively). Phenolic compounds such as thymol, carvacrol and eugenol, have been shown to have antifungal activity against *A. niger* by disrupting the fluidity of the cell membrane (Ansari *et al*., 2013). Thymol disrupts ergosterol production in *Candida* species by disrupting the cell vesicles and damaging the cell membrane (Hyldgaard *et al*., 2012). Phenolics have antifungal activity against *A. flavus* and *A, parasiticus* (Hua *et al*., 1999) and their presence in both the crude extract and most predominantly in HEA1 indicate that they may be contributing to tne antifungal nature of the plant extract. These indicators then lead us to the next stage of the study which was to determine the effect of the plant extract on sterol production (chapter 4).

The most prominent phytochemical present in the plant extract was saponins. Their mechanism of action on *A. flavus* can be investigated further, by isolating them from the plant extract and identifying those with antifungal activity using various chromatographic techniques which include TLC, bioautography, HPLC and LC-MS. Studies which involve enhancement and higher accumulation of the secondary metabolites in plants should be investigated to enhance the antifungal nature of the plant extract and its compounds. Other phytochemicals which should be investigated for their antifungal activity are phenolics. This will include separation from the plant extract with TLC using the appropriate mobile phase, identification of the active compound by TLC-bioautography and identification and characterization with RP-HPLC and NMR analyses. The type of solvent used for the extraction process may also influence the type of phytochemicals present in the extract. Water has been shown to produce a higher yield of saponins (Engelberth *et al*., 2010), while phenolics are extracted maximally with methanol (Proestos *et al*., 2005). This may also explain the low levels or total absence of other phytochemicals in the plant extract.

From the results obtained above, the antifungal nature of *T. violacea* aqueous plant extract against *A. flavus*, may be due to either saponins, phenolics or both possibly through a synergistic interaction between them. Further investigations into the exact chemical composition of these phytochemicals will shed light into their identity. Since various types of saponins and phenolics

are found in plant extracts, it would be most useful to isolate and identify each one to determine which is responsible for the antifungal nature of HEA1. This is essential to further develop the responsible agent into a chemotherapeutic agent.

Tulbaghia violacea is taken as a form of traditional medicine in its crude extract form to treat various ailments (Soyingobe *et al*., 2013). In this study, the majority of phytochemicals tested were found in the crude extract and may contribute to its antifungal nature. Further investigations on the effect of this plant extract on sterol production by *A. flavus* (chapter 4) and on its cell wall (chapters 5) were carried out with the crude extract.

3.5 References

Ahmed, D.B., Chaieb, I., Salah, K.B., Boukamcha, H., Jannet, H.B., Mighri, Z. and Daami-Remadi, M. 2012. Antibacterial And Antifungal Activities of Cestrum Parqui saponins: Possible Interaction with Membrane Sterols. *International Research Journal of Plant Science* **3**(1):001-007.

Augustin, J.M., Kuzina, V., Andersen, S.B. and Bak, S. 2011. Molecular Activities, Biosynthesis and Evolution of Triterpenoid Saponins. *Phytochemistry* **72**:435-457.

Ankri, S. and Mirelman, D. 1999. Antimicrobial Properties of Allicin from Garlic. Microbes and Infection 2: 125-129

Ansari, M.A., Anurag, A., Fatima, Z.and Hameed, S. 2013. Natural Phenolic Compounds: A Potential Antifungal Agent. *Microbial Pathogens and Strategies for Combating Them: Science, Technology and Education*. A. Mendez-Vilas Edition.

Barile, E., Bonanomi, G., Antignani, V., Zolfaghari, B., Sajjadi, S.E., Scala, F. and Lanzotti, V. 2007.Saponins from Allium minutiflorum with Antifungal Activity. *Phytochemistry* **68**:596-603.

Belewa, X.V. 2009. The Effect of *Tulbaghia violacea* Aqueous Plant Extract on the Growth of *Aspergillus* Species. MSc Dissertation., Nelson Mandela Metropolitan University

Belewa, V., Baijnath, H. and Somai, B.M. 2011. Aqueous Extracts from the Bulbs of *Tulbaghia violacea* are Antifungal against *Aspergillus flavus*. *Journal of Food Safety*. **31**:176-184.

Blainski, A., Lopes, G.S. and de Mello, J.C.P. 2013. Application and Analysis of the Folin - Ciocalteu Method for the Determination of the Total Phenolic Content from *Limonium Brasiliense* L. *Molecules* **18**: 6852-6865.

Bourgaud, F., Gravot, A., Milesi, S. and Gontier, E. 2001. Production of Plant Secondary Metabolites: A historical Perspective. *Plant Science* **161**: 839-851.

Ciesla, L. 2012. Biological Fingerprinting Of Herbal Samples By Means Of Liquid Chromatography. *Chromatography Research International* **2012**: 1-9.

Davison, C., Levendal, R.A. and Frost, C.L. 2012. Cardiovascular Benefits of an Organic Extract of *Tulbaghia violacea*: Its Anticoagulant and Anti-Platelet Properties. *Journal of Medicinal Plant Research* **6**(33):4815-4824

Deo, S.S., Inam, F. and Mahashabde, R.P. 2011. Antimicrobial Activity and HPLC Fingerprinting of Crude *Ocimum* Extracts. *E-Journal of Chemistry* **8**(3): 1430-1437.

Eloff, J.N. 1998.Which Extractant Should be Used for the Screening and Isolation of Antimicrobial Components from Plants? *Journal of Ethnopharamcology* **60**:1-8.

Eloff, J.N., Ntloedibe, D.T. and van Brummelen, R. 2011. A Simplified but Effective Method for the Quality Control of Medicinal Plants by Planar Chromatography. *African Journal of Traditional and Complementary Alternative Medicine* **8**:1-12.

Engelberth, A.S., Clausen, E.C. and Carrier, D.J. 2010. Comparing extraction methods to recover ginseng saponins from American ginseng (*Panax quinquefolium*), followed by purification using fast centrifugal partition chromatography with HPLC verification. *Separation and Purification Technology* **72**: 1-6.

Gunalan, G., Saraswathy, A. and Vijayalakshmi, K. 2012. HPLTC Fingerprinting Profile of *Bauhinia variegata* Linn. Leaves. *Asian Pacific Journal of Tropical Disease*: 521-525.

Hagerman, A.E. 2002. Vanillin Assay. *Tannin Chemistry*.

Hoai, N.N., Dejaegher, B., Tistaert, C., Thi Hong, V.N., Riviere, C., Chataigne, G., Van, K.P., Van, M.C., Quietin-Leclercq, J. and Heyden, Y.V. 2009. Development of HPLC fingerprints for Mallotus Species Extracts and Evaluation of the Peaks Responsible for their Antioxidant Activity. *Journal of Pharmaceutical and Biomedical Analysis* **50**:753-763.

Hua, S-S.T., Grosjean, O-K. and Baker, J.L. 1999. Inhibition of Aflatoxin Biosynthesis by Phenolic Compounds. *Letters in Applied Microbiology* **29**: 289-291

Hue, S-M., Boyce, A.N. and Somasundram, C. 2012. Antioxidant Activity, Phenolic and Flavonoid, Contents in the Leaves of Different Varieties of Sweet Potato (*Ipomoea batatas*). *Australian Journal of Crop Science* **6**(3): 375-380.

Hyldgaard, M., Myngind, T.and Meyer, R.L. 2012. Essential Oils in Food Preservation: Mode of Action, Synergies and Interactions with Food Matrix Components. *Frontiers in Microbiology* 3(12): 1-24.

Jäger, A.K. and Stafford, G.I. 2012. Quality Assessment of *Tulbaghia* Rhizomes. *South African Journal of Botany* **82**:92-98

Jayashree, D. 2013. Phytochemical Analysis and TLC Fingerprinting of Methanolic Extracts of Three Medicinal Plants. *International Research Journal of Pharmacy* **4**(6): 123-126.

Jiang, F., Tao, Y. and Shao, Y. 2007. Fingerprinting Quality Control of Qianghuo by High Performance Liquid Chromatography-Photodiode Array Detection. *Journal of Ethnopharamcology* **111**: 265-270.

Khoddami, A., Wilkes, M.A. and Roberts, T.H. 2013. Techniques for Analysis of Plant Phenolic Compounds. *Molecules* **18**: 2328-2375.

Kubec, R., Velisek, J. and Musah, R.A. 2002. The Amino Acid Precursors and Odour Formation in Society Garlic (*T. violacea Harv*). *Phytochemistry* **60**: 21-25

Lanzotti, V., Romano, A., Lanzuise. S., Bonanomi, G. and Scala, F. 2012(a). Antifungal Saponins from Bulbs of White Onion, *Allium cepa L. Phytochemistry* **74**: 133-139.

Lanzotti, V., Barile, E., Antignani, V., Bonanomi, G. and Scala, F. 2012(b). Antifungal Saponins from Bulbs of Garlic, *Allium sativum* L. var. *Voghiera*. *Phytochemistry* **78**: 126-134

Lim, S.H., Darah, I. And Jain, K. 2006. Antimicrobial Activities of Tannins Extracted from *Rhizophora Apiculata* Barks. *Journal of Tropical Forest Science* **18(**1): 59-65

Lyantagaye, S.L. 2011. Ethnopharmacological and Phytochemical Review of *Allium* Species (Sweet Garlic) and *Tulbagh*ia Species (Wild Garlic) from Southern Africa. *Tanzanian Journal of Science* **37**: 59-72.

Lyantagaye, S.L. 2013. Methyl-α-D-Glucopyranoside from *Tulbaghia violacea* Extract Induces Apoptosis *in vitro* in Cancer Cells. *Bangladesh Journal of Pharmacology* **8**: 93-101.

Maoela, M.S. 2005. Studies on Some Biologically Active Natural Products from *Tulbaghia alliacea* MSc Dissertation, University of the Western Cape

Masoko, P. and. Eloff, J.N. 2006. Bioautography Indicates the Multiplicity of Antifungal Compounds from Twenty-Four Southern African *Combretum* Species (Combretaceae). *African Journal of Biotechnology* **5**(18): 1625-1647.

Mir, M.A., Sawhney, S.S. and Jassal, M.M.S. 2013. Qualitative and Quantitative Analysis of Phytochemicals of *Taraxucum officinale. Wudpecker Journal of Pharmacy and Pharmacology* **2**(1): 001-005.

Motsei, M.L., Lindsey, K.L., van Staden, J. and Jager, A.K 2003. Screening of Traditionally Used South African Plants for Antifungal Activity against *Candida albicans*. *Journal of Ethnopharmacology* **86**: 235-241.

Nakamura, Y., Tsuji, S. and Tonogai, Y. 2003. Analysis of Proanthocyanidins in Grape Seed Extract, Health Foods and Grape Seed Oils. *Journal of Health Science* **49**(1): 45-54.

Ncube, B., Ngunge, V.N.P., Finnie, J.F. and van Staden, J. 2011. A comparative Study of The Antimicrobial and Phytochemical Properties between Outdoor Grown and Propagated *Tulbaghia violacea Harv*. Plants. *Journal of Ethnopharamcology* **134**: 775-780.

Proestos, C., Chorianopoulos, N., Nychas, G-J.E. and Komaitis, M. 2005. RP-HPLC Analysis of the Phenolic Compounds of Plant Extracts. Investigation of their Antioxidant Capacity and Antimicrobial Activity. *Journal of Agriculture and Food Chemistry* **53**: 1190-1195.

Ribera, A.E. and Zuniga, G. 2012. Induced Plant Secondary Metabolites for Phytopathogenic Fungi Control: A Review. *Journal of Soil Science and Plant Nutrition* **12**(4): 893-911.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K.M. and Latha, L.Y. 2011. Extraction, Isolation and Characterization of Bioactive Compounds from Plant Extracts. *African Journal of Traditional, Complementary and Alternative Medicines* **8**(1):1-10.

Schofield, P., Mbugua, D.M. and Pell, A.N. 2001. Analysis of Condensed Tannins: A Review. *Animal Feed Science and Technology* **91**: 21-40.

Scorzoni, L., Benaducci, T., Almeida, A.M.F., Silva, D.H.S., Bolsan, da Silva.V. and Gianinni, M.J.S.M. 2007. The Use of Standard Methodology for the Detection of Antifungal Activity of Natural Products against Medical Yeasts *Candida* sp and *Cryptococcus* sp.*Brazilian Journal of Microbiology* **38**: 391-397.

Sharma, A., Patel, V.K. and Ramteke, P. 2009. Identification of Vibriocidal Compounds from Medicinal Plants Using Chromatographic Fingerprinting. *World Journal of Microbiology and Biotechnology* **25**: 19-25.

Sindhu, C.G. 2011. Phytochemical Screening of *Mirabilus jalap* Linn Leaf Extract by TLC. *Research Journal of Pharmaceutical, Biological and Chemical Sciences* **2**(1): 521-524.

Sirikatitham, A., Chuchom, T. and Itharat, A. 2007. Development of the Chromatographic Fingerprint Analysis of Dioscorealides and Dioscoreanone from *Dioscorea membranacea* Piere. *Songklanakarin Journal of Science and Technology* **29**(1).

Somai, B.M. and Belewa, V. 2011. Aqueous Extracts of *Tulbaghia violacea* Inhibit Germination of *Aspergillus flavus* and *Aspergillus parasiticus* Conidia. *Journal of Food Protection* **74**(6):1007-1011

Soyingobe, O.S., Oyedeji, A.O., Basson, A.K., Singh, M. and Opoku, A.R. 2013. Chemical Composition, Antimicrobial and Antioxidant Properties of the Essential Oils of *Tulbaghia violacea* Harv L.F. *African Journal of Microbiology Research* **7**(18): 1787-1793.

Sulaiman, S., Ibrahim, D., Kassim, J. and Sheh-Hong, L. 2011. Antimicrobial and Antioxidant Activities of Condensed Tannin from *Rhizophora Apiculata* Barks. *Journal of Chemical and Pharmaceutical Research* **3**(4): 436-444.

Sun, B., Ricardo-da-Silva, J-M. and Spranger I. 1998. Critical Factors of Vanillin Assay for Catechin and Proanthocyanidins. *Journal of Agriculture and Food Chemistry* **46**: 4276-4274.

Taganna, J.C., Quanico, J.P., Perono, R-M.G., Amor, E. and Rivera, W.L. 2011. Tannin-Rich Fraction from *Terminalia catappa* Inhibits Quorum Sensing (QS) in Chromobacter violaceum and the QS-Controlled Biofilm Maturation and LasA Staphylotic Activity in *Pseudomonas aeruginosa*. *Journal of Ethnopharamcology* **134**: 865-871.

Tang, D., Yang, D., Tang, A., Gao, Y., Jiang, X., Mou, J. and Yin, X 2010. Simultaneous Chemical Fingerprint and Quantitative Analysis of *Ginko biloba* extract by HPLC-DAD. *Analytical Bioanalytical Chemistry* **396**: 3087-3095.

Teshima, Y., Ikeda, T., Imada, K., Sasaki, K., El-Sayed, M.A., Shigyo, M., Tanaka, S. and Ito, S-I. 2013. Identification and Biological Activity of Antifungal Saponins from Shallot (*Allium cepa* L Aggregatum Group). *Journal of Agriculture and Food Chemistry* **61**: 7440-7445.

Thamburan, S., Klaasen, J., Mabusela, W.T., Cannon, J.F., Folk, W. and Johnson, Q. 2006. *Tulbaghia alliacea* Phytotherapy: A Potential Anti-Infective Remedy for Candidiasis. *Phytotherapy Research* **20**:844-850.

Webster, D., Taschereau, P., Belland, R.J., Sand, C. and Rennie, R.P. 2008. Antifungal Activity of Medicinal Plant Extracts; Preliminary Screening Studies. *Journal of Ethnopharmacology* **115**:140-146

Wendakoon, C., Calderon, P. and Gagnon, D. 2012. Evaluation of Selected Medicinal Plants Extracted in Different Ethanol Concentrations for Antibacterial Activity against Human Pathogens. *Journal of Medicinally Active Plants* **1**(2): 60-68.

Wettasinghe, M., Shahidi, F., Amarowicz, R. and Abou-Zaid, M.M. 2001. Phenolic Acids in Defatted Seeds of Borage (*Borago officinalis* L.). *Food Chemistry* **75**: 49-56.

Zhou, Y., Jiang, S.O.Y., Ding, L-S., Cheng, S-W., Xu, H-X., But, P.P-H. and Shaw, P-C. 2008. Chemical Fingerprinting of Medicinal Plants "Giu-jiu" by LC-ESI Multiple-Stage MS. *Chromatographia* **68**(9/10): 781-789.

Internet references

http://vidverto.info/index.files/Page4763.htm/saponins, 20 July 2014

http://www.chem.agilent.com/en-US/products-services/Columns-Sample-Preparation/LC-LC-MS-Columns/Analytical-HPLC-UHPLC/; 20 July 2014

https://www.phenomenex.com/Kinetex/; 20July 2014

CHAPTER 4

THE EFFECT OF *Tulbaghia violacea* AQUEOUS PLANT EXTRACT ON ERGOSTEROL PRODUCTION

4.1 Introduction

Sterols are a crucial component of eukaryotic cell membranes and play an important role in cell growth and viability, regulation of membrane fluidity and normal functioning of the cell (Alcazar-Fouli and Mellado, 2013; Chemler *et al*., 2006; Joffrion and Cushion, 2010). Ergosterol is the predominant sterol component that is found in the cell membrane of yeasts and mycelial fungi and plays an important role in regulating membrane permeability and integrity as well as regulating the functioning of membrane-bound enzymes (Alcazar-Fuoli *et al*., 2006, Liu, *et al*., 2013). The ergosterol biosynthesis pathway is regulated by key enzymes and these have often been investigated as potential targets for antifungal agents used in the treatment of many fungal infections (Liu *et al*., 2011).

Polyenes, allylamines and azoles are antifungal agents that target either ergosterol or enzymes involved in the ergosterol biosynthetic pathway. Allylamines inhibit squalene epoxidase (Alcazar-Fuoli and Mellado, 2013), causing an accumulation in the amount of squalene produced. This is toxic to fungal development resulting in a disruption in ergosterol production, which ultimately results in cellular death (Muller *et al*., 2013). Azoles block the enzyme 14α demethylase, which is involved in the conversion of lanosterol to other sterol intermediates and ultimately ergosterol (Rodriguez *et al*., 2014). The accumulation of the toxic sterol intermediates result in the inhibition of ergosterol production. Polyenes such as amphotericin B bind directly to ergosterol present in the plasma membrane. They form channels in the cell membrane, causing leakage of cellular components, cell permeability and osmotic lysis. This disturbance in the cell membrane potentially results in cell death (Jackson *et al*., 2009; Muller *et al*., 2013).

Although these antifungal agents affect ergosterol production, some are fungistastic rather than fungicidal and others such as amphotericin B are toxic to the host. Fungal resistance is also on the increase and has rendered some antifungal agents ineffective on occasion. Natural products

such as essential oils have been shown to have antifungal activity with a broad spectrum of action and no reported side effects (de Lira Mota *et al*., 2011; Sajjad *et al*., 2011). Essential oils have been shown to inhibit ergosterol production in *Aspergillus, Candida, Saccharomyces* and other fungal species, by binding directly to ergosterol at the plasma membrane (Ahmad *et al*., 2011a and b); Pinto *et al*., 2013; Yamamoto-Ribeiro *et al*., 2013).

The aqueous plant extract of *T. violacea* has been shown to have antifungal activity against *A. flavus, Candida* and other fungal plant pathogens (Motsei *et al*., 2003; Belewa *et al*., 2011, Ncube *et al*., 2011). However, its mechanism of action has not been fully investigated. In this chapter, the aim was to investigate the effect of the plant extract on the elements of the ergosterol biosynthetic pathway in *A. flavus.*

4.2 Materials and methods

4.2.1 Growth conditions

The *A. flavus* strain (2527) was maintained in glucose minimal medium (GMM 10 g glucose, 50 ml nitrate salts, 1 ml Trace Elements, 5g Yeast extract, pH to 6.5 with NaOH pellets) at 30°C. The conidia were resuspended by flooding a plate with 0.1% Tween 20 and the spores scraped off the surface of the plate with a sterile glass rod, counted under a haemocytometer and 1 x 10^6 conidia was used for each assay. Cultures of *A. flavus* were grown in the presence of various concentrations (2, 5; 5, 7.5 10 and 12.5 mg/ml) of plant extract or positive control (Terbinafine). The minimal inhibitory and minimal fungicidal concentration for the plant extract was determined in a previous study (Belewa 2009) and the sub-inhibitory concentrations were based on these findings.

4.2.2 Reagents and equipment used

The reagents that were used in this study were purchased from Sigma Aldrich and Merck Millipore South Africa. Ergosterol, hexane, 2, 3-oxidosqualene, lanosterol and squalene were purchased from Sigma Aldrich. Analytical TLC plates, ethanol, ethyl acetate, methanol, potassium hydroxide, sulphuric acid and vanillin were purchased from Merck Millipore. The Agilent HPLC system 1100 series consisting of a degasser, binary pump, autosampler, fraction collector and a Diode array detector (DAD) was used for sterol analyses. An Agilent scalar prep-C18 column (100mm x 4.6mm; 5µm) was used for the HPLC analysis.

4.2.3 Isolation of total sterols

Ergosterol and other sterol components were extracted from *A. flavus* in the presence and absence of the plant extract and in the presence and absence of terbinafine (positive control), using modified protocols of Arthington-Skaggs *et al*., (2000) and Alcazar-Fuoli *et al*., (2008). Briefly, 1 x 10^6 conidia were inoculated into YES (1g yeast extract, 10g sucrose) in the presence and absence (negative control) of the plant extract and was incubated at 30°C for 4 days. The mycelial mat was removed, rinsed with sterile double distilled water, dried with paper towel and weighed. Five millilitres of 25% alcoholic KOH (10 g KOH, 65 ml 100% ethanol, 35 ml double

distilled sterile water) was added to 100 mg of mycelium, vortexed for 1min and saponified at 85°C for 1 hr to release the esterified ergosterol from the other lipid components. This was followed by an extraction of ergosterol, squalene, oxidosqualene and lanosterol by the addition of 2 ml of double distilled water and 5 ml of n-hexane. The tubes were then centrifuged at 3500 *xg* for 10 min. The hexane layer was removed to a clean glass tube and the extraction with water and hexane was repeated twice. The hexane layers were pooled and evaporated to dryness, and stored at -20°C until further use (Chiocchio and Matkovic, 2011; Gesner and Schmitt 1996; Tardieu *et al*., 2007).

4.2.4 TLC detection of total sterols

The extracted and dried samples were resuspended in 150 µl of methanol and 30 µl was spotted onto an analytical silica gel F_{254} TLC aluminium sheet together with suitable standards. Ergosterol and the other sterol components present in the extract were then separated with hexane: ethyl acetate (4:1) as the mobile phase. The plate was then dried, first viewed under UV light and then stained with vanillin sulphuric acid reagent (2 g vanillin in 20 ml ethanol; 2 ml sulphuric acid) and heated at 100°C for 5 min or until spots developed (Cabral *et al*., 2013). Ergosterol, oxidosqualene and lanosterol standards were dissolved in 100% methanol at a concentration of 100µg/ml and squalene was dissolved in 100% acetonitrile.

4.2.5 Quantification of ergosterol by RP-HPLC

Thirty microliters of each isolated sample was also subjected to RP-HPLC analysis (Agilent 1100 series) with the Agilent scalar prep C-18 column (5µm; 100mm x 5.4mm) at a temperature of 30°C. An isocratic mobile phase used for ergosterol detection was composed of methanol: water (97:3), the analysis was done at a flow rate of 1ml/min and compounds detected via UV detection at 282nm (Alcazar-Fuoli *et al*., 2008). Ergosterol standard was used as a reference to detect and identify ergosterol in the mixture based on similar peak retention times. A standard curve was constructed using the peak areas obtained after injection of the diluted standard and this was used for quantification of ergosterol produced in the presence and absence of the plant extract and terbanifine (Appendix 2, A6). Triplicate results were completed and used for

statistical analysis. A student t-test was completed and a p value less than 0.05 was considered to be significant.

4.2.6 Quantification of squalene, oxidosqualene and lanosterol by RP-HPLC

A similar procedure as described in section 4.2.5 was used for analysis of squalene, oxidosqualene and lanosterol except that the isocratic mobile phase was methanol: water (99:1) and UV detection was done at 210nm (Spanggord *et al*., 2006; Xu *et al*., 2010). Standard curves for squalene, oxidosqualene and lanosterol were constructed and used for the quantification of the respective sterol component (Appendix 1, A7-A9).

4.3 Results

4.3.1 TLC analysis of the crude sterol fraction

Ergosterol was successfully extracted from *A. flavus* cultures that were treated with various sub-inhibitory concentrations of the plant extract, as previously determined (Belewa 2009) and detected byTLC. Thin layer chromatography is a faster and more efficient method for detecting a wide range of compounds by using various visualisation techniques and staining reagents. These include UV fluorescent indicators and general staining reagents and allow for the quantitative analysis of the compounds (Zarzaycki *et al*., 2007).Vanillin-sulphuric acid reagent is used for the detection of a wide variety of compounds which include triterpenes, steroids and sterol components. That reagent will result in a wide variety of coloured spots that will indicate the presence of these compounds (Jockovic *et al*., 2007).

Staining of the TLC plate with vanillin-sulphuric acid showed the presence of ergosterol in all samples that were tested (Fig 4.1). The ergosterol band from each extract was identified by comparison to the R_f value obtained with the ergosterol standard (R_f = 0.5). It was difficult to determine the identity of the other sterol intermediates present in the extracts with TLC, as their standards (oxidosqualene, squalene and lanosterol) were not clearly visible after visualization of the bands with UV light and after staining (data not shown). Although densitometry could have been used to quantify the ergosterols present on the TLC plate, if co-migration of other lipid components with ergosterol had occurred, it would have not given a precise result. This would then require repeated purification steps and a high volume of sample would be required to ensure a high yield. A low recovery of the end product may be a result of this. Therefore, it was necessary to use RP-HPLC for the separation, identification and quantification of ergosterol and the other sterol components. High performance liquid chromatography would enable not only the detection of ergosterol at its optimal wavelength, but enable separation of the other sterol components that are present in the sample and which may have co-migrated with ergosterol during TLC analysis.

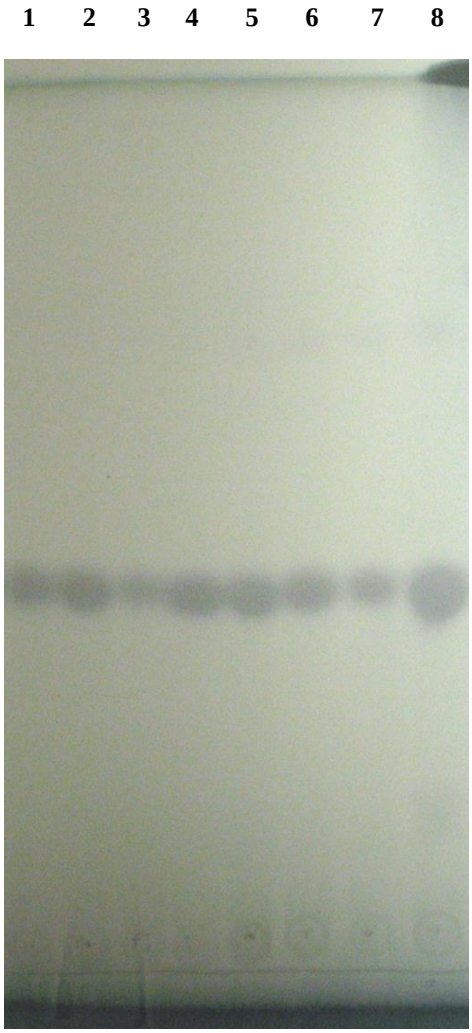


Figure 4.1: A TLC analysis of sterols extracted from *A. flavus* cultures treated with various concentrations (1 to 6) of *Tulbaghia violacea* plant extract or terbanifine (7). The TLC plate was developed with hexane:ethyl acetate (4:1) as the mobile phase. The plate was stained with vanillin-sulphuric acid to visualise the different sterols. Standards were used to locate the different sterols present in each *A. flavus* extract. 1 = 0mg/ml; 2.5 mg/ml; 3 = 5mg/ml; 4 = 7.5mg/ml; 5 = 10mg/ml; 6 = 12.5mg/ml; 7 = Terbinafine; 8 = ergosterol standard

4.3.2 RP-HPLC analysis of ergosterol

Ergosterol in the crude sterol fractions of both treated and non-treated *A. flavus* samples was separated and quantified by RP-HPLC. This allowed separation of the ergosterol from other sterol fractions and permitted its quantification from the ergosterol standard curve. The DAD

spectrum of the ergosterol standard showed a characteristic curve (Fig 4.2) with ergosterol detectable at 282nm and ergosterol esters at 270nm and 292nm (Yuan 2007). A DAD spectrum of ergosterol isolated from *A. flavus* in the presence and absence of the plant extract was identical to that obtained for the ergosterol standard as determined by an overlay of the two spectra. The identical overlay of these spectra (Fig 4.3) provided additional confirmation that ergosterol was present in the isolated extract of *A. flavus*.

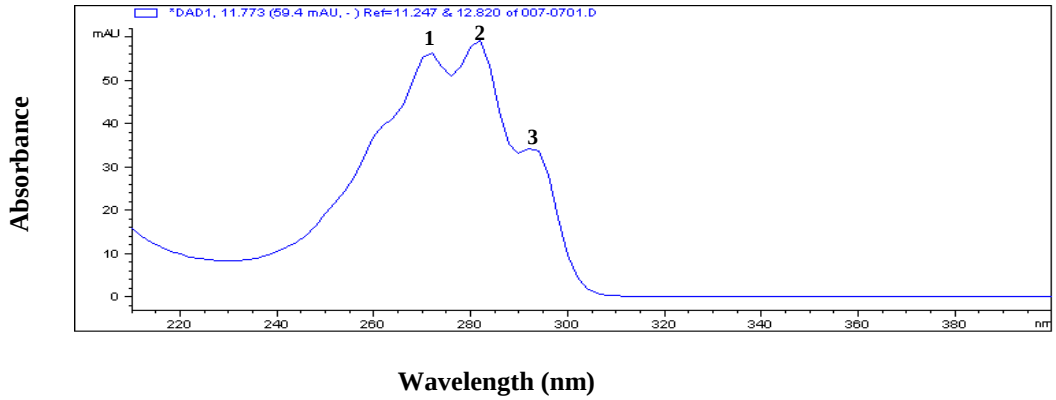


Figure 4.2: **Diode array spectrum of the ergosterol standard. Ergosterol is detectable at a wavelength of 282nm. Peak 1 = ergosterol ester/s; peak 2 = ergosterol standard; peak 3 = ergosterol ester/s.**

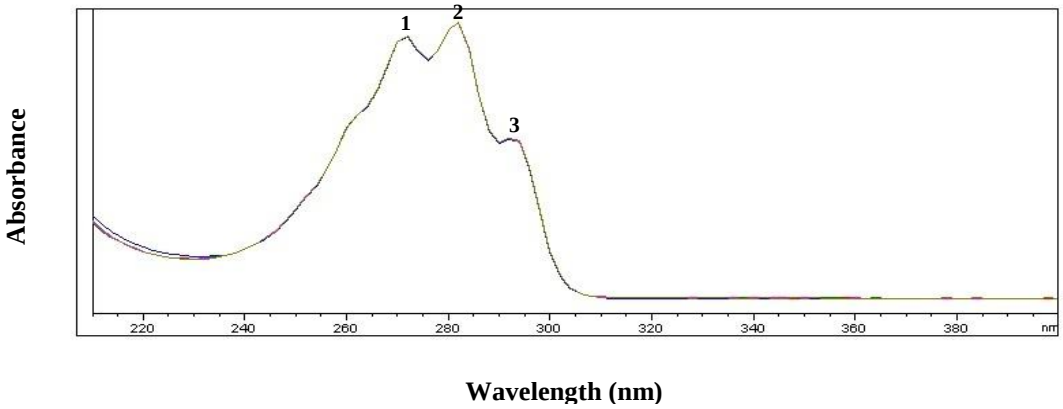


Figure 4.3: **Overlay of diode array spectra of ergosterol standard and crude sterol fraction extracted from *Aspergillus flavus* exhibiting identical spectra with 3 distinctly identifiable peaks**

The RP-HPLC chromatogram of the ergosterol standard showed the presence of ergosterol at 282nm with a retention time of 11.17min (Fig 4.4). Other distinct peaks were detectable on the HPLC chromatogram, but they were present at low quantities. These peaks could be ergosterol esters (or breakdown products) which occur together with the authentic ergosterol (Yuan 2007). The ergosterol standard curve was constructed using the peak areas of the diluted ergosterol for a range of ergosterol concentrations (1-50µg/ml) (Appendix 1, Fig A6).

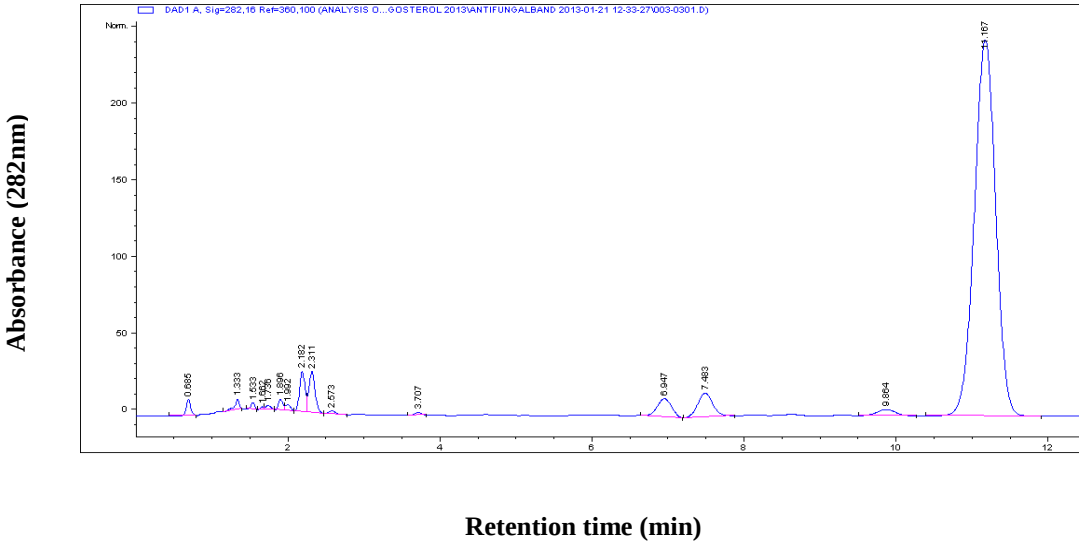


Figure 4.4: RP-HPLC chromatogram of ergosterol standard detectable at a wavelength of 282nm and eluting at 11.2min.

The RP-HPLC chromatograms of the ergosterol isolated from *A. flavus* treated and untreated with *T. violacea* plant extract, showed the presence of other sterol components, which were co-isolated with ergosterol (Fig 4.5A-C). These were distinctly separated from the ergosterol peak and could possibly be sterol intermediates. The compounds eluted from the column between 0 and 10min as opposed to ergosterol, which eluted at 11.29min. As the plant extract concentration was increased, it was found that a decline in the ergosterol peak intensity was observed and that this occurred in a dose dependent manner at a range of 2.5 -12.5 mg/ml plant extract

concentration. Treatment of the fungus with the highest extract concentration (12.5mg/ml) exhibited the lowest ergosterol peak intensity.

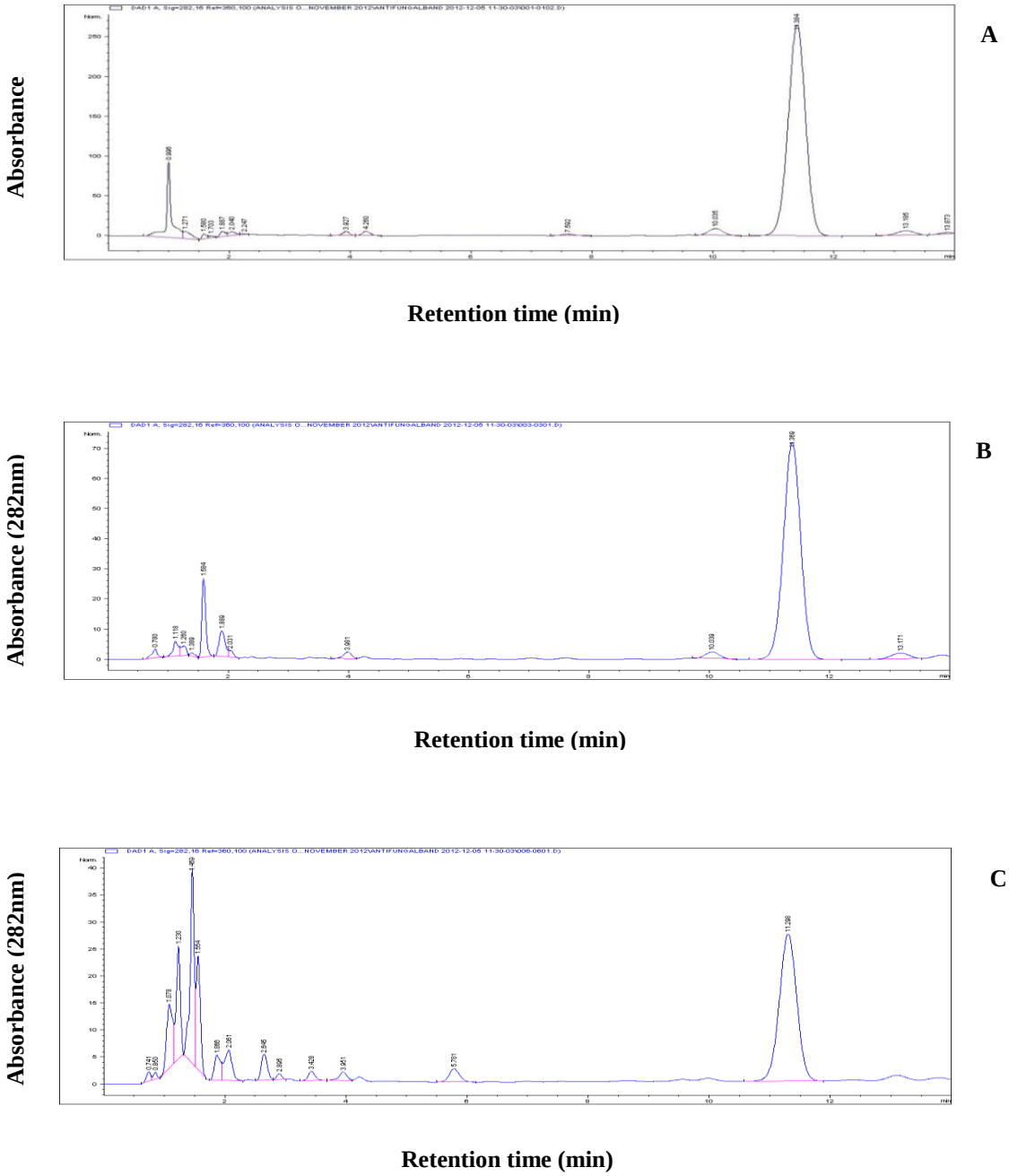


Figure 4.5: **Reverse phase-HPLC chromatograms of extracted sterol fractions isolated from *Aspergillus flavus* in the absence (A) and the presence (B, C) of *Tulbaghia violacea* plant extract, with ergosterol eluting at 11.3min at a wavelength of 282nm. The letters A to C represent the different concentrations of plant extract used to treat *A. flavus*. A = 0mg/ml; B = 5mg/ml; C = 12.5mg/ml plant extract.**

4.3.3. Quantification of ergosterol content

When *A. flavus* was treated with the plant extract there was a significant decline in ergosterol production relative to the untreated sample ($p<0.01$; Fig 4.6). A dose dependent decrease in ergosterol production was observed when *A. flavus* was grown in the presence of increasing (2.5; 5.0; 7.5; 10 and 12.5 mg/ml) plant extract concentrations. At a plant extract concentration of 2.5 mg/ml, approximately 78µg of ergosterol was produced as opposed to 121µg of ergosterol in the absence of the plant extract. Thus at 2.5mg/ml, ergosterol production was reduced to 64.5% relative to the untreated sample. A more pronounced decline was observed at plant extract concentrations of 5 and 7.5 mg/ml with only 40.5% (49µg) and 20.6% (25 µg) of ergosterol being produced respectively relative to the untreated sample. In the presence of 10mg/ml and 12.5mg/ml plant extract concentrations, *A. flavus* only managed to produce 23 µg and 16.8 µg of ergosterol respectively which is 19% and 13.9% of the untreated sample.

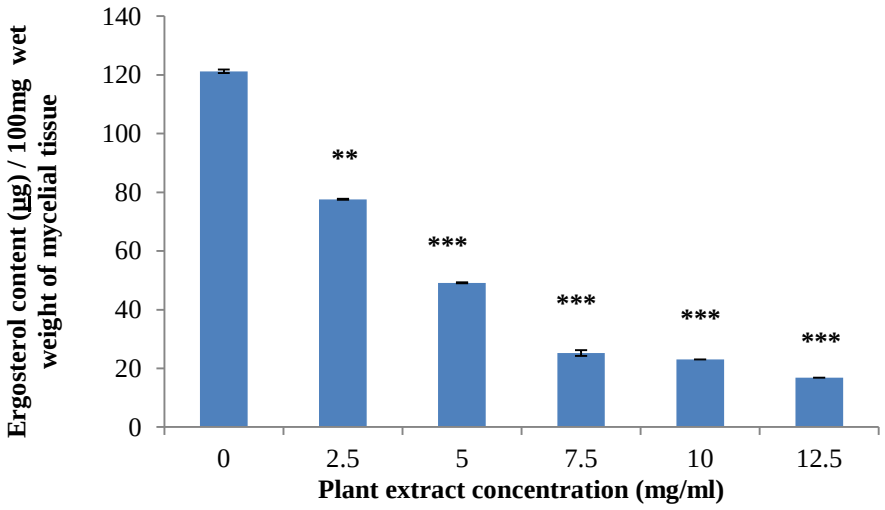


Figure 4.6: The effect of *T. violacea* plant extract on ergosterol production by *A. flavus*. Error bars represent the standard deviation for each sample (n=3). Asteriks represent the significance level relative to the control (0mg/ml). ** = $p<0.01$; *** = $p< 0.001$

When *A. flavus* was treated with terbinafine, the positive control antifungal agent, a significant decline in the amount of ergosterol produced by *A. flavus* was also observed ($p < 0.001$; Fig 4.7). Lower concentrations of terbinafine had a greater inhibitory effect on ergosterol production with concentrations ranging between 0.0156 to 0.25 µg/ml reducing ergosterol production from 23.9 to 2.7µg.

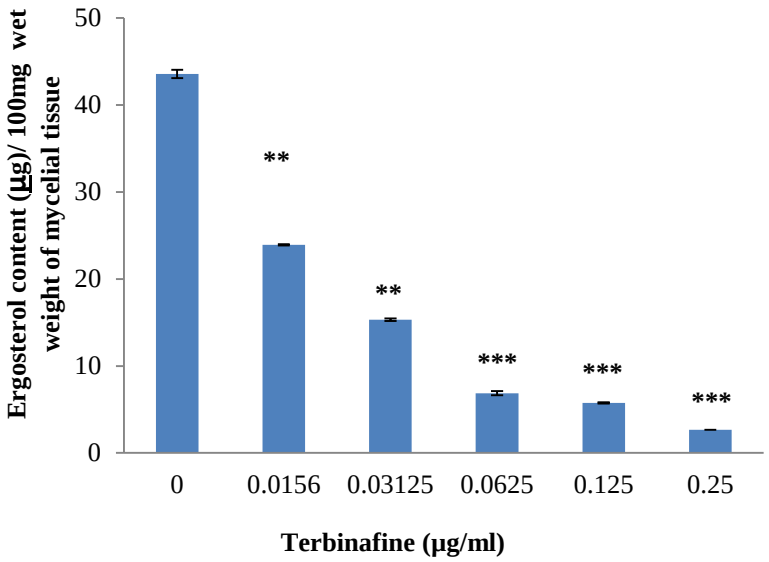


Figure 4.7: Effect of terbinafine on ergosterol production by *A. flavus*. Error bars represent the standard deviation for each sample (n = 3). Asterisks represent the significance level relative to the control (0mg/ml). ** = $p<0.01$; *** = $p< 0.001$

4.3.4 HPLC analysis of squalene, oxidosqualene and lanosterol

To determine the point in the ergosterol biosynthetic pathway that was inhibited by *T. violacea* plant extract, levels of key pathway intermediates were determined. i.e. 2,3-oxidosqualene, lanosterol and squalene levels were then analysed by RP-HPLC. Higher peak sensitivities for the tested standards were obtained at a detection wavelength of 210 nm and also allowed for the simultaneous detection of all three sterols in one HPLC run within 20 min. For squalene, 2,3-oxidosqualene and lanosterol standards retention times were 15.49, 7.55 and 9.36 min respectively (Fig. 4.8A-C). The retention times and similarities of the peak spectra were then

used to identify similar components present in the crude fractions isolated from *A. flavus* (data not shown). Commercial standards were used to construct standard curves for each sterol by plotting their peak areas against their respective concentrations (Appendix 1, Fig A7-A9). The standard curves of each sterol intermediate had good correlation coefficiencies (R^2 = 0.99) between the peak area and the different sterol concentrations that were used (Appendix 1 Fig A7-A9). The standard curves were then used to quantify the concentration of each sterol component present within the crude fractions.

The RP-HPLC analysis of the crude components of *A. flavus* after treatment and non-treatment with the plant extract showed a variety of sterols or sterol intermediates present (Fig 4.9A-C). A comparison between the retention times of the standards with the sterols isolated from *A. flavus* showed that 2,3-oxidosqualene (peak 1) had a retention time of 7.9 min and an increased peak height and absorbance was observed in cultures treated with the plant extract as opposed to the untreated sample (control). This was more pronounced for the higher plant extract concentrations that were tested. Lanosterol had a retention time of 10 min (peak 2) but exhibited variable peak heights in the presence of the plant extract, when compared to the untreated sample. Squalene had a retention time of 15.4 min (peak 3). The peak height of squalene was very low in the absence (Fig 4.9A) and presence (Figs 4.9B and 4.9C) of the plant extract and this was noticeable for all plant extract concentrations tested.

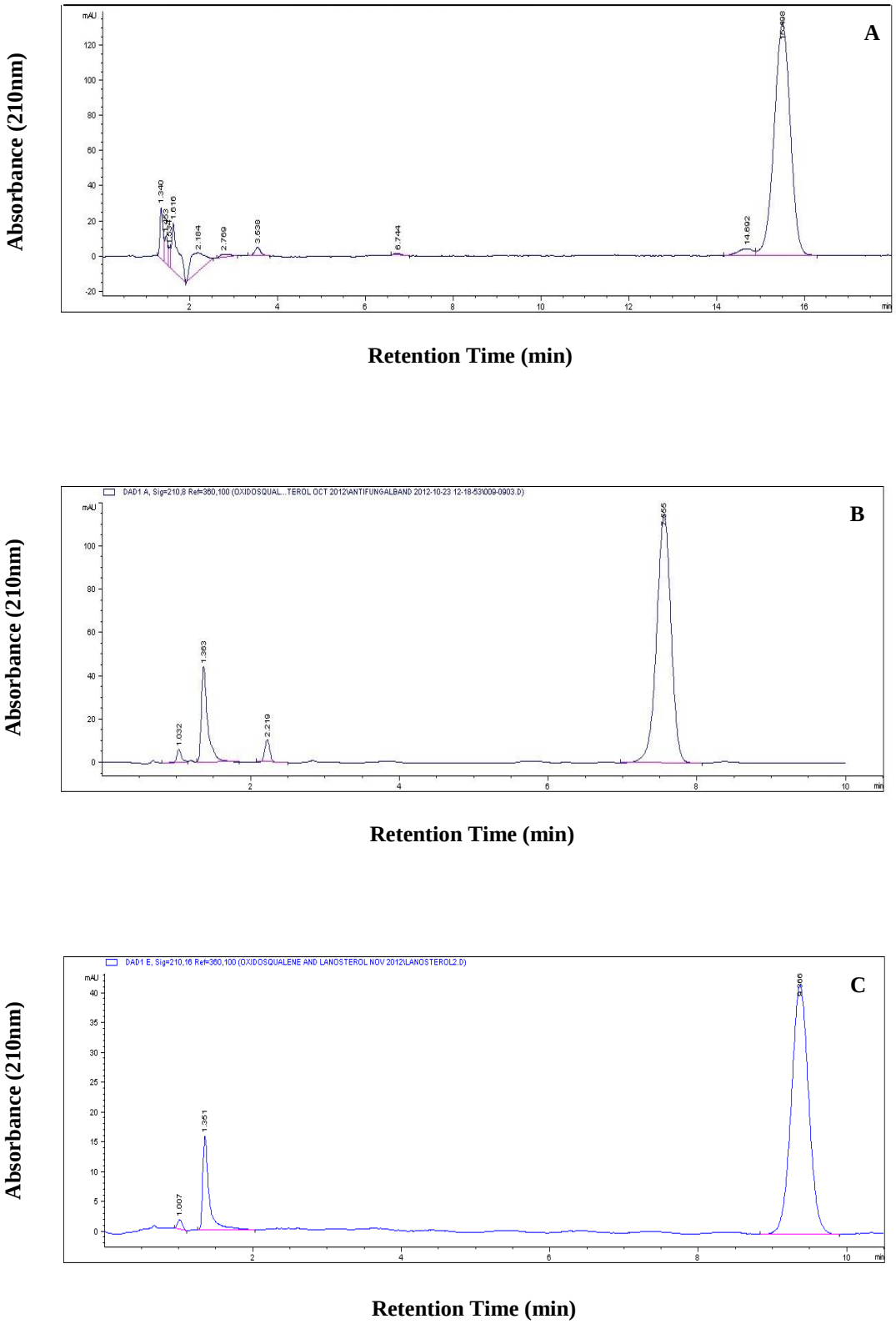


Figure 4.8: Reverse phase-HPLC chromatograms of (A) squalene, (B) 2,3-oxidosqualene and (C) lanosterol standards at a detection wavelength of 210nm.

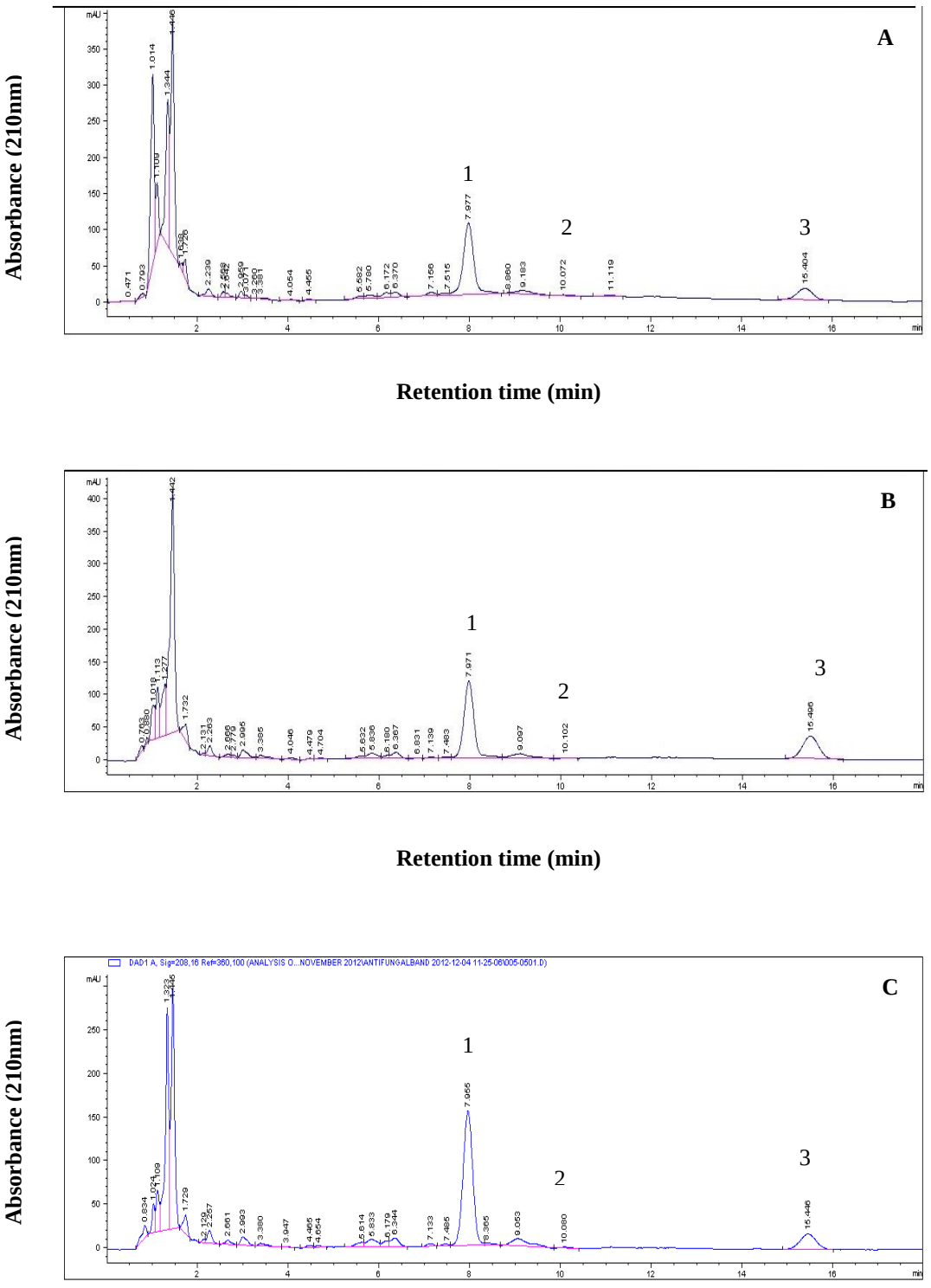


Figure 4.9: Reverse phase-HPLC chromatograms of the fractions extracted from *A. flavus* in the absence (A) and presence (B and C) of 0mg/ml (A), 5mg/ml (B) and 12.5mg/ml (C) plant extract showing the presence of 2,3-oxidosqualene (peak 1), lanosterol (peak 2) and squalene (peak 3).

4.3.5 Quantification of the sterol components extracted from *A. flavus*

Quantification of the squalene content in the *A. flavus* extract, showed that there was a significant increase in the amount of squalene produced in plant extract-treated *A. flavus* cultures relative to the untreated control ($p<0.05$; Fig 4.10). However this increase was at extremely low levels and fell in the lower range of the standard curve. In the absence of the plant extract only 0.1µg squalene was produced by *A. flavus*. At a treatment concentration of 2.5 and 12.5 mg/ml of the plant extract, the squalene content was between 0.15µg and 0.27µg respectively. The low levels of squalene may indicate that squalene is rapidly being converted to 2,3 oxidosqualene.

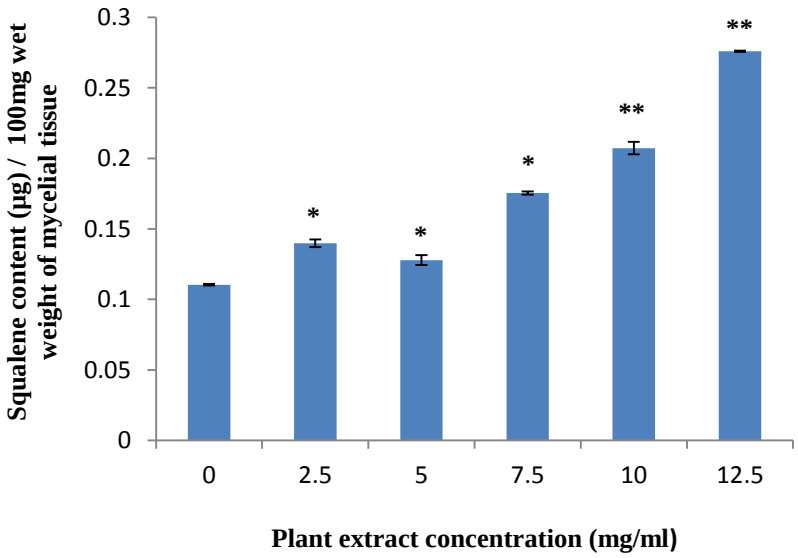


Figure 4.10: The effect of *T. violacea* plant extract on squalene production in *A. flavus*. Error bars represent the standard deviation for each sample (n=3). Asterisks represents the level of significance relative to the control (0 mg/ml). * = p< 0.05; ** = p< 0.01.

There was a significant increase in the amount of 2,3-oxidosqualene produced by the fungus in the presence of the plant extract when compared to the untreated sample ($p<0.05$; Fig 4.11). The increase was in a dose dependent manner, with almost double (192µg) the amount of 2,3-oxidosqualene being produced at a plant extract concentration of 12.5mg/ml when compared to

the absence of any extract (106µg). A more pronounced increase in 2,3-oxidosqualene was observed for concentrations above 5mg/ml plant extract. The increase in oxidosqualene levels was much higher than that observed for squalene.

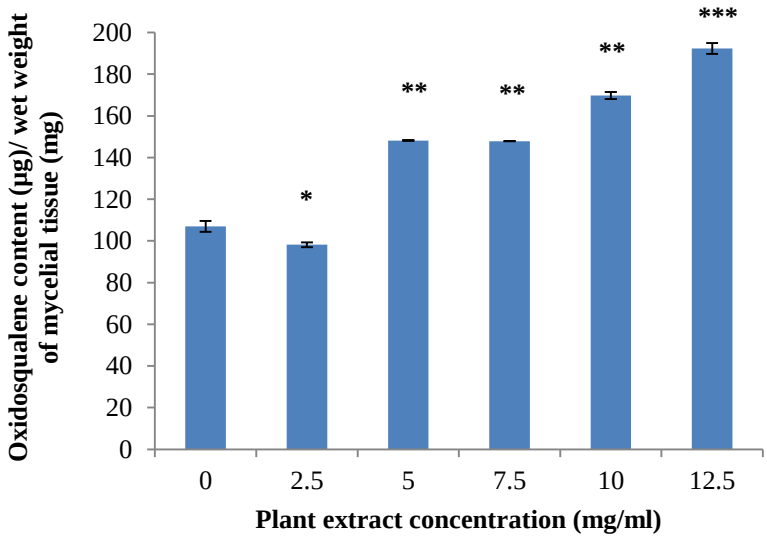


Figure 4.11: The effect of *T. violacea* plant extract on 2,3-oxidosqualene production by *A. flavus*. Error bars represent the level of significance for each sample (n= 3). Asterisks represent the level of significance relative to the control (0mg/ml). * = p< 0.05; ** = p< 0.01, *** = p< 0.005

The concentration of lanosterol indicated that there was an initial increase in the amount of lanosterol produced by *A. flavus* in the presence of 2.5mg/ml (32µg) and 5mg/ml (46µg) plant extract when compared to the untreated sample (24.6µg). Lanosterol was produced maximally at a concentration of 5mg/ml (46µg; Fig 4.12). As the plant extract concentration increased above 7.5mg/ml, lanosterol levels decreased (29µg). At plant extract concentrations of 10mg/ml and 12.5mg/ml, lanosterol levels (32.5µg and 26.8µg) were similar to those produced at 2.5mg/ml and 0mg/ml plant extract concentrations respectively. Thus at plant extract concentrations between 0mg/ml and 5mg/ml, lanosterol levels increased and at plant extract concentrations above 5mg/ml, lanosterol levels decreased. The variation in lanosterol levels could be attributed

to the fact that lanosterol is one of the intermediates for other pathways in the ergosterol biosynthetic pathway

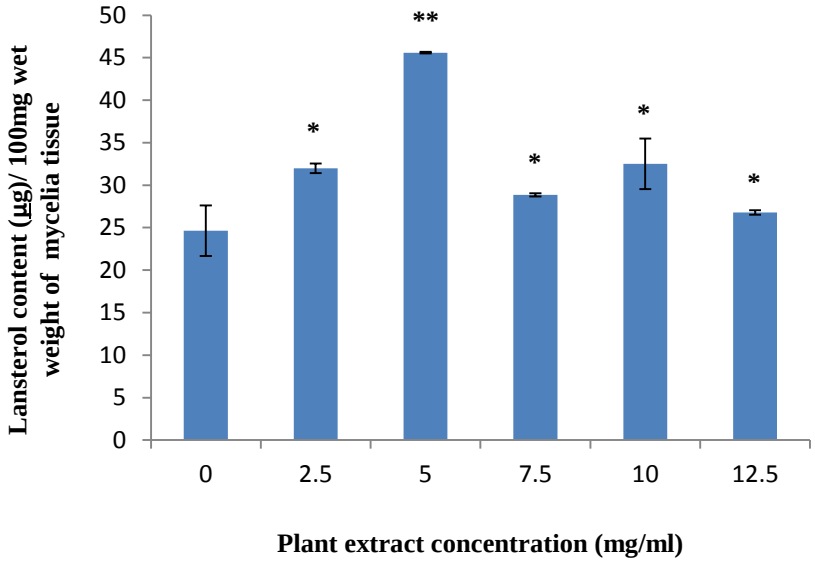


Figure 4.12: The effect of *T. violacea* plant extract on lanosterol production by *A .flavus*. Error bars represent the level of significance for each sample (n= 3). Asterisk represents the level of significance relative to the control (0mg/ml). * = p< 0.05; ** = p< 0.01.

4.4 Discussion

The importance of ergosterol in the regulation of cellular metabolism and in the functioning of membrane bound enzymes makes it and its biosynthetic pathway an ideal target for antifungal agents. Most antifungal agents that are currently used target the ergosterol pathway by either binding to metabolic intermediates, causing an accumulation of intermediate products or by inhibiting the pathway-specific enzymes themselves preventing conversion of the intermediates into ergosterol, resulting ultimately in the inhibition of ergosterol production (Oliaro-Bosso *et al*., 2005). Commercial terbinafine is an allylamine that causes accumulation of squalene by blocking the conversion of squalene to 2,3-oxidosqualene by squalene epoxidase. This results in the inhibition of ergosterol production and can result in cell death (Vickers *et al*., 1999). Fluconazole as well has been shown to cause an accumulation of lanosterol in *C. albicans, C. tropicalis* and *C. krusei* resulting in a decline in ergosterol production (Pfaller and Riley 1992). The accumulation of sterol intermediates and the inhibition of ergosterol production make such antifungal agents toxic towards their host. Our repertoire of antifungal agents is limited and some fungi have begun to develop resistance towards them. Multiple drug resistant fungal strains are also emerging (Morschhauser 2010).

Many plant derived natural products have been shown to inhibit ergosterol production in various fungal species (Yamamoto-Ribeiro *et al*., 2013). These include essential oils extracted from various plants that are capable of inhibiting ergosterol production in *A. flavus*, *Candida* and other fungal pathogens and in so doing disrupt cell membrane integrity (Ahmad *et al*., 2010; Ahmad *et al*., 2011a and b; Tian *et al*., 2012a and b; Yamamoto-Ribeiro *et al*., 2013). Saponins have also been shown to interact with the fungal cell membrane by binding directly to ergosterol to form sterol-saponin complexes. This results in disruption of cell membrane integrity through inhibition of ergosterol production (Avis, 2007; Bernards *et al*., 2011). In *Saccharomyces cerevisiae*, the steroidal saponin of tomato (α-tomatine) is known to target the ergosterol biosynthetic pathway. It inactivates the C_{24} methyltransferase and prevents the progression of zymosterol to fecosterol and ultimately to ergosterol (Simons *et al*., 2006).

In the current study ergosterol were isolated and detected by TLC. Ergosterol was found to be present in *A. flavus* samples that were untreated and treated with sub inhibitory concentrations of

the plant extract. With TLC analysis, no direct correlation could be established between plant extract concentration and ergosterol production by *A. flavus*. This is possibly because the sensitivity and resolving power of TLC is not as strong as RP-HPLC and co-elution of other lipid components or sterol intermediates with ergosterol, present in *A. flavus*, could have occurred. It was not possible to detect other sterol intermediates with TLC, again indicating that this technique may not be sensitive enough for the detection of these sterol intermediates. Reverse phase-HPLC analysis proved that during the isolation of ergosterol, other sterol components were present in the isolated samples (Fig 4.5). Thus RP-HPLC was found to be a much more sensitive technique than TLC for the detection of *A. flavus* sterols.

Reverse phase HPLC analysis showed that there was significant reduction in ergosterol production by *A. flavus* in the presence of the various concentrations of the plant extract when compared to the untreated sample. There was a dose-dependent decrease in ergosterol production with an increase in plant extract concentration (Fig 4.6). To validate the various assays and results obtained during this study, terbanifine was used as a positive control. When *A. flavus* cultures were subjected to terbanifine treatment, an identical trend was observed as with the *T. violacea* plant extract i.e. a significant decrease in ergosterol production in *A. flavus* was found to occur in a dose dependent manner upon exposure to terbanifine. Terbanifine is known to inhibit squalene epoxidase and therefore, in fungi, the conversion of squalene to 2,3-oxidosqualene is prevented (Alcazar-Fuoli and Mellado, 2013) resulting in a decrease in ergosterol production. The results obtained with terbanifine validated the assays used in this study and proved that the reduction in ergosterol production upon exposure of *A. flavus* to *T. violacea* plant extract was valid. It was therefore concluded that the plant extract either bound to ergosterol pathway intermediates or negatively affected key enzyme(s) responsible for ergosterol production resulting in a decrease in *A. flavus* ergosterol production.

Antifungal agents targeting cell membranes often affect the ergosterol biosynthetic pathway inhibiting ergosterol synthesis (Borreli *et al*., 2008; Alcazar-fuoli and Mellado, 2013). Squalene is the first stable sterol intermediate in the ergosterol biosynthetic pathway and its conversion to 2,3 oxidosqualene by squalene epoxidase (Veen *et al*., 2003; Gauwerky *et al*., 2009) is inhibited by terbinafine. The net result is depletion in ergosterol production (Oliaro-Bosso *et al*., 2005;

Vandeputte *et al*., 2011). Owing to the similarity between the effect of *T. violacea* plant extract and terbanifine on ergosterol production, squalene production was first quantified. In the present study, although significant increases in squalene production was observed in the presence of increasing concentrations of the plant extract, the quantity of squalene isolated after each treatment was extremely low (nanogram quantities). A low concentration of squalene was also noted in *A. flavus* cultures grown in the absence of the plant extract i.e. similar levels of squalene were produced in the presence and absence of the plant extract with no increasing or decreasing trends being observed. Taken together, this suggests that at this point in the ergosterol pathway, squalene was probably consumed during the formation of the next sterol intermediate, 2,3-oxidosqualene, in the pathway. Treatment of *A. flavus* cultures with varying concentrations of terbinafine also yielded low levels of squalene (results not shown), similar to that observed with *T. violacea* plant extract.

The 2,3-oxidosqualene intermediate produced between squalene and lanosterol in the ergosterol biosynthesis pathway is converted to lanosterol by oxidosqualene/lanosterol cyclase (Joffrion and Cushion 2010). Inhibition of this enzyme will prevent lanosterol production and ultimately ergosterol production. In this study, a progressive accumulation of 2,3-oxidosqualene with increasing concentrations of *T. violacea* plant extract was observed. This observation was more prevalent at plant extract concentrations of 5mg/ml and higher. A definitive and pronounced increase in 2,3-oxidosqualene production during treatment with increasing plant extract concentrations indicates that the plant extract was potentially preventing the conversion of 2,3-oxidosqualene to lanosterol by inhibiting oxidosqualene cyclase.

An investigation of the next intermediate in the ergosterol biosynthetic pathway, lanosterol, after treatment with different plant extract concentrations indicated that oscillations in lanosterol production in *A. flavus* had occurred. In *Aspergillus fumigatus*, ergosterol formation from the lanosterol intermediate, involves the use of alternative biochemical pathways, which at the end merge to form ergosterol (Alcazar-Fuoli *et al*., 2008). The fungus uses these pathways as a defense mechanism to adapt to and overcome non-favourable growth conditions, to ensure uninterrupted production of ergosterol thereby allowing for its survival. These alternative pathways make it possible for the fungus to overcome loss of some enzymes within the

ergosterol biosynthetic pathway (Luppetti *et al*., 2002; Alcazar-Fuoli *et al*., 2008). Oscillations in ergosterol production have been reported in *Fusarium verticilliodes*, where an increase in ergosterol was observed at lower concentrations of *Zinger officinale* extract, followed by a decline and then complete inhibition at the higher concentration of the essential oil (Yamoto-Ribeiro *et al*., 2013). Lucini *et al*. (2006) reported that at lower concentrations of the essential oils, the fungus adopted a defence mechanism to protect its cell wall structure by increasing ergosterol production. In the present study, the oscillation observed in lanosterol production could also be a defence mechanism of the fungus in response to exposure to sub-inhibitory levels of *T. violacea* plant extract to allow for the production of ergosterol. It is also possible that lanosterol may be produced by an alternative compensatory pathway not entirely affected by *T. violacea* plant extract resulting in variations in lanosterol levels in the presence of the plant extract.

Bulbs of *Allium sativum* contain a variety of saponins exhibiting antifungal activity (Lanzotti *et al*., 2012a and b; Teshima *et al*., 2013). They cause disruption of the cell membrane and loss in its structural integrity by binding directly to the cell membrane to form saponin-sterol complexes, which block ergosterol production by the fungus (Ahmed *et al*., 2012; Augustin *et al*., 2011). Since the *T. violacea* plant extract has been found to contain saponins, it is possible that these saponins may have a high affinity for sterols in the cell membrane and may be one of the contributing factors causing the reduction of ergosterol production by *A. flavus*. Further investigations on the role of saponins isolated from *T. violacea* plant extract on ergosterol production need to be done.

These findings show that *T. violacea* aqueous plant extract affect the ergosterol biosynthetic pathway of *A. flavus*. The results indicate that the plant extract (possibly saponins) acts on the ergosterol biosynthetic pathway by causing an accumulation of 2,3-oxidosqualene. It therefore appears that this inhibition may be brought about by the extract interfering with 2,3 oxidosqualene cyclase preventing the formation of downstream intermediate products resulting in inhibition of ergosterol production.

4.5 References

Ahmad, A., Khan, A., Manzoor, N. and Khan, L.A. 2010. Evolution of Ergosterol Biosynthesis Inhibitors as Fungicidal Against Candida. *Microbial Pathogenesis* **48**: 35-41.

Ahmad, A., Khan, A., Akhtar, F., Yousuf, S., Xess, I., Khan, L.A. and Manzoor, N. 2011(a). Fungicidal Activity of Thymol and Carvacrol by Disrupting Ergosterol Biosynthesis and Membrane Integrity Against *Candida*. *European Journal of Clinical Microbiology and Infectious Diseases* **30**: 41-50.

Ahmad, A., Khan, A., Kumar, P., Bhatt, R.P. and Manzoor, N. 2011(b). Antifungal activity of *Coriaria nepalensis* essential oil by disrupting ergosterol biosynthesis and membrane integrity against *Candida*. *Yeast* **28**: 611-617.

Alcazar-Fuoli, l., Mellado, E., Garcia-Effron, G., Buitrago, M.J., Lopez, J.F., Grimalt, J. O.,Cuenca-Estrella,J.M. and Rodriguez-Tudela, J.L. 2006. *Aspergillus fumigatus* C-5 Sterol Desaturases Erg3A and Erg3B: Role in Sterol Biosynthesis and Antifungal Drug Susceptibility. *Antimicrobial Agents and Chemotherapy* **50**(2): 453-460.

Alcazar-Fuoli., L., Mellado, E., Garcia-Effron, G., Lopez J.F., Grimalt, J.O., Cuensa-Estrella, J.M and Rodriguez-Tudela, J.L. 2008. Ergosterol Biosynthesis Pathway in *Aspergillus fumigatus*. *Steroids* **73**: 339-347.

Alcazar-Fouli, L. and Mellado, E. 2013. Ergosterol Biosynthesis in *Aspergillus fumigatus*: Its Relavance as An Antifungal Target and Role in Antifungal Drug Resistance. *Frontiers in Microbiology* **3**(439): 1-6.

Arthington-Skaggs, B. A., Warnock, D.W. and Morrison, C. 2000. Quantitation of *Candida albicans* Ergosterol Content Improves the Correlation between *In Vitro* Antifungal Susceptibility Test Results and In Vivo Outcome after Fluconazole Treatment in a Murine Model of Invasive Candidiasis. *Antimicrobial Agents and Chemotherapy* **44**: 2081-2085.

Augustin, J.M., Kuzina, V., Andersen, S.B. and Bak, S. 2011. Molecular Activities, Biosynthesis and Evolution of Triterpenoid Saponins. *Phytochemistry* **72**: 435-457.

Avis, T.J. 2007. Antifungal Compounds that Target Fungal Membranes: Applications in Plant Disease Control. *Canadian Journal of Plant Pathology* **29**: 323-329.

Belewa, V., Baijnath, H. and Somai, B.M. 2011. Aqueous Extracts from the Bulbs of *Tulbaghia violacea* are Antifungal Against *Aspergillus flavus*. *Journal of Food Safety* **31**: 176-184.

Bernards, M. A., Ivanov, D.A., Neculai, M.A. and Nicol, R.W. 2011. Ginsenosides: Phytoanticipins or Host Recognition Factors? *The Biological Activity of Phytochemicals*. D. R. Gang. Canada, Springer Science and Business Media. **41**.

Borelli, C., Schaller, M., Niewerth, M., Nocker, K., Baasner, B., Berg, D., Tiemann, R., Tietjen, K., Fugmann, B., Lang-Fugmann, S. and Korting, H.C. 2008. Mode of Action of the New Arylguanidine Abufungin beyond Interference with Ergosterol Biosynthesis and *In Vitro* Activity against Medically Important Fungi. *Chemotherapy* **54**: 245-259.

Cabral, M.E., Figueroa, L.I.C. and Farina, J.I. 2013. Synergistic Antifungal Activity of Statin-Azole Associations as Witnessed by *Saccharomyces cerevisiae* and *Candida utilis*-Bioassay and Ergosterol Quantification. *Revista Iberoamericana de Micologia* **30**(1): 31-38.

Chemler, J.A., Yan, Y. And Koffas, M.A.G. 2006. Biosynthesis of Isoprenoids, Polyunsaturated Fatty acids and Flavonoids in *Saccharomyces cerevisiae*. *Microbial Cell Factories* **5**(20): 1-9.

Chiocchio, V.M. and Matkovic, L. 2011. Determination of Ergosterol in Cellular Fungi by HPLC. A Modified Technique. *The Journal of the Argenbtine Chemical Society* **98**: 10-15.

de Lira Mota, K. M., de Oliviera Pereira, F., de Oliviera, W.A., Oliviera Lima, I. and de Oliviera Lima, E. 2012. Antifungal Activity of *Thymus vulgaris* L. Essential Oli and Its Constituent Phytochemicals Against *Rhizopus oryzae*: Interaction with Ergosterol. *Molecules* **17**: 14418-14433.

Gauwerky, K., Borelli, C. and Korting, H.S. 2009. Targeting Virulence: A New Paradigm for Antifungals. *Drug Discovery Today* **14**(3/4): 214-222.

Gessner, M.O. and Schmitt, A.L. 1996. Use of Solid-Phase Extraction to Determine Ergosterol Concentration in Plant Tissue Colonized by Fungi. *Applied and Environmental Microbiology* **62**(2): 415-419.

Jackson, C., Agboke, A. and Nwoke, V. 2009. *In vitro* Evaluation of Antimicrobial Activity Of Combinations of Nystatin and *Euphorbia Hirta* Leaf Extract against *Candida albicans* by the Checkerboard Method. *Journal of Medicinal Plants Research.* **3**: 666-669.

Jockovic, N., Pavlovic, M., Sabovljevic, M. and Kovacevic, N. 2007. Contribution to the Chemical Constituents of Balkan Bryophytes: Phenolic Acids, Flavonoids, Triterpenes and Alkaloids. *Natura Montenegrina, Podgorica* **6**: 123-129.

Joffrion, T.M. and. Cushion., M.T. 2010. Sterol Biosynthesis and Sterol Uptake in the Fungal Pathogen *Pneumocystis carinii*. *FEMS Microbiology Letters.* **3**(11): 1-9.

Lanzotti, V., Romano, A., Lanzuise, S., Bonanomi, G. and Scala, F. 2012(a). Antifungal Saponins from Bulbs of White Onion, *Allium cepa* L. *Phytochemistry* **24**: 133-139.

Lanzotti, V., Barile, E., Antignani, V., Bonanomi, G. and Scala, F. 2012(b). Antifungal Saponins from Bulbs of Garlic, *Allium sativum L. var. Voghiera*. *Phytochemistry* **78**: 126-134.

Liu, X., Fung, J., Yun, Y., Yin, Y. and Ma, Z. 2011. A Sterol C-14 Reductase Encoded by *FgERG24B* is Responsible for the Intrinsic Resistance of *Fusarium graminearum* to Amine Fungicides. *Microbiology* .**157**: 1665-1675.

Liu, X., Jianga, H., Yin, Y. and Ma, Z. 2013. Involvement of *FgERG4* in Ergosterol Biosynthesis, Vegitative Differentiation and Virulence in *Fusarium graminearum*. *Molecular Plant Pathology* **14**(1): 71-83.

Lucini, E.L., Zunino, M., Lopez, M. and Zygadlo, J.A. 2006. Effect of Monoterpenes on Lipid Composition and Slerotial Development of *Slerotium cepivorum Berk*. *Journal of Phytopathology* **154**: 441-446.

Lupetti, A., Danesi, R., Campa, M., Del Tacca, M. and Kelly, S. 2002. Molecular Basis of Resistance to Azole Antifungals.*Trends in Molecular Medicine* **8**(2): 76-81.

Morschhäuser, J. 2010. Regulation of Multidrug Resistance in Pathogenic Fungi. *Fungal Genetics and Biology*.**47**:94-106

Motsei, M. L., Lindsey, K.L., van Staden, J. and Jäger,A.K. 2003. Screening of Traditionally used South African Plants for Antifungal Activity Against *Candida albicans*. *Journal of Ethnopharmacology* **86**: 235-241.

Muller, C., Staudacher, V., Krauss, J., Giera, M. and Bracher, F. 2013. A Convenient Cellular Assay for the Identification of the Molecular Target of Ergosterol Biosynthesis Inhibitors and Quantification of their Effects on Total Ergosterol Biosynthesis. *Steroids* **78**: 483-493.

Ncube, B., Ngunge, V.N.P., Finnie, J.F. and Van Staden, J. 2011. A Comparative Study of the Antimicrobial and Phytochemical Properties Between Outdoor Grown and Micropropagated *Tulbaghia violacea* Harv. Plants. *Journal of Ethnopharmacology* **134**: 775-780.

Oliaro-Bosso, S., Ceruti, M., Balliano, G., Milla, P., Rocco, F. and Viola, F. 2005. Analogs of Squalene and Oxidosqualene Inhibit Oxidosqualene Cyclase of *Trypanosoma cruzi* Expressed in *Saccharomyces cerevisiae*. *Lipids* **40**(12): 1257-1262.

Pinto, E., Hrimpeng, K., Lopes, G., Vaz, S., Goncalves, M.J., Cavaleiro, C. and Salgueiro, L. 2013. Antifungal Activty of *Ferulago capillaris* Essential Oil Against *Candida, Cryptococcus, Aspergillus* and Dermatophyte Species. *European Journal of Clinical Microbiology and Infectious Diseases*: **32**:1311-1320.

Pfaller, M. and Riley, J. 1992. Effects of Fluconazole on the Sterol and Carbohydrate Composition of Four Species of *Candida*. *European Journal of Clinical Microbiology and Infections and Disease* **11**(2): 152-155.

Rodriguez, C.F., Silva, S. and Henriques, M. 2014. *Candida glabrata*: A Review of its Features and Resistance. *European Journal of Clinical Microbiology* **33**:673-688.

Sajjad, M., Khan, A. and Ahmad, I. 2011. Antifungal Activity of Essential Oils and their Synergy with Fluconazole Against Drug-Resisitant Strains of *Aspergillus fumigatus* and *Trichophyton rubrum*. *Applied Microbial Biotechnology* **90**: 1083-1094.

Simons, V., Morrisey, J.P., Latijnhouwers, M., Csukai, M., Cleaver, A., Yarrow, C. and Osbourn, A. 2006. Dual Effects of Plant Steroidal Alkaloids on *Saccharomyces cerevisiae*. *Antimicrobial Agents and Chemotherapy* **50**(8): 2732-2740.

Spanggord, R.J., Sun, M., Lim, P. and Ellis, W.Y. 2006. Enhancement of an Analytical Method for the Determination of Squalene in Anthrax Vaccine Adsorbed Formulations. *Journal of Pharmaceutical and Biomedical Analysis* **42**: 494-499.

Tardieu, D., Bailly, J.D., Benard, G. and Guerre, P. 2007. Comparison of Two Extraction Methods for Ergosterol Determination in Vegetal Feeds. *Revuede Medecine Veterinaire* **159**(8-9): 442-446.

Teshima, Y., Ikeda, T., Imada, K., Sasaki, K., El-Sayed, M.A., Shigyo, M., Tanaka, S. and Ito, S-I. 2013. Identification and Biological Activity of Antifungal Saponins from Shallot (*Allium cepa* L. Aggregatum Group). *Journal of Agriculture and Food Chemistry* **61**: 7440-7445.

Tian, J., Haung, B., Luo, X., Zeng, H., Ban, X., He, J. and Wang, Y. 2012(a). The Control of *Aspergillus flavus* with *Cinnamomum jensenianum* Hand-Mazz Essential Oil and Its Potential Use As a Food Preservative.*Food Chemistry* **130**: 520-527.

Tian, J., Ban, X, Zeng, H., He, J., Chen, Y. and Wang, Y. 2012 (b)) The Mechanism of Antifungal Action of Essential Oil from Dill (*Anethum graveolens L.*) on *Aspergillus flavus*. *PloS ONE* **7**(1): 1-10.

Vandeputte, P., Ferrari, S. and Coster, A.T. 2011. Antifungal Resistance and New Strategies to Control Fungal Infections. *International Journal of Microbiology* **2012**: 1-26.

Veen, M., Stahl, U. and Lang, C. 2003. Combined Overexpression of Genes of the Ergosterol Biosynthetic Pathway Leads to Accumulation of Sterols in *Saccharomyces cerevisiae*. *Yeast Research* **4**: 87-95.

Vickers, A.E., Sinclair, J.R., Zollinger, M., Heitz, F., Glanzel, U., Johanson, L. and Fischer, V. 1999. Multiple Cytochrome P-450s Involved in the Metabolism of Terbinafine Suggest a Limited Potential for Drug-Drug Interactions. *Drug Metabolism and Disposition* **27**(9): 1029-1037.

Xu, J.-W., Xu, Y-N. and Zhong, J-J. 2010. Production of Individual Ganoderic Acids and Expression of Biosynthetic Genes in Liquid Static and Shaking Cultures of *Ganoderma lucidum*. *Applied Microbial Biotechnology* **85**: 941-948.

Yamamoto-Ribeiro, M.M.G., Grespan, R., Kohiyama, C.Y., Ferreira, F.D., Mossini, S.A.G., Silva, E.L., de Abreu Fiho, B.A., Mikcha, J.M.G. and Machinski Junior, M. 2013.

Effect of *Zinger officinale* Essential Oil on *Fusarium verticilliodes* and Fumonisin Production.*Food Chemistry* **141**: 3147-3152.

Yuan, J-P., Wang, J-W., Liu, X., Kuang, H-C. and Zhao, S-Y. 2007. Simultaneous Determination of Free Ergosterol and Ergosteryl Esters in *Cordyceps sinensis* by HPLC. *Food Chemistry* **105**:1755-1759.

Zarzycki , P. K., Baran , M. Włodarczyk, E. and Bartoszuk,M. A. 2007. Improved Detection of Ergosterol, Stigmasterol, and Selected Steroids on Silica Coated TLC Plates using Phosphomolybdic Acid Staining. *Journal of Liquid Chromatography and Related Technologies* **30**(17): 2629-2634.

CHAPTER 5

THE EFFECT OF *Tulbaghia violacea* AQUEOUS EXTRACT ON THE FUNGAL CELL WALL of *Aspergillus flavus*

5.1 Introduction

The fungal cell wall is a dynamic structure that is crucial to the survival and growth of fungi. It provides the fungus with shape and integrity, allows interaction with its surroundings and regulates membrane fluidity. The rigid structure allows the organism to withstand and survive unfavourable conditions (Fujioka *et al*., 2007; Latge, 2007). The cell wall is a complex cross-linked network of polysaccharides made up of chitin, glucans and mannans and is held together by cell wall proteins (Guerriero *et al*., 2013). Chitin is a linear homopolymer of linked β-(1,4) N-acetyl glucosamine. It is a major component of the cell wall which maintains the structural integrity of the cell and provides a link between the fungus and the host cell (Rogg *et al*., 2012). The glucan component of the cell wall is an important component and aids in the survival of the fungus. It is made up of glucose monomers, which are linked by β-(1,3) or β-(1,6)-bonds. β-(1,3) glucan is synthesized from UDP-glucose by the membrane bound enzyme, β-(1,3)-glucan synthase (Latge *et al*., 2005). It serves as the attachment surface for other cell wall components and provides the cell wall with structural integrity (Cowen and Steinbach 2008). The cell wall is made up of N- and O-linked oligosaccharides, whose structure varies amongst fungi. In some fungi, the oligosaccharide chains are composed of galactommanans made up of mannose and galactose residues. This offers protection against unfavourable conditions and maintains the structure and rigidity of the cell (Bowman and Free 2006).

The complex arrangement and the uniqueness of the fungal cell wall makes it an ideal target for antifungal agents. Most of these antifungal agents target cell wall enzymes such as chitin and β-glucan synthases to inhibit the production of cell wall components. The glucan synthase complex is made up of two membrane-bound protein subunits: (a catalytic (Fksp) and a regulatory (Rho) subunit that regulate glucan synthesis in the cell (Garcia-Effron *et al*., 2009). Echinocandins are

able to bind to these subunits and inactivate them resulting in inhibition of glucan synthase activity (Staab *et al*., 2010; Walker *et al*., 2013). Nikkomycin inhibits chitin synthesis by binding to chitin synthase complexes thereby inhibiting their activity (Fortwendel *et al*., 2009). Although antifungal agents such as echinocandins have been effective in inhibiting the synthesis of cell wall components (β-glucans), the fungus develops a compensatory mechanism for the loss of one component by increasing the production of another (e.g. chitin) and this can result in development of resistance towards the antifungal agent (Fortwendel *et al*., 2009; Lee *et al*., 2011; Rogg *et al*., 2012). Plant extracts too have been shown to be effective in inhibiting the production of cell wall components such as chitin (Maoz and Neeman, 2000; Souza *et al*., 2010; Khan *et al*., 2013). The essential oil, *trans*-cinnamaldehylde, for example, is known to specifically target β-(1,3) glucan synthase and chitin synthase (Bang *et al*., 2000). Recently, *T. violacea* aqueous plant extract has been shown to have antifungal activity against *A. flavus* (Belewa *et al*., 2011), but its mechanism of action on the cell wall components has not been studied. The aim of the current study, was to ninvestigate the effect of *T. violacea* aqueous plant extract on chitin and β-(1,3) glucan synthesis, together with its effect on β-(1,3) glucan synthase, chitin synthase and chitinases.

5.2 Materials and methods

5.2.1 Growth conditions

Aspergillus flavus strain (2527) was maintained in GMM at 30°C. When required, conidia were resuspended by flooding a plate with 0.1% Tween 20 and the spores resuspended by scraping the surface of the plate with a sterile glass rod. For each assay, *A. flavus* was grown in the presence of various concentrations (2.5, 5, 7.5, 10 and 12.5mg/ml) of plant extract or positive control (caspofungin or nikkomycin Z). stock solutions of caspofungin and nikkomycin Z were prepared in water for the construction of standard curves.

5.2.2 Materials and equipment used

The reagents used for this study were purchased either from Sigma Aldrich or Merck Millipore South Africa. 4 -Methylumbeliferone, 4-methylumbeliferyl-N-Acetyl-β-D-glucosaminide (4-MU-GlcNAc); 4-methylumbeliferyl-β-D-N-N-diacetylchitobioside (4MU-$(GlcNAc)_{2)}$; 4-methylumbeliferyl-β-D-N-N-N-triacetylchitotriose (4MU-$(GlcNAc)_{3)}$; D+ glucose, NaOH, aniline blue, bovine serum albumin (BSA), glycine, nikkomycin Z, curdlan, cellite 303, $NaNO_2$, $KHSO_4$, ammonium sulphamate, 3-methyl-2-benzothiazolinone hydrozone hydrochloride hydrate (MBTH), $FeCl_3$, glucosamine, phenylmethylsulfonyl fluoride [PMSF], Bradford reagent, GTP, EDTA, Brij 35, glycerol, UDP-Glc, wheat germ agglutin (WGA), magnesium acetate, β-mercaptoethanol, GlcNAc, UDP-GlcNAc, trypsin, glass beads, WGA-horseradish peroxidase conjugate, 3,3',5,5'-tetramethylbenzidine (TMB) and dithiothreitol (DTT) were purchased from Sigma Aldrich, South Africa. Tris-HCl, EGTA, hydrogen peroxide, ethanol and calcofluor white were purchased from Merck-Millipore South Africa. Caspofungin was purchased from Aspen Pharmacare, South Africa. The equipment used included a Synergy MX Fluorescence reader (Biotek), Power wave XS 96 well microtiter plate reader (Biotek) and an ultracentrifuge (Beckman Coulter).

5.2.3 Quantification of β-(1, 3) glucan

Quantification of total β-glucan content in the presence and the absence of the plant extract was done with the aniline blue flourescence assay using a modified procedure as described by Fortwendel *et al*., (2009) and Shedletzky *et al*., (1997). Aniline blue binds to β-(1,3) glucan to form a β-(1,3) glucan/aniline blue flourochrome complex. The unbound fluorescent dye is then decolourized at room temperature and the bound β-(1,3) glucan complex emits fluorescence at an excitation of 366nm and an emission wavelength of 460nm (Shedletzky *et al*., 1997; Ko and Lin 2004). One million conidia per milliliter were inoculated into GMM containing 5% yeast extract, in the presence or absence of various concentrations (2.5, 5, 7.5, 10 and 12.5mg/ml) of *T. violacea* plant extract. As a positive control, 1 x 10^6 conidia were also grown in the presence of increasing concentrations (3.125, 6.25, 12.5, 25 and 50µg/ml) of caspofungin (commercially available antifungal agent). Cultures were incubated at 30°C with continuous shaking at 200 rpm for 4 days. Mycelia were harvested and washed with 0.1M NaOH, snap frozen in liquid nitrogen and freeze dried for 24h. Five milligrams of lyophilized mycelium was resuspended in 250µl 1M NaOH and incubated at 52°C for 30min. Fifty microlitres of each sample extract was transferred to 3 wells of a 96 well microtiter plate. The 3 samples represented a replicate. Two hundred and ten microlitres of aniline blue mix (40 volumes of 0.1% aniline blue, 21 volumes of 1N HCl, 59 volumes 1M glycine NaOH; pH 9.5) was added to each well and the plate was incubated for a further 30min at 52°C. After the incubation period, the plate was allowed to cool for 30min at room temperature to allow for decolourization of the unbound dye. The fluorescence of the bound dye complex was detected using a Synergy MX Fluorescence reader (Biotek) at an excitation of 450nm and emission wavelength of 460nm. A standard curve was also constructed using various concentrations of curdlan ranging between 10- 50µg/ml (Appendix 1, Fig A10). This standard curve was used to quantify the amount of β-(1, 3) glucan present in each sample. The experiment was completed in triplicate and statistical analysis was performed using the student t-test.

5.2.4 Glucan synthase activity assay

The effect of the plant extract on β-glucan synthase activity was investigated using the aniline blue-fluorescence assay once again. The assay involves the detection of β-(1,3) glucan synthase

from crude microsomal membranes. During this process the mycelia are disrupted with glass beads in the presence of a homogenisation buffer made up of sucrose, EDTA, EGTA, DTT, β-mercaptoethanol, GTP and Tris/HCl. Tris/HCl acts as the stabilizing agent of the extracted microsomal membranes, EGTA and EDTA chelate the divalent cations, with EGTA having a high affinity for calcium ions. Beta-mercaptoethanol acts as a reducing agent and breakes disulphide bonds between proteins. The presence of GTP in the buffer is important as it activates glucan synthases. DTT increases the activity and stability of the enzyme and also permealizes the membrane to allow for easier accessibility of the substrate (Frost *et al*., 1994). Once the microsomal membranes have been isolated the protein content is quantified with the Bradford assay, with BSA as the positive control. The Bradford assay is based on the ability of the Bradford reagent to bind to the protein to form a protein-dye complex. This is indicated by a colour change of the Bradford from red to a blue colour in the presence of the protein and this is measured at 595nm (Bradford, 1976). The BSA is used to construct the standard curve, which was used to quantify the amount of protein present in the sample (Appendix 1, Fig A11).

5.2.4.1 Preparation of microsomal proteins

Aspergillus flavus cultures were grown in GMM containing 5% yeast extract, in the presence or absence of various concentrations of the plant extract and in the presence of increasing concentrations (3.125µg/ml, 6.25µg/ml, 12.5µg/ml, 25µg/ml and 50µg/ml) of caspofungin at 30°C with continuous shaking at 200rpm. One hundred milligrams wet mycelia was mixed with 1mm diameter glass beads and resuspended in 5ml of homogenisation buffer (250 mM sucrose, 3 mM EDTA, 3 mM EGTA, 3 mM DTT, 20 mM β-mercaptoethanol, 25 µM GTP, 70 mM Tris-HCl pH 8.0). These were vortexed for 20min at 5min intervals. The homogenised mixture was transferred to a clean tube and the beads were washed with 5ml of washing buffer (1 mM EDTA, 1 mM EGTA, 4 mM DTT, 25 µM GTP, 50mM Tris-HCl pH 8.0) to remove any microsomal material that may have been clinging to them. The fractions were combined and the suspension was centrifuged at 3,000x*g* at 4°C for 20min. The supernatant was centrifuged at 100,000x*g* for 1h at 4°C to recover the microsomal membranes. The pellet was resuspended in 500µl storage buffer (1 mM EDTA, 1 mM DTT, 33% (v/v) glycerol, 50 mM Tris-HCl pH 7.5) and stored at -

80°C until further use (Frost *et al.,* 1994). The protein content of the microsomal membrane was quantified with the Bradford assay (Bradford 1976, Appendix 1, A11).

5.2.4.2 Glucan synthase assay

A non-radioactive assay was used to quantify the amount of β-glucan synthase that was produced by *A. flavus* when grown in the presence and the absence of the plant extract. The reaction mixture was made up of 50 µl 50mM Tris-HCl pH 7.5, 20 µM GTP, 4 mM EDTA, 0.5% Brij 35, 6.6% glycerol, 2 mM UDP-Glc and 100 µg of the isolated microsomal protein. This was incubated at 25°C for 30min and the reaction was stopped by adding 10 µl of 6N NaOH. The glucan produced by the reaction was then solubilised by incubating at 80°C for 30min and was quantified with the aniline blue fluorescence assay (section 5.2.3). Fluorescence of the bound dye complex was detected using a Synergy MX Fluorescence reader (Biotek) at an excitation of 450nm and an emission wavelength of 460nm. The curdlan standard curve (Appendix 1, A12) was used for the quantification of β-glucan formed in the presence and the absence of the plant extract or caspofungin (positive control). The assay was completed in triplicate (Shedletsky *et al*., 1997). Statistical analysis was performed using the student t-test.

5.2.5 Quantification of total chitin content

A chitin assay was used to determine the amount of chitin produced by *A. flavus* in the presence and absence of either the plant extract or nikkomycin Z. This assay involves the deacylation of chitin into an insoluble chitosan by KOH. This is followed by the depolymerisation and deamination of chitosan into a soluble aldehyde by $NaNO_2$ and $KHSO_4$. The aldehyde forms a dark blue reaction with ferric chloride and MBTH (Lehmann and White, 1975).

The growth, harvesting and lyophilization of conidia was done as previously mentioned for β-glucan quantification except that serial dilutions of nikkomycin Z (1.56, 3.125, 6.25, 12.5 and 25) instead of caspofungin were used. The chitin assay was done using modified procedures of Fortwendel *et al*., (2009) and Staab *et al*., (2010). Five milligrams of lyophilized mycelium was resuspended in 3 ml 1M KOH and this was incubated at 130°C for 1h. The mixture was then cooled to room temperature and 8ml 70% ethanol was added. This was then vortexed until a single phase was formed and was incubated on ice for 15min, after which 0.3ml freshly

prepared 13.3% (w/v) Cellite was added. The tubes were centrifuged at 1,500x*g* for 5min at 2°C. The supernatant was discarded and the pellet was washed once with 10ml 40% ethanol and twice with distilled water. After the washing steps, the pellet was resuspended in 0.5ml sterile double distilled water and this was mixed with 0.5ml 5% (w/v) $NaNO_2$ and $KHSO_4$, respectively. The tubes were gently mixed at room temperature for 15min, followed by centrifugation at 1500x*g* for 2min at 2°C. Each sample (100µl) was mixed with 400µl of sterile double distilled water. Two hundred microliters of 12.5% (w/v) ammonium sulfamate was added to each sample and vortexed for 5min. This was followed by the addition of 0.2ml freshly prepared 5mg/ml of 3-methyl-2-benzothiazolinone hydrozone hydrochloride hydrate (MBTH) and an incubation at 130°C for 3min. The mixture was allowed to cool to room temperature and 0.2ml 0.49% $FeCl_3$ was added to each tube. This was incubated at room temperature for 25min and the absorbance of the samples was taken at 650nm. A negative control containing water, 5% (w/v) $NaNO_2$ and $KHSO_4$, MBTH and $FeCl_3$ was prepared to determine the background fluorescence of the reagents. Various standard concentrations of glucosamine (ranging between 5µg/ml and 25µg/ml) in water was used to construct a standard curve (Appendix 1; Fig A13) in order to calculate the amount of glucosamine released from chitin in the presence and absence of the plant extract or nikkomycin Z (positive control). This experiment was done three times and statistical analysis was done using the student t-test.

5.2.6 Chitin synthase activity assay

A non-radioactive chitin synthase assay was used for the detection of chitin synthase in the presence and absence of the plant extract and nikkomycin Z. The assay involves binding of chitin to WGA, a chitin-binding lectin with a high affinity for glucosamine and N-acetyl-D-neuranimic acid. This is followed by binding of the immobilised chitin to WGA-conjugated to horseradish peroxidase. After a washing step, horseradish peroxidase (HRP) activity is monitored by the TMB reagent. During this reaction HRP catalyses oxidation of TMB by H_2O_2 into two products. The first product is a partially oxidized blue charged-transfer complex, which has an absorption spectrum ranging from 575nm-725nm and absorbs maximally at 625nm. The second product is a yellow diimine compound which is produced by further oxidation of the charge-transfer complex. This product has an absorption spectrum ranging from 400nm-500nm and a maximum

absorption wavelength at 450nm (Lucero *et al*., 2002; Li *et al*., 2009). In the presence of an acid such as H_2SO_4 the yellow diimine becomes more stable with a maximum absorption wavelength of 450nm and this increases the sensitivity of the reaction (Lucero *et al*., 2002).

5.2.6.1 Preparation of the microsomal membranes

The microsomal membranes were prepared using modified protocols of Fortwendel *et al*., (2010) and Mellado *et al*., (2003). Cultures of *A. flavus* were grown in the presence or absence of plant extract as described for the isolation of microsomal membranes for β-glucan analysis (section 5.2.4.1). Each mycelial sample was filtered and washed three times with sterile double distilled water. The wet mycelium was resuspended in 5ml extraction buffer (50 mM Tris-HCl pH 7.5; 50 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF]) and glass beads were added to each sample tube. This was vortexed for 20min at 5min intervals until the culture was completely pulverized. The suspension was centrifuged at 10,000x*g* for 20min. The supernatant was centrifuged at 100,000x*g* for 1h to recover the microsomal proteins. The resulting pellet was washed with 5 ml wash buffer (50mM Tris-HCl, pH 7.5, and 5mM magnesium acetate) and was centrifuged at 100,000 x*g* for 1 h. The pellet was resuspended in 50mM Tris-HCl, pH 7.5 containing 30% (v/v) glycerol and stored at -80°C until further use. The protein content of the microsomes was quantified with the Bradford assay.

5.2.6.2 Chitin synthase assay

A non-radioactive chitin synthase assay was performed using a modified version of Lucero *et al*., (2002); Fortwendel *et al*., (2010) and Magellan *et al*., (2013).

A stock solution of Wheat germ agglutin (WGA) (1mg/ml in 50mM Tris-HCl pH 7.5) was diluted to 50µg/ml in sterile double distilled water. One hundred microliters of the 50µg/ml WGA was added to each well and incubated for 24h at room temperature. The wells were washed three times with double distilled water, with shaking in between each wash to remove any unbound WGA. Thirty microliters of blocking buffer (20 mg/ml BSA, 50 mM Tris-HCl pH 7) was added to each well and incubated for 3h at room temperature or stored at -20°C until further use.

The blocking buffer was removed from the well by vigorous shaking and 100µl of 2x reaction mixture (32 mM Tris-HCl pH 7.5, 4.3 mM magnesium acetate, 32 mM GlcNAc, 1.1 mM UDP-

GlcNAc,4 µg trypsin) containing 100µg of microsomal protein was added to each well. The plates were incubated at room temperature for 90min and after the incubation period 20µl of 50mM EDTA pH 8.0 was added to each well. The plate was vortexed for 30s and this was followed by the addition of 100µl of 1µg/ml WGA-horseradish peroxidase conjugate (in 20mg/ml BSA) to each well to detect the amount of bound chitin in the wells. This was followed by incubation at room temperature for 15min. After the incubation period, the plate was emptied by vigorous shaking and washing 5 times with double distilled water. Once the unbound chitin was removed from the plate, 100µl of TMB peroxidase reagent (1 ml 20% H_2O_2, 9 ml sterile double distilled water, 1 mg of TMB in 10ml of sterile water with 20% H_2O_2) was added to each well and incubated at room temperature for 5min. The reaction was stopped by the addition of 100µl 1N H_2SO_4 and absorbance was measured at 450nm. Negative controls consisting of the 2x reaction mixture with no UDP-GlcNAc, and with no trypsin were prepared (for background absorbance). A standard curve of chitin azure in 1.7M acetic acid at a range of 10µg/ml -70µg/ml was constructed to quantify the amount of chitin synthase in each sample (Fortwendel *et al*., 2010) (Appendix 1; Fig A14). The experiment was done in triplicate and . Statistical analysis was performed using student t-test.

5.2.7 Microscopic analysis

The effect of the plant extract on fungal morphology was assessed. *Aspergillus flavus* spores were inoculated into liquid YES medium, in the presence and absence of various concentrations of plant extract (2,5mg/ml; 5mg/ml; 7.5mg/ml; 10mg/ml and 12.5mg/ml) and grown as standing cultures at 30°C for 48h. Fifty milligrams wet weight of mycelia was mixed with sterile water and vortexed for 5min. One hundred microliters of the supernatant containing the spores was then transferred to a microscope slide and allowed to dry for 3min. A coverslip was then placed on the slide and the mycelial preparation viewed on the light microscope at 400x magnification for any changes in fungal physiology. The effect of the plant extract on chitin and β-glucan production was also assessed by calcofluor and aniline blue staining. One hundred microliters of the supernatant was mixed with 10µl of calcofluor white stain (100µg/ml), placed onto a microscope slide and was incubated at room temperature for 5min. The slide was then rinsed with double distilled water and a coverslip was placed on the slide. The slides were then

visualized under the fluorescence microscope for chitin deposition on the hyphal cross walls. The same procedure was followed for the staining with aniline blue for the visualization of β-glucan on the hyphal tips and along the hyphal structure (Fortwendel *et al*., 2010).

5.2.8 Chitinase assay

The chitinolytic activity of chitinases involved in cell wall synthesis was determined with a flourimetric assay using fluorescent 4-methylumbeliferone N-acetyl glucosamine (4MU-$(GlcNAc)_{1-3}$) oligosaccharides as substrates. The substrates function as dimeric, trimetric and tetrametic compounds and the reaction is based on the hydrolysis of the substrates resulting in the release of the fluorescent product, 4MU, which can be excited at 355nm and detected at an emission wavelength of 460nm (Haran *et al*., 1995; Hodge *et al*., 1995). The effect of the plant extract on the chitinase activity of *A. flavus* with emphasis on chitobiosidase, β-N-acetylglucosaminidase (exochitinases) and endochitinase activities was investigated. The three substrates that were used were 4-methylumbelliferyl N,N′-diacetyl-β-D-chitobioside, 4-methylumbelliferyl N-acetyl-β-D-glucosaminide and 4-methylumbelliferyl β-D-N,N′,N′′-triacetylchitotroise. The activity of each substrate was expressed as the amount of 4-methylumbeliferone (4-MU) that was released.

5.2.8.1 Preparation of microsomal protein

Aspergillus flavus cultures were grown in the presence or absence of various concentrations of the plant extract (0, 2.5l, 5, 7.5, 10 and 12.5 mg/ml) and also in the presence of the positive control, acetazolamide, at concentrations of 1.25, 2.5, 5, 10l and 20µg/ml respectively. One hundred milligrams wet mycelia was mixed with 10ml of protein extraction buffer (1 mM EDTA, 10 mM Tris-HCl (pH 7.4), 1 µm PMSF) and mycelial tissue was disrupted by vortexing in the presence of glass beads for 5 min at 4°C. This was repeated twice and the resulting homogenates were pooled. These were centrifuged at 17,000 x*g* for 20 min and the supernatant used for further analyses. The protein content in the supernatant was quantified using the Bradford assay (Guthrie *et al*., 2005).

5.2.8.2 Enzyme activity assay

A flourimetric chitinase activity assay was done with 4-methylumbeliferyl-N-Acetyl-β-D-glucosaminide (4-MU-β-GlcNAc), 4-methylumbeliferyl-β-D-N-N-diacetylchitobioside (4MU-β-$(GlcNAc)_2$) and 4-methylumbeliferyl-β-D-N-N-N-triacetylchitotriose (4MU-β-$(GlcNAc)_3$) as substrates. A reaction mixture made up of 100 µg of microsomal protein, 50 mM citrate-phosphate buffer pH 6.0 (257 ml of 0.2 M Dibasic sodium phosphate, 243 ml of 0.1 M Citric acid, 500 ml double de-ionized water) and 20 µM of substrate in a total volume of 100 µl was incubated at 37°C for 1h. The reaction was stopped by adding 50 µl 0.2 M Na_2CO_3 followed by incubation at room temperature for 5min. Standard curves (Appendix 1, Figs A15-A17) were constructed using various concentrations of 4MU to quantify the amount of 4MU released for each substrate. The amount of MU released was measured at an excitation of 355nm and an emission wavelength of 460nm (Alcazar-Fuoli *et al*., 2011).

5.3 Results

5.3.1 Quantification of β-(1,3) glucan content

β-(1,3) glucan content was quantified with the aniline blue fluorescence based assay. Curdlan was used as the standard for the quantification of the β-glucan content of *A. flavus* (Appendix 1, Fig A10).

From Fig 5.1 it was observed that there was a significant decline in the amount of β-glucan content produced by *A. flavus* in the presence of the plant extract when compared to the untreated sample ($p<0.05$). The reduction in β-glucan content increased when the fungus was treated with an increasing plant extract concentration. Although significant decreases in β-glucan production occurred at extract concentrations of 2.5mg/ml and 5mg/ml, a significant and dramatic decline in β-glucan content was observed ($p<0.01$) at an extract concentration of 7.5mg/ml. Although there was a decrease in β-glucan production at an extract concentration of 10mg/ml, the decrease was not significant when compared to that produced in the presence of an extract concentration of 7.5mg/ml ($p = 0.38$). The decrease in β-glucan production was more significant at an extract concentration of 12.5mg/ml ($p< 0.01$) when compared to that produced in the presence of 10mg/ml plant extract concentration.

When *A. flavus* was treated with caspofungin (positive control), there was a significant decline in the β-glucan content of the fungus as well, when compared to the untreated sample ($p<0.05$) (Fig 5.2). Increasing caspofungin concentrations resulted in corresponding decreases in β-glucan production by the fungus with significant differences in β-glucan content being observed at all five caspofungin concentrations tested ($p <0.01$).

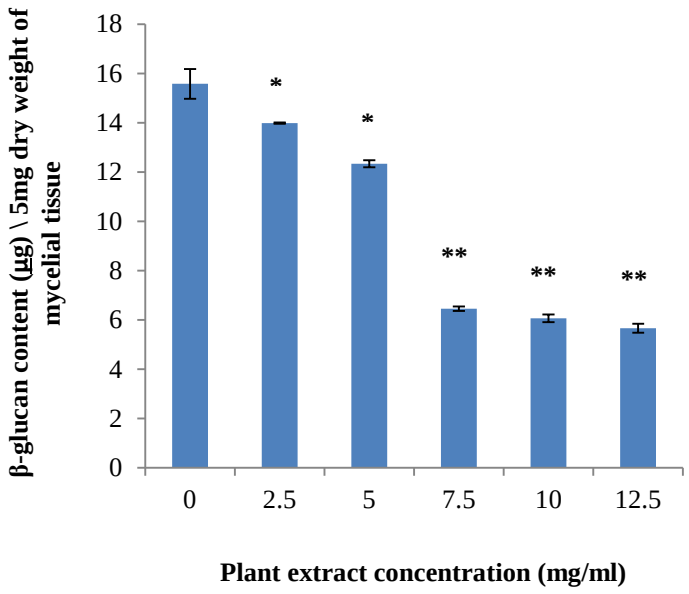


Figure 5.1: **The effect of *T. violacea* plant extract on β-glucan production by *A. flavus*. Error bars represent the standard deviation between each sample (n=3) and the asterisks represent the level of signifance relative to the untreated sample (0mg/ml). * = p <0.05; ** = p < 0.01.**

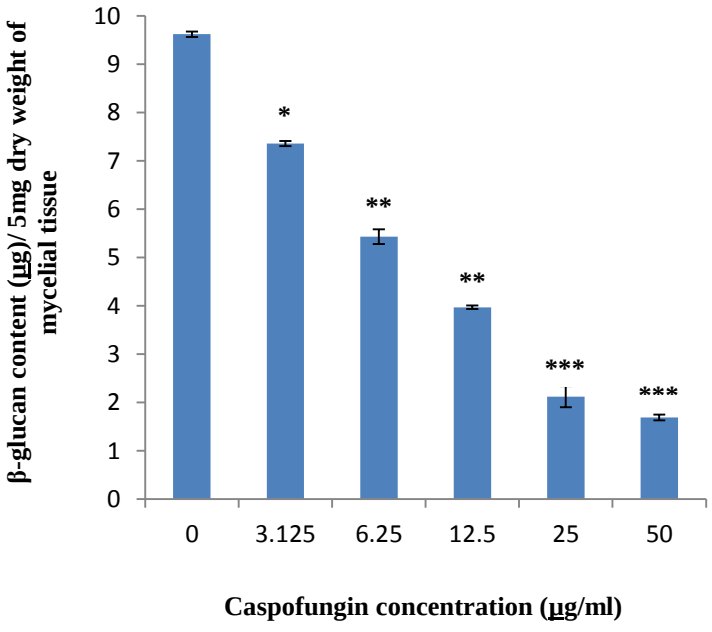


Figure 5.2: **The effect of caspofungin on β-glucan production by *A. flavus*. Error bars represent the standard deviation between each sample (n=3) and the asterisks represent the level of significance relative to the untreated sample (0µg/ml). * = p < 0.05; ** = p<0.01; *** = p< 0.001.**

5.3.2 β-glucan synthase quantification

For the determination of β-(1,3) glucan synthase activity, the aniline blue assay was used. Aniline blue binds to the glucan synthesized in the presence of the substrate UDP-glucose, to form a fluorescent complex. Quantification of β-(1,3) glucan synthase activity in the presence and absence of the plant extract showed that there was a significant decline in enzyme activity in the presence of the plant extract when compared to its absence ($p<0.05$; Fig 5.3). With an increasing plant extract concentration, the enzyme activity decreased in a dose dependent manner. Between treatments, the observed decrease was significant ($p<0.01$). Curdlan, which was used to generate a standard curve, showed a linear response with good correlation (R^2 =0.994) between the different concentrations of curdlan and the fluorescence intensity (Appendix 1, Fig A12).

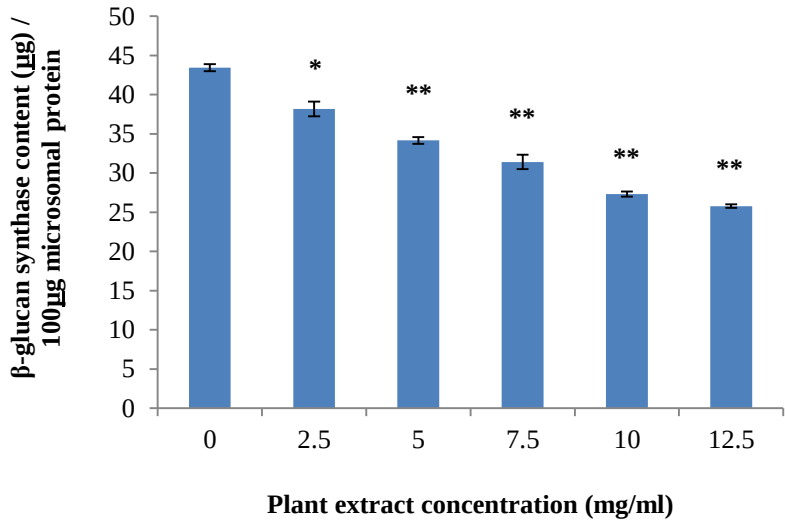


Figure 5.3: The effect of *T. violacea* plant extract on β-(1,3) glucan synthase activity of *A. flavus*. Error bars denote the standard deviation between each sample (n = 3) and the asterisks represent the level of significance relative to the untreated sample (0mg/ml). * = p < 0.05; ** = p<0.01.

Treatment of *A. flavus* with caspofungin showed a similar trend in β-(1,3) glucan synthase activity to that with the plant extract, with a significant reduction in enzyme activity in the presence of caspofungin when compared to the untreated sample (Figure 5.4) ($p<0.01$). The various concentrations of caspofungin also showed significant differences in their inhibitory

effect on β-(1,3) glucan synthase levels when compared to one another ($p<0.001$), with caspofungin concentrations of 12.5µg/ml, 25µg/ml and 50µg/ml causing the more pronounced declines.

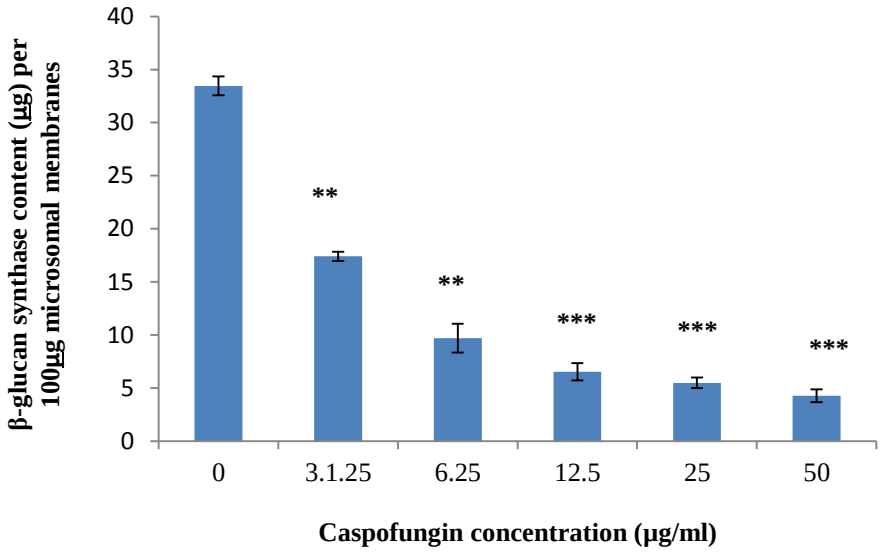


Figure 5.4: The effect of caspofungin on β-(1,3) glucan synthase activity of *A. flavus*. Error bars denote the standard deviation between each sample (n = 3) and the asterisks represent the level of significance relative to the untreated sample (0µg/ml). ** = p < 0.01; *** = p<0.001.

5.3.3 Quantification of total chitin content

It was observed that there was a significant decline in the amount of chitin produced by *A. flavus* in the presence of the plant extract relative to the untreated sample ($p<0.05$; Fig.5.5). It was found that when exposed to increasing concentrations of the plant extract, the chitin content progressively decreased accordingly from a concentration of 2.5mg/ml up to the highest plant extract concentration of 12.5mg/ml. Glucosamine was used as the standard for the quantification of the chitin content and was expressed as glucosamine equivalents. The standard curve indicated that there was a linear response with good correlation ($R^2 = 0.995$) between the various concentrations of glucosamine that was used and the absorbance readings obtained at 650nm (Appendix 1, Fig A13). Treatment of *A. flavus* with nikkomycin Z also showed a highly significant decline in total chitin content relative to the untreated sample ($p<0.01$; Fig 5.6). In a

similar manner to the plant extract, the various dilutions of nikkomycin Z also caused a progressive decline in the chitin concentration of *A. flavus*.

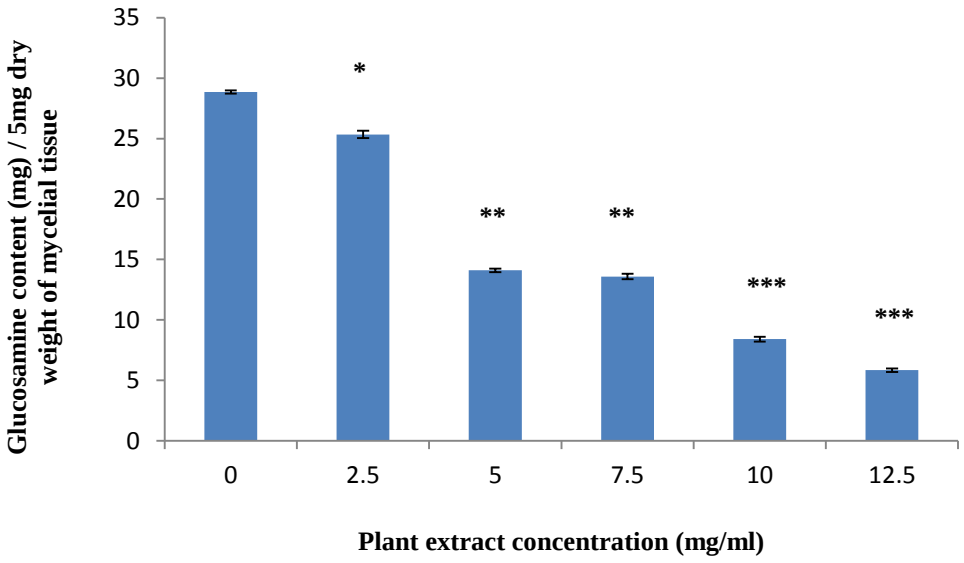


Figure 5.5: **The effect of *T. violacea* plant extract on chitin production by *A. flavus*. Error bars represent the standard deviation for each sample (n = 3) and the asterisks represent the level of significance relative to the untreated sample (0mg/ml). * = p < 0.05; ** = p<0.01; *** = p< 0.001.**

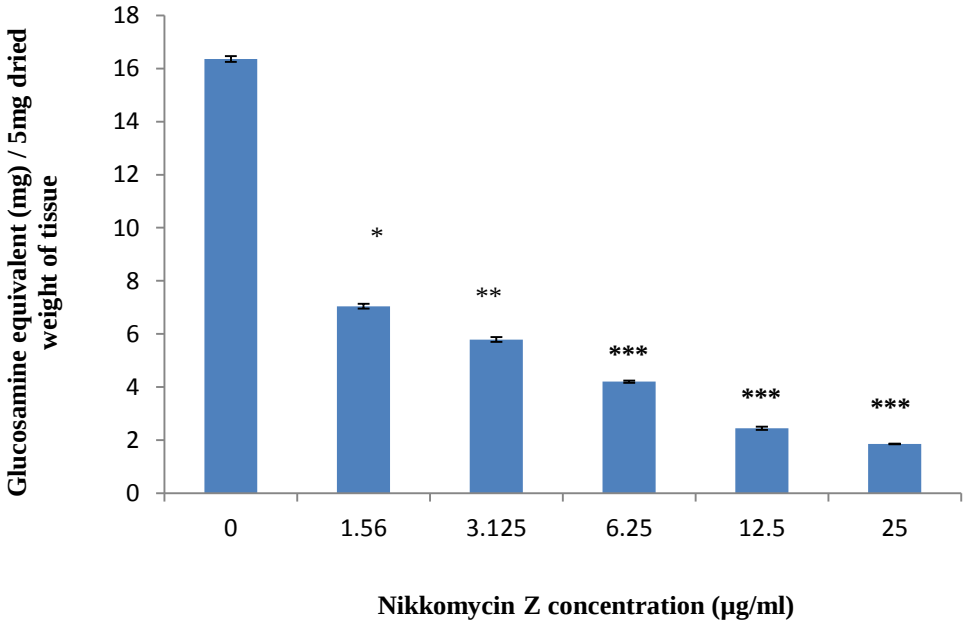


Figure 5.6: **The effect of nikkomycin Z on chitin production by *A. flavus*. Error bars denote the standard deviation between each sample (n = 3) and the asterisks represent the level of significance relative to the untreated sample (0µg/ml). ** = p < 0.01; *** = p<0.001.**

5.3.4 Chitin synthase activity assay

The standard curve that was constructed with the different chitin concentrations yielded good correlation between the range of chitin used and the corresponding absorbance that was obtained (Appendix 1, Fig A14). This was then used for the quantification of chitin synthase activity present in *A. flavus* mycelia. The chitin synthase assay revealed a similar trend to that of the chitin content assay, in that a significant decline in chitin synthase activity in the presence of the plant extract, relative to the untreated sample ($p<0.05$) was observed. The reduction was significant between the different plant extract concentrations that were used for each treatment (Fig 5.7). Although there was a progressive decline in chitin synthase between the plant extract concentrations tested, a larger decrease was observed at a plant extract concentration of 7.5 and 10 mg/ml when compared to the 2.5 and 5 mg/ml concentrations.

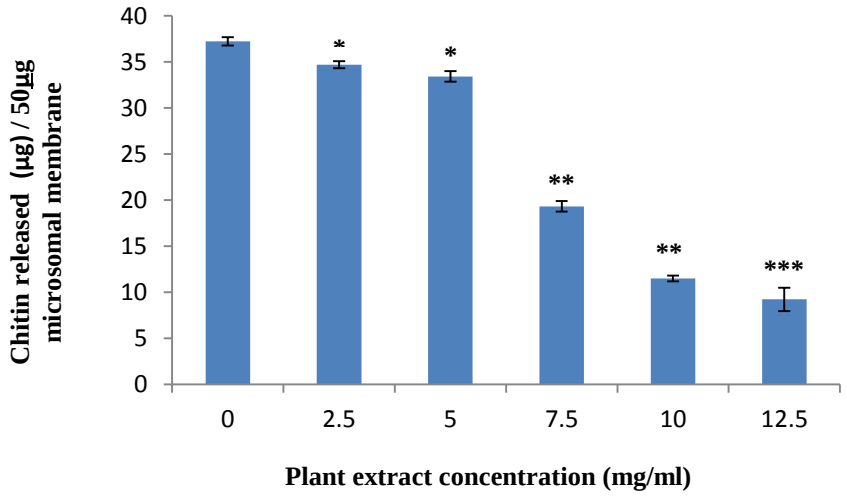


Figure 5.7: The effect of *T. violacea* plant extract on chitin synthase activity of *A. flavus*. Error bars denote the standard deviation between each sample (n = 3) and the asterisks represent the level of significance relative to the untreated sample (0mg/ml). * = $p < 0.05$; ** = $p < 0.01$; *** = $p<0.001$

Similarly, there was a significant decline in chitin synthase activity of *A. flavus* when cultures were subjected to nikkomycin Z treatment as opposed to the untreated sample ($p<0.01$; Fig 5.8). As the nikkomycin Z concentrations were increased, a significant ($p<0.001$) corresponding decrease in chitin synthase activity was obtained in a dose dependent manner.

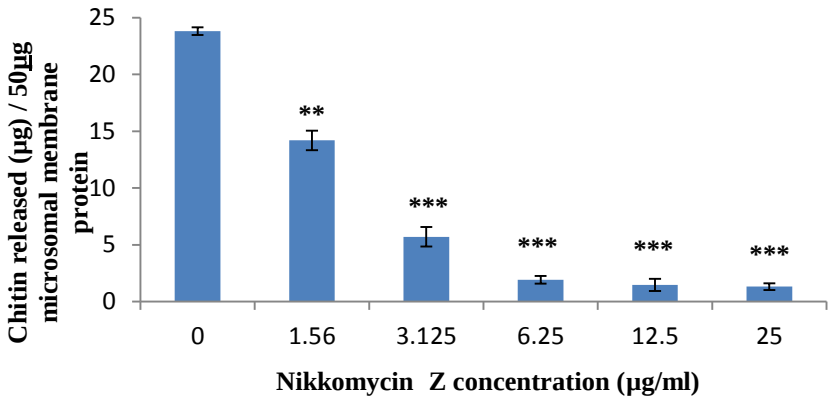


Figure 5.8: The effect of nikkomycin Z on chitin synthase activty of *A. flavus*. Error bars denote the standard deviation between each sample (n = 3) and the asterisks represent the level of significance relative to the control (0µg/ml). ** = p < 0.01; *** = p<0.001.

5.3.5 Microscopic analysis and staining procedures

Microscopic analysis on *A. flavus* plant extract-treated and non-treated mycelia was carried out to investigate the physiological effects of the extract on fungal morphology, β-glucan and chitin production. *Aspergillus flavus* treated with increasing concentrations of the plant extract showed no observable differences in morphology when compared to the untreated samples. All samples had intact conidiophores bearing swollen vesicles on apices and which radiated out multiple conidia. It appeared that in the presence of plant extract concentrations of 7.5 mg/ml and upward, a visual increase in hyphal branching was observable (Fig 5.9).

5.3.5.1 Aniline blue staining

Aniline blue staining of *A. flavus* mycelia showed that all samples did produce β-glucan, as evident by the blue fluorescing hyphal tips and mycelial walls (Fig 5.10) irrespective of whether or not the mycelia was treated with *T. violacea* plant extract. It was visually evident that at a

plant extract concentration of 2.5mg/ml, there were no observable differences in β-glucan content when compared to the untreated sample. In both instances β-glucan deposits were seen to occur in the cytoplasm within the hyphae and at the septal cross walls. It was noted that there was a visible decline in the amount of β-glucan present in fungal mycelia that was exposed to the plant extract concentration of 5 mg/ml and higher with the most prevalent decrease being observed at extract concentrations of 7.5 mg/ml up to the highest extract concentration of 12.5 mg/ml that was tested. In contrast, staining of *A. flavus* mycelia after treatment with various concentrations of caspofungin showed an absence of β-glucans within the fungal mycelia (Fig 5.11). Furthermore, caspofungin treated mycelia exhibited highly branched, shortened, rounded and swollen morphologies, which was not evident with the plant extract-treated samples.

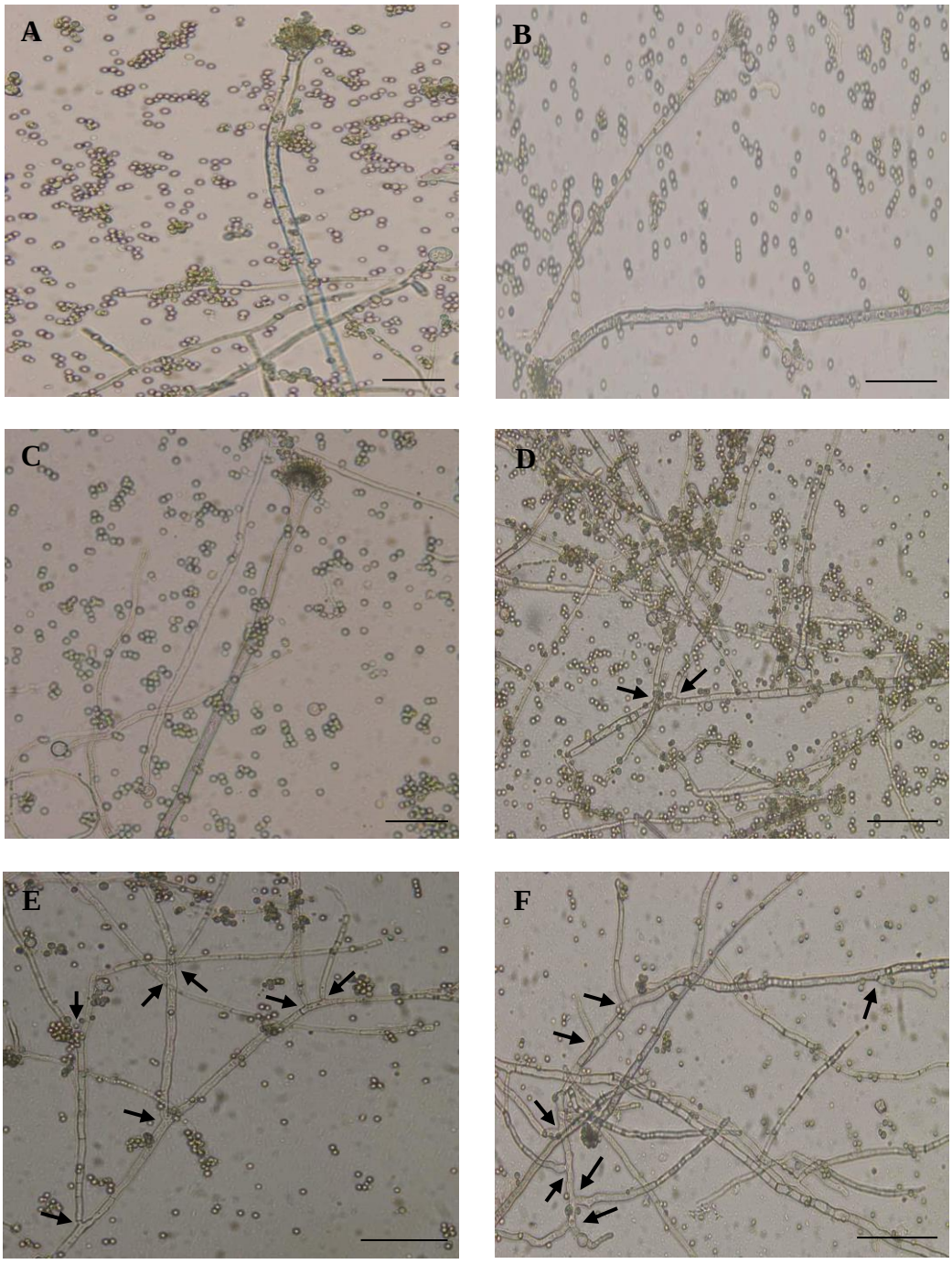


Figure 5.9: **Light micrographs of *A. flavus* grown in the absence (A) and presence (B-F) of *T.violacea* plant extract. In all samples conidia were attached to vesicles and the conidiophores remained intact. The arrows in D-F show excessive branching of mycelia which is most prominent in E and F. A = 0mg/ml ; B = 2.5mg/ml; C = 5mg/ml; D = 7.5mg/ml; E = 10mg/ml; F = 12.5mg/ml. Magnification is at 200x. The scale bar is 50µm**

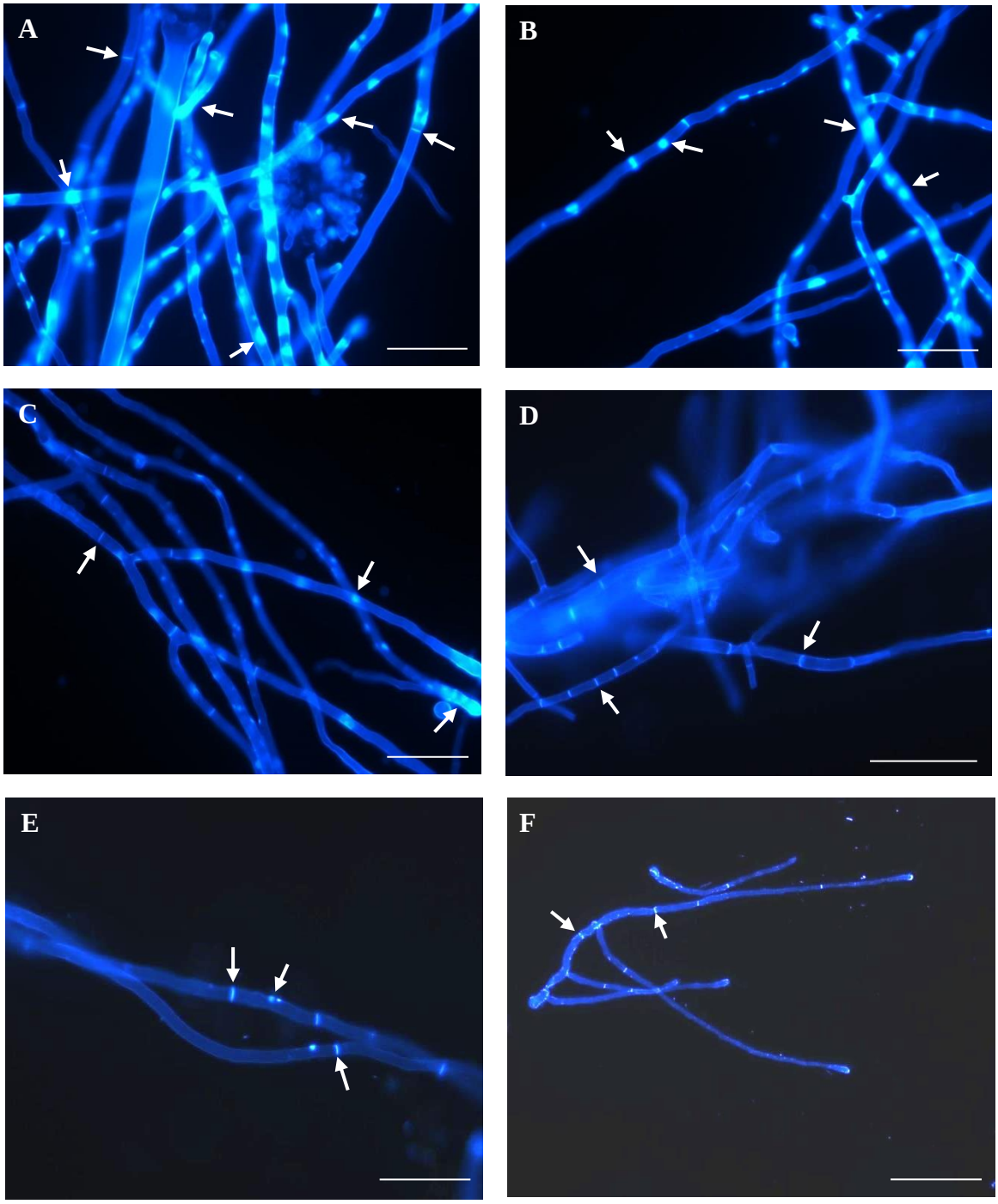


Figure 5.10: Aniline blue staining of *A. flavus* mycelia treated with various concentrations (A to F) of *Tulbaghia violacea* plant extract. The arrows indicate the aniline blue stained β-glucans on the fungal mycelia. A = 0mg/ml; B = 2.5mg/ml; C = 5mg/ml; D = 7.5mg/ml; E = 10mg/ml; F = 12.5mg/ml. Magnification is at 400x. The scale bar is 100µm

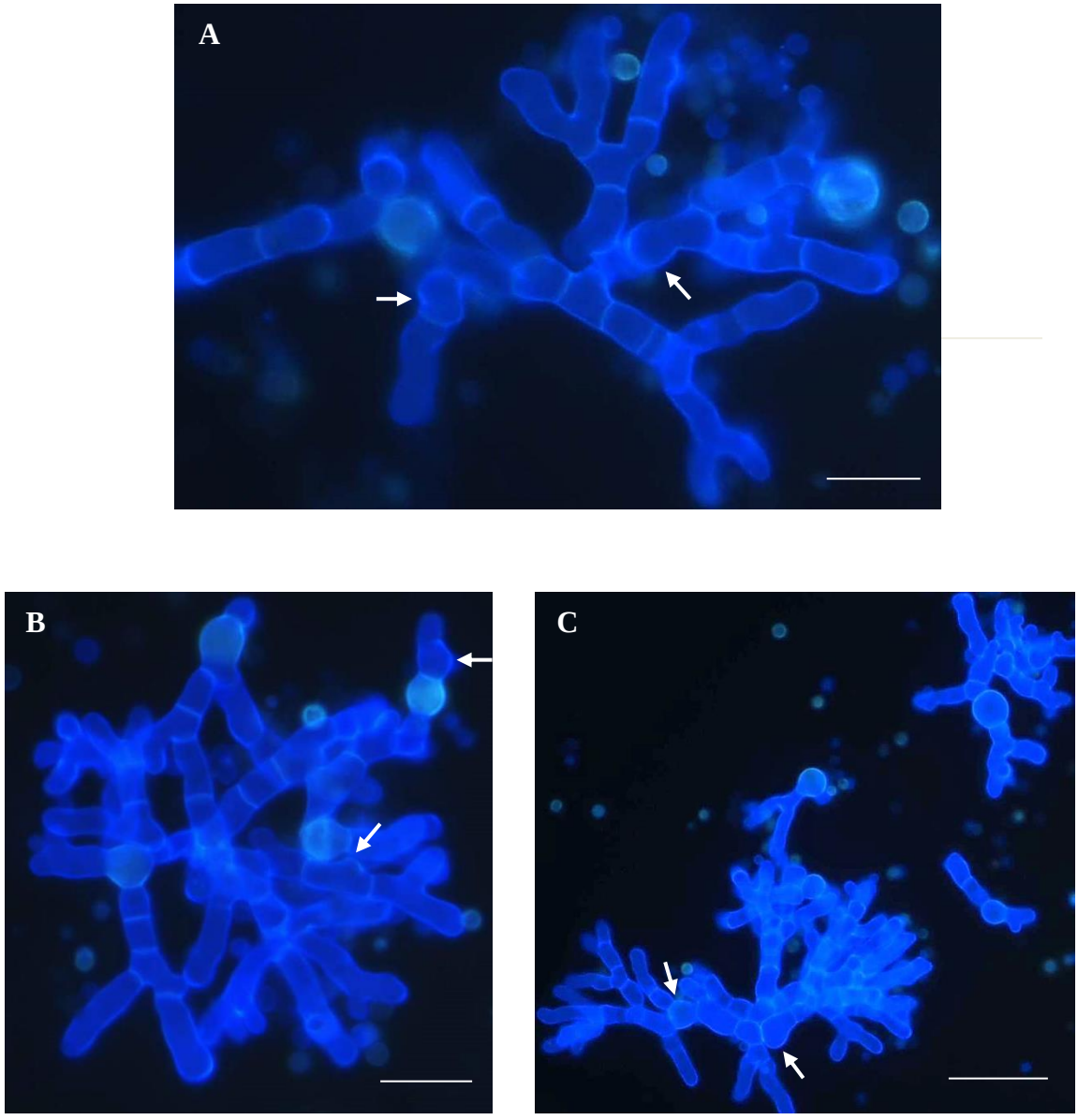


Figure 5.11: Aniline blue staining of *A. flavus* mycelia treated with various concentrations (A to C) of caspofungin. Mycelia are short and stubby with sometimes swollen and misshapen hyphal segments (arrows) formed in clusters in the presence of caspofungin. A = 6.25µg/ml; B = 25µg/ml and C = 50µg/ml. Magnification 400x.The scale bar is 100µm.

5.3.5.2 Calcofluor white staining

Calcofluor staining of the mycelia after treatment with and without the plant extract confirmed the presence of chitin in both the extract-treated and non-treated samples as evidenced by the blue fluorescence of the cross walls in the mycelia. It was, however, noted that there was a visible decline in the intensity of the staining to the cross walls in mycelia treated with the plant extract, especially at concentrations of 10 and 12.5 mg/ml when compared to the untreated sample (Fig 5.12). Also, the occurrence of cross walls appeared to decrease as the concentration of the plant extract was increased with the least amount of septa being visible at a plant extract concentration of 12.5mg/ml. With the positive control, nikkomycin Z, cross walls were not evident within the fungal mycelium and there was a lack of chitin accumulation within the hyphae. In addition, the conidia took on a rounded, more swollen appearance (Figure 5.13) which was not visible with the plant extract-treated samples.

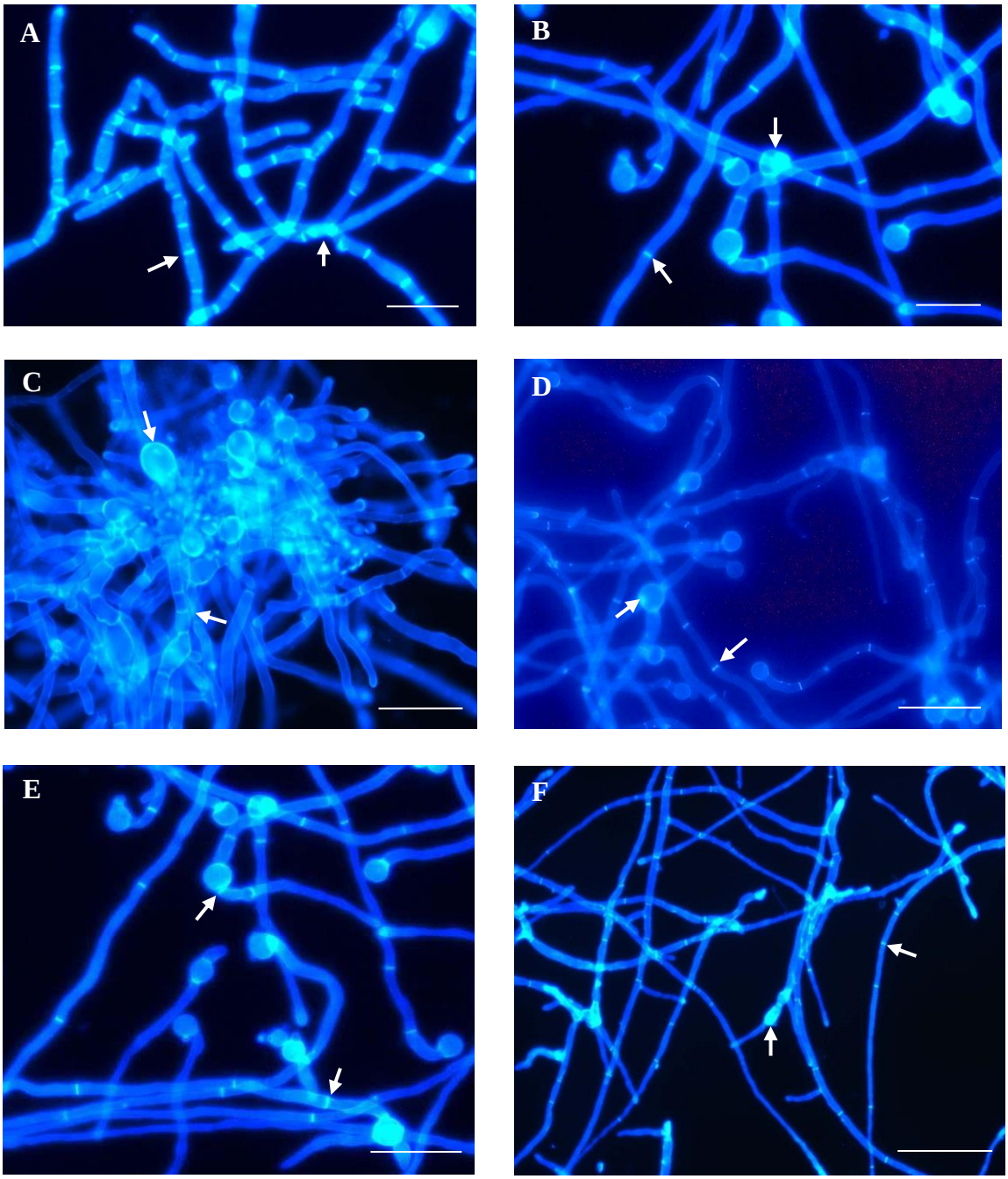


Figure 5.12: **Calcofluor white staining of *A. flavus* mycelia treated with various concentrations (A to F) of *T. violacea* plant extract. A = 0mg/ml; B = 2.5mg/ml; C = 5mg/ml; D = 7.5mg/ml; E = 10mg/ml; F = 12.5mg/ml. Arrows indicate the fluorescent chitin-containing depositions at the cross walls and sometimes witin the cytoplasm. Magnification is at 400x. The scale bar is 100µm**

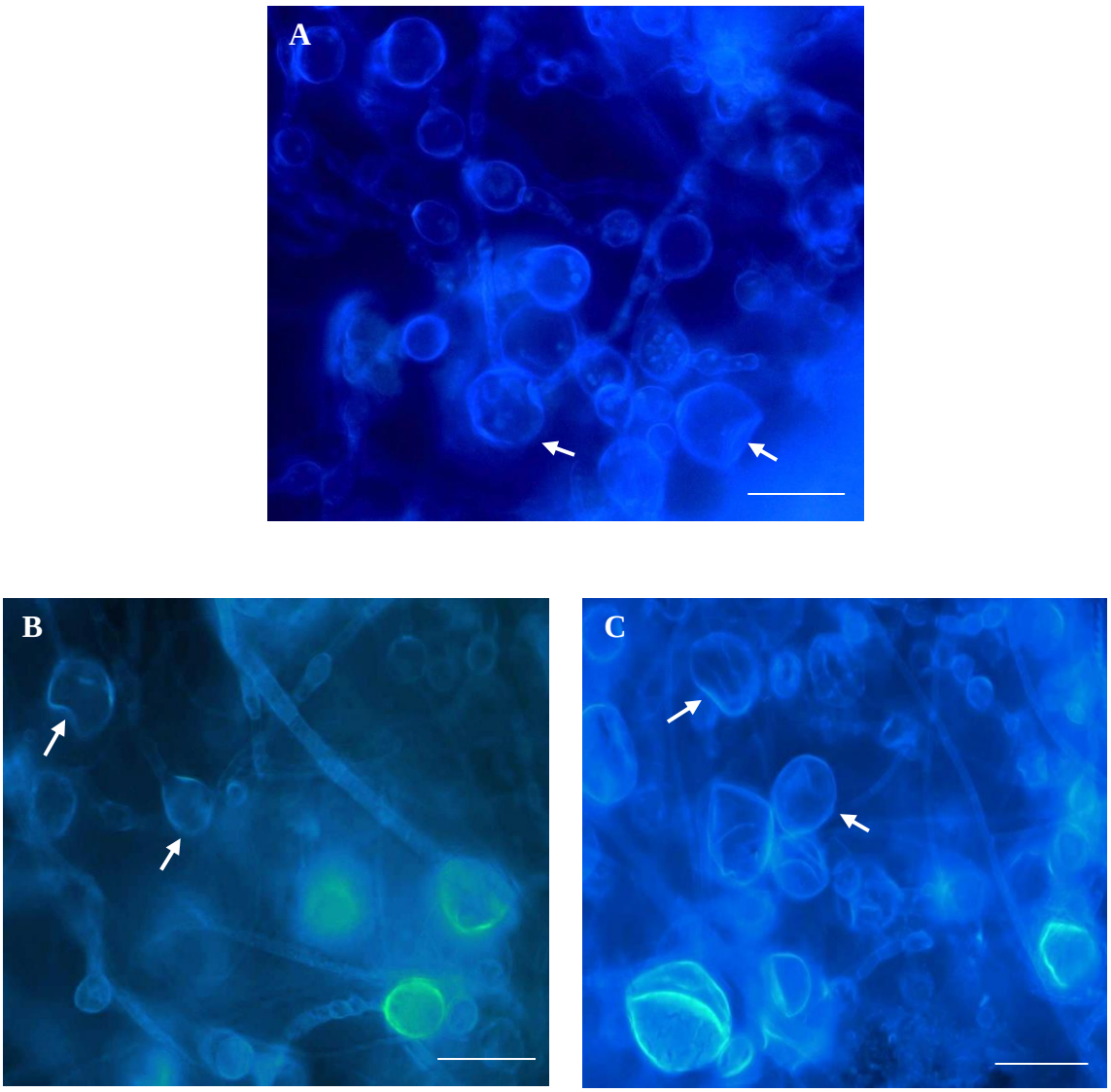


Figure 5.13: **Calcofluor white stain of *A. flavus* after growth in the presence of various concentrations of nikkomycin Z. The arrows indicate the swollen misshapen globose vesicles, with distorted mycelia. A = 3.125µg/ml; B = 12.5µg/ml; C = 25µg/ml. Magnification is at 400x. The scale bar is 100µm.**

5.3.6 Chitinase activity assay

The effect of the plant extract on the chitinase activity of *A. flavus* with emphasis on chitobiosidase, β-N-acetylglucosaminidase (exochitinases) and endochitinase activities was investigated. The three substrates that were used were 4-methylumbelliferyl N,N′-diacetyl-β-D-chitobioside, 4-methylumbelliferyl N-acetyl-β-D-glucosaminide and 4-methylumbelliferyl β-D-N,N′,N″-triacetylchitotroise. The activity of each substrate was expressed as the amount of 4-methylumbeliferone (4-MU) that was released. The standard curves constructed with various concentrations of 4-MU exhibited a linear response and good correlation ($R^2 = 0.99$) between the amount of 4-MU released at various concentration ranges and the fluorescence intensity that was obtained for each of the tested substrates (Appendix 1; Figs A15-17).

The chitobiosidase activity assay using 4MU-$(GlcNAc)_2$ as the substrate indicated that there were significant increases in chitobiosidase activity in the presence of all concentrations of the plant extract ($p< 0.05$), when compared to the untreated sample (Figure 5.14A). In contrast the positive control, acetazolamide, caused a significant decline in chitobiosidase activity of *A. flavus*, when compared to the untreated sample ($p<0.05$). Comparisons between the various concentrations that were used showed that the decline in chitobiosidase activity was in a dose-dependent manner from a concentration of 1.25l up to 20µg/ml acetazolamide (positive control) (Fig 5.14B).

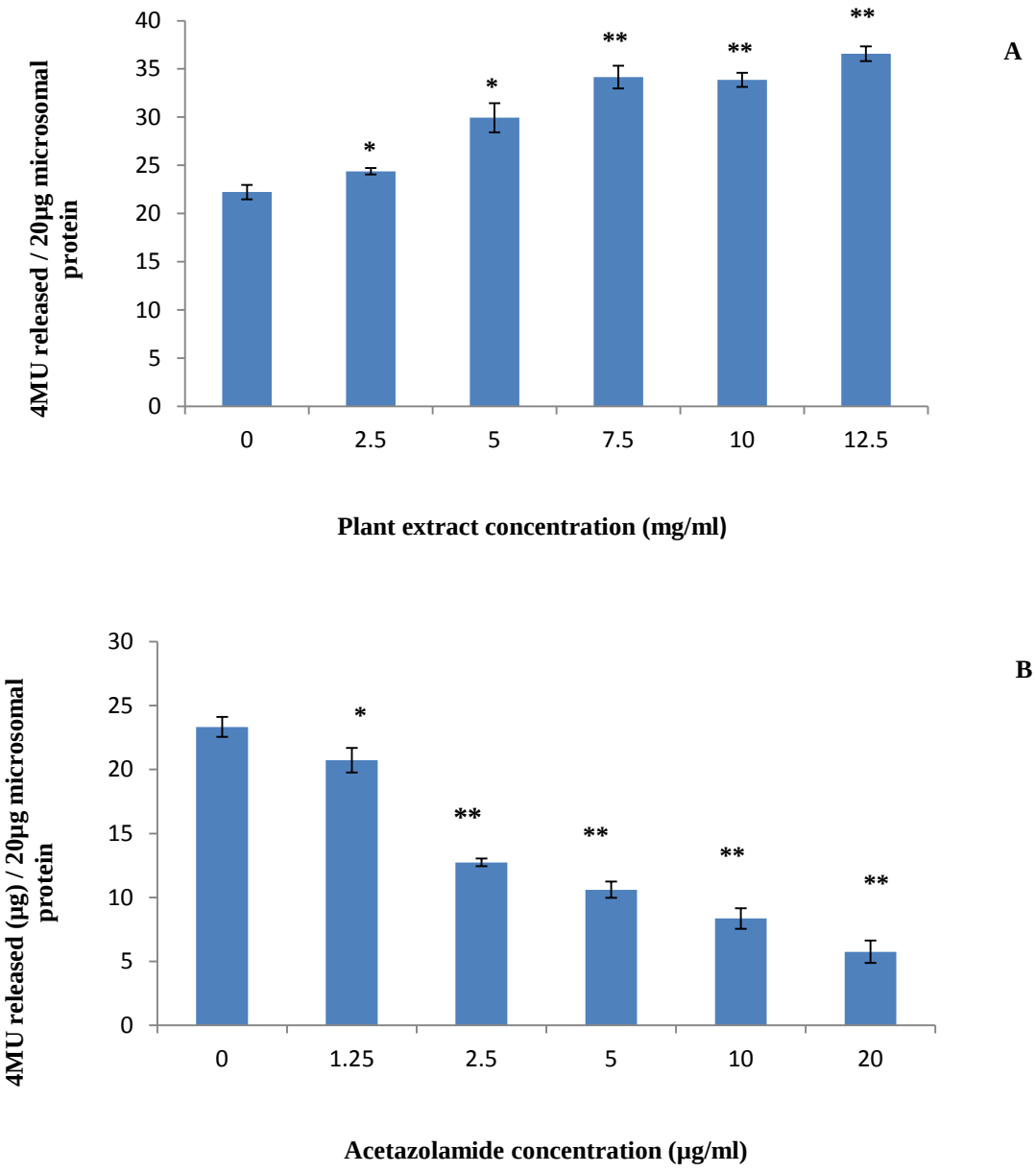


Figure 5:14. The effect of *T.violacea* plant extract (A) and acetazolamide (B) on chitobiosidase activity of *A. flavus* with 4-MU-$(GlcNAc)_2$ as the substrate. The error bars represent the standard deviation between each sample (n = 3). Asterisks represent the levels of significance relative to the untreated sample (0mg/ml and 0µg/ml for plant extract and acetazolamide respectively). * = p <0.05, ** = p < 0.01.

There was a significant decline in β-N-acetylglucosaminidase activity of *A. flavus* in the presence of the plant extract when compared to the untreated sample ($p<0.05$) for all extract concentrations tested (Fig 5.15A). Treatment with acetazolamide caused a significant decline in β-N-acetylglucosaminidase activity of *A. flavus* when compared to the untreated sample ($p< 0.05$) (Fig 5.15B). This reduction in β-N-acetylglucosaminidase activity was observed to occur in a dose-dependent manner throughout the concentrations tested from 1.25µg/ml up to the highest concentration of 20µg/ml

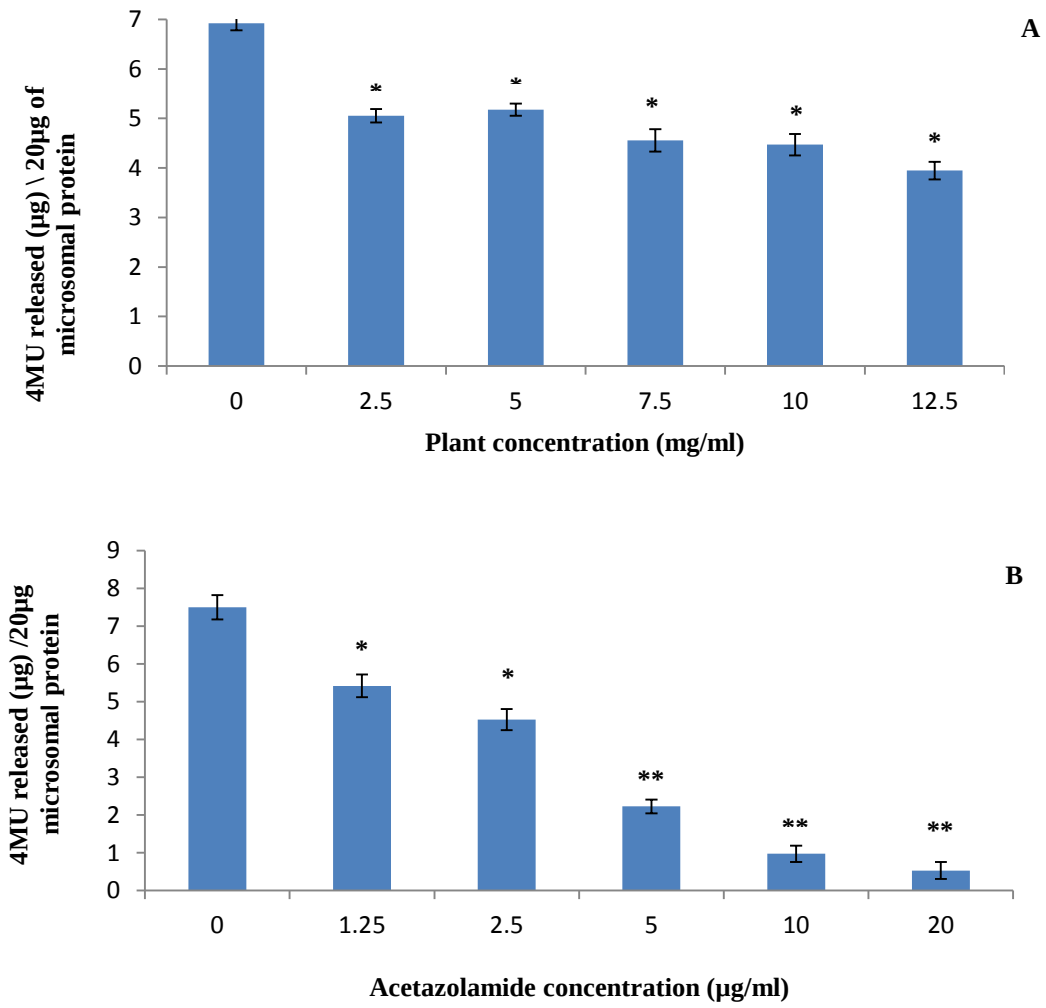


Figure 5.15: The effect of *T. violacea* plant extract (A) and acetazolamide (B) on β-N-acetylglucosaminidase activity of *A. flavus*. Error bars represent the standard deviation for each sample (n = 3). Asterisks represent the level of significance relative to the untreated sample (0mg/ml and 0µg/ml plant extract and acetazolamide respectively). * = p <0.05, ** = p < 0.01.

Endochitinase activity of *A. flavus* showed a significant dose dependent increase in the presence of the plant extract from an extract concentration of 5mg/ml and above relative to the untreated sample ($p<0.05$) (Fig 5.16A). Acetazolamide on the other hand caused a significant decline in a dose dependent manner in endochitinase activity when compared to the untreated sample at all the concentrations tested (Fig 5.16B).

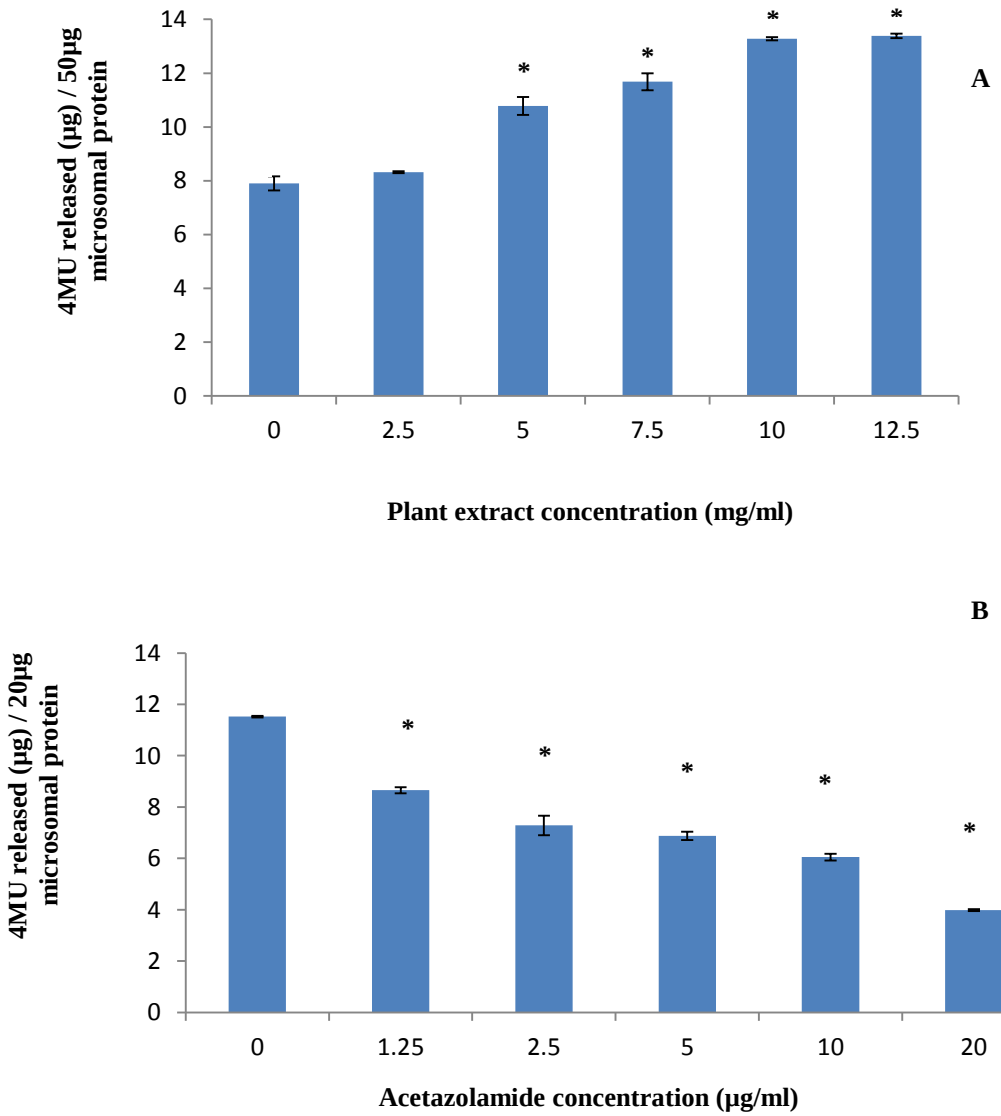


Figure 5.16: The effect of *T. violacea* plant extract (A) and acetazolamide (B) on endochitinase activity of *A. flavus*. Error bars represent the standard deviation for each sample (n = 3). Asterisks represent the level of significance relative to the untreated sample (0mg/ml and 0µg/ml for plant extract and acetazolamide respectively). * = p <0.05.

5.4 Discussion

Fungi have evolved mechanisms to counteract or resist the effects of many antifungal agents. The fungal cell wall is an important component since it acts as an effective barrier against invasion by foreign components (Souza *et al*., 2010). It contains essential components such as β-glucans, chitin and mannoproteins, which are crucial to the survival of the fungus and therefore enzymes that synthesize these components are ideal targets for antifungal agents (Okada *et al*., 2010; Souza *et al*., 2010). βeta-(1,3) glucan is crucial to the fungal structure and together with chitin plays an essential role in providing the cell wall with structure and rigidity. Any disturbance in either chitin or β-glucan synthesis can result in distortion of the fungal cell wall, a disturbance in cell wall integrity (Walker *et al*., 2013) leading to possible death of the fungus.

Previously, *T. violacea* aqueous plant extracts have been shown to have antifungal activity against *A. flavus* and *C. albicans* (Motsei *et al*., 2003; Belewa *et al*., 2011). However its mechanism of action has not been fully investigated. It is known that the extracts inhibit spore germination (Somai and Belewa, 2011) and that fungal lipid content decreases with exposure to increasing extract concentrations (Belewa 2009). This indicated that the extract probably affected the cell membrane and/or cell wall of *A. flavus*. Therefore, the effect of the plant extract on the fungal cell wall with emphasis on β-glucan and chitin synthesis was investigated.

The plant extract caused a significant decline in the amount of β-(1,3) glucan produced by *A. flavus* in a dose dependent manner (Fig 5.1). Staining of fungal mycelia with aniline blue also showed that there was a decline in the amount of β-glucan present within mycelia that had received treatment with the plant extract when compared to the untreated samples (Fig 5.10). An investigation of the *A. flavus* glucan synthase activity in the presence of the plant extract showed that the extract had a negative effect on fungal glucan synthase activity (Fig 5.3). Again, there was a dose dependent response to the plant extract. These results suggested that the plant extract had targeted the *A. flavus* glucan synthase complex thereby inhibiting its activity which directly caused the decline in fungal glucan synthesis. In literature, similar results had been found with other plant derived products such as the essential oil *trans*-cinnamaldehylde which was reported to have a similar effect on *S. cereviciae*, where it inhibited β-glucan production by inhibiting β-glucan synthase activity (Bang *et al*., 2000).

The use of caspofungin yielded very similar results to that of *T. violacea* plant extract. The antifungal agent caused inhibition of β-(1,3) glucan synthesis and β-(1,3) glucan synthase (Fig 5.4). Caspofungin belongs to the echinocandins and is a lipopeptide that inhibits β-glucan production by targeting the β-glucan synthase complex. It has fungicidal activity against *Candida* species and is fungistatic against *Aspergillus* species (Plummer *et al*., 2007; Walker *et al*., 2008). With *Aspergillus* species (*A. flavus, A. terreus* and *A. nidulans*), it targets β-(1,3) glucan synthase activity (Bowman *et al*., 2006). In this study, staining with aniline blue after caspofungin treatment, showed that caspofungin induced the formation of short, rounded swollen structures and branched chains with reduced β-glucan production. This was similar to that previously observed in *A. fumigatus* hyphae treated with caspofungin, where highly branched, shortened, stubby and broad based hyphae were formed (Fortwendel *et al*., 2010; Verwer *et al*., 2012). The results obtained here with caspofungin validated the findings with the *T. violacea* plant extract which acted in a very similar manner.

In filamentous fungi, the glucan synthase complex is found in the apical region of fungal mycelia and is detected by staining with aniline blue (Beauvais *et al*., 2001). The synthase complex is made up of two subunits: a catalytic (Fksp) and a regulatory (Rho) subunit. The Fskp subunit is found in the plasma membrane and catalyses the transfer of sugars from UDP glucose to the specific acceptor β-(1,3)-D-glycosyl (N) resulting in the formation of glycosidic bonds (Garcia-Effron *et al*., 2009). Rho1p is a Ras-like GTP binding protein and is responsible for the regulation of glucan synthase activity (Beauvais *et al*. 2001; Walker *et al*., 2011). Although this study found that the extract inhibited β-glucan synthase activity, further investigations will need to be considered on the β-glucan synthase complex. This would determine which of the two subunits are negatively affected thereby resulting in an inhibition of β-glucan synthesis.

Beta-(1,3) glucans are interlinked to chitin and together these components play an important role in maintaining the structure and integrity of the cell wall. It has been shown that fungi possess a compensatory mechanism for the loss of β-(1,3) glucans by increasing the amount of chitin that they produce (Ueno *et al*., 2011; Verwer *et al*., 2012). Antifungal agents targeting β-glucans or chitin stimulate the production of the other to compensate for the loss of the one. Caspofungin, inhibits β-glucan production in *Candida* and *Aspergillus* species but this stimulates the fungus to

increase the chitin content of its cell walls in response to the decrease in β-glucan content (Walker *et al*., 2008 and 2013; Fortwendel *et al*., 2009 and 2010; Staab *et al*., 2010). Similarly, nikkomycin Z inhibits chitin synthesis but causes an increase in β-glucan production (Verwer *et al*., 2012). This compensatory increase of either chitin or β-glucan is a survival mechanism for fungi and allows them to escape death (Fortwendel *et al*., 2009) by maintaining the thickness of their cell walls in order to maintain effective turgor pressure needed for growth.

Chitin synthesis is a multistep process that involves at least nine enzymes that play a role in the conversion of glucose to N-acetylglucosamine. Further down in the pathway, chitin synthase catalyses the conversion of UDP-N-acetylglucosamine to chitin. Chitin is then degraded during cell wall remodelling by chitinases to N-N'-diacetylchitobiose, which is converted to N-acetylglucosamine by N-acetylglucominidase (Chaudary *et al*., 2013). In this study, biochemical analysis of chitin indicated that there was a decline in chitin content (Fig 5.5) when fungal tissue was exposed to *T. violacea* plant extract. This was further confirmed by morphological analysis (staining) where extract-treated mycelia exhibited less calcofluor fluorescent material (Fig 5.12). Again, an analysis of chitin synthase activity revealed that there was a corresponding decrease in enzyme activity with an increase in plant extract concentration (Fig 5.7). Thus the extract negatively affected chitin synthase resulting in a decrease in chitin content.

In order to validate the results, each biochemical assay was repeated with a known inhibitor of chitin synthesis, nikkomycin Z. Nikkomycin Z caused inhibition of chitin synthesis as indicated by a reduction in fungal chitin content when exposed to the drug (Fig 5.6). This was further confirmed by morphological analysis of nikkomycin Z-treated samples which showed swollen, hollow, rounded conidial structures with little to no chitin production (Fig 5.13) typical of the mechanism of action of nikkomycin Z in fungi (Verwer *et al*., 2012). Again, further investigation implicated the inhibition of chitin synthase activity as the cause of the reduction in fungal chitin content.

Aspergillus species contain 8 chitin synthase genes which are further divided into seven classes of chitin synthases. Each of them plays a different role in fungal development and regulation of chitin synthesis (Specht *et al*., 1996; Rogg *et al*., 2011; Jiminez-Ortigosa *et al*., 2012). Classes I,

II, IV and VII, together with their respective genes contribute to the formation of chitin in the cell wall and may have synergistic interactions with the other chitin synthase enzymes in the regulation of cell wall synthesis and in the maintenance of cell wall integrity (Lenardon *et al*., 2010; Rogg *et al*., 2012). Chitin synthase enzymes of *A. fumigatus* and *A. nidulans* are well known and their mechanism of action has been investigated (Specht *et al*., 1996; Bernard and Latge 2001; Mellado *et al*., 2003; Latge 2007; Lenardon *et al*., 2010; Rogg *et al*., 2011 and 2012; Jiminez-Ortigosa *et al*., 2012). Currently there is no literature on chitin synthase genes of *A. flavus* and their role in cell wall synthesis. Further studies in this regard need to be carried out to investigate whether specific chitin synthesis genes are affected by the plant extract and the exact mechanism of action. This would involve transcriptional analysis of these genes by quantitative real time PCR (Larson *et al*., 2011).

Morphological analysis of *A. flavus* cultures treated with the plant extract showed excessive branching at high extract concentrations. It has previously been suggested that any changes in the morphology of fungi treated with antifungal agents indicates that such agents are targeting the fungal cell wall and its components (Plaine *et al*., 2008; Souza *et al*., 2010; Spampinato and Leonardi 2013). Taken together, the excessive branching is an indication that the extract had potentially induced excessive weakening of the cell wall at each branch point with the resultant turgor pressure causing extrusion of the cell membrane leading to branch formation. Increased branch formation is also a consequence of cell wall remodelling that normally occurs during unfavourable conditions or during cell wall stresses (Staab *et al*., 2010) and which this study has done by forcing the fungus to grow in the presence of *T. violacea* plant extract.

Chitinases are found in a wide variety of chitin-containing microorganisms such as bacteria, fungi, insects and plants and perform a variety of functions (Dahiya *et al*., 2006; Matsumoto 2006). Their role in fungi involves degradation of exogenous chitin of fungal cell walls, cell wall remodelling during growth and morphogenesis and defence against other pathogens and unfavourable conditions (Adams 2004; Seidl 2008). Chitinases are classified into endochitinases, chitobiosidases and β-N-acetylglucosaminidase.

An analysis of chitobiosidase activity of *A. flavus* in the presence of various concentrations of the plant extract showed that the plant extract stimulated the release of diacylchitobiose (Fig 5.14A). A similar trend was observed with endochitinase activity, where an increase in the release of the GlcNAc oligomers was observed in the presence of the plant extract (Fig 5.15A). In *A nidulans*, an increase in endochitinase and β-N-acetylglucosaminidase activity has been reported to occur during autolysis (Yamazaki *et al*., 2007). Therefore, the elevation in endochitinase and chitobiosidase activities could be a consequence of either increased autolysis or increased cell wall remodelling that was forced to occur during exposure to the plant extract. Autolysis can also form part of programmed cell death triggered by unfavourable conditions, which include nutrient depravation and physical stress (Yamazaki *et al*., 2007; Emri *et al*., 2008) most probably induced by exposure to the plant extract resulting in increased chitinase and proteinase activity (Shin *et al*., 2009). In this study, *A. flavus* may be using autolysis or cell wall re-modelling as a survival mechanism to compensate for the loss of other fungal components to enable it to grow in the presence of the plant extract.

The degradation of chitin by chitinases occurs in two steps. It is first broken down by exo- and endo-chitinases into oligomers of β-N-acetylglucosamine. These are further broken down into glucosamine monomers by β-N-acetylglucosaminidases (Matsumoto 2006; Shin *et al*., 2009). During this study, a decline in exochitinase activity of β-N acetylglucosaminidases was observed in the presence of the plant extract (Fig 5.13A). This could be as a result of direct inhibition by the plant extract itself or as an indirect result of the inhibition of chitin by the plant extract. Chitin has been shown to be an inducer of β-N-acetylglucosaminidase (Haran *et al*., 1995; Li and Li 2009). Therefore, a reduction in the *A. flavus* chitin levels induced by the plant extract, would also have contributed to the inhibition of the β-N-acetylglucosaminidase activity. In contrast analysis of chitinase activity with acetazolamide which is known to be effective against *A. fumigatus* (Schuettelkopf *et al*., 2010), showed that this compound inhibited chitinase activity for all three substrates that were used in a dose dependent manner. This difference may probably be due to differences in the doses utilized or in the fact that the plant extract was composed of a mixture of compounds which could have induced multiple effects which may have obscured any direct similarities to acetazolamide.

Beta-N-acetylglucosaminidases, chitobiosidases and endochitinases can be detected as dimers, trimers and tetramers after separation by SDS-PAGE and detection of enzyme activity using different fluorescent substrates (Hodge *et al*., 1995; Guthrie *et al*., 2005). Attempts to detect the specific types of chitinases present in *A. flavus* after treatment with the plant extract were unsuccessful. The low yield of the crude protein that was obtained after extraction was insufficient for detection on SDS-PAGE. Chitinases of various microorganisms are often extracted in low levels and their activity may be due to the synergistic interaction of the different types found in each isoform (Li and Li 2009). A higher yield of the protein in a more purified form would be required for the separation and detection of the various chitinases.

The antifungal activity of *T. violacea* on β-glucan and chitin production of *A. flavus* may also be triggering interactions between exochitinase and endochitinase activity of the fungus as a survival mechanism in response to the plant extract. The thickening of the fungal cell wall which was observed in previous studies in the presence of the plant extract could be caused by the accumulation of the exochitinases. Since endochitinases function on the inner cell wall, their accumulation could be a compensatory mechanism for the reduction of β-(1, 3) glucans and chitin found in the presence of the plant extract. The antifungal activity of the plant extract on various components of the fungal cell wall makes it a potential antifungal agent for treatment of *Aspergillus* infections.

In many instances, medicinal plants have been found to target the cell wall by causing thickening of and damage to the cell wall components. *Trans*-cinnamaldehyde inhibits the β-glucan synthase and chitin synthase cell wall synthesizing enzymes of *S. cereviciae* (Bang *et al*., 2000) while the leaf extract of *Inusa viscosa* inhibits chitin synthesis in *C. albicans* (Maoz and Neem 2000). Cinamaldehyde and eugenol target cell wall components, destroy intracellular components and cause leakage of cellular components (Khan *et al*., 2013). In this study, it was shown that *T. violacea* targets the cell wall of *A. flavus*, by inhibiting the production of β-glucan and chitin synthases resulting in the decline of β-glucan and chitin synthesis respectively. The different compounds within the plant extract contribute to the efficacy and the antifungal nature of the plant extract. Possible synergistic interactions between the different compounds of the plant extract may enable it to target multiple components of the cell wall and this could serve as

an advantage to limit the chances of the fungus developing a tolerance or resistance mechanism towards it, as has been shown to be the case with other known antifungal agents.

5.5 References

Adams, D.J. 2004. Fungal Cell Wall Chitinases and Glucanases. *Microbiology* **150**: 2029-2035.

Alcazar-Fuoli, L., Clauvad, C., Lamarre, C., Aimanianda, V., Seidl-Seiboth, V, Mellado, E. and Latge, J-P. 2011. Functional Analysis of the Fungal/Plant Class Chitinase Family in *Aspergillus fumigatus*. *Fungal Genetics and Biology* **48**: 418-429.

Bang, K.-H., Lee, D-W., Park, H-M. and Rhee, Y-H. 2000. Inhibition of Fungal Cell Wall Synthesizing Enzymes by *trans*-Cinnamaldehyde. *Bioscience, Biotechnology and Biochemistry* **64**(5): 1061-1063.

Beauvais, A., Bruneau, J.M., Mol, P.C., Buitrago, M.J., Legrand, R. and Latge, J-P. 2001. Glucan Synthase Complex of *Aspergillus fumigatus*. *Journal of Bacteriology* **183**(7): 2273-2279.

Belewa, X.V. 2009. The Effect of *Tulbaghia violacea* Aqueous Plant Extract on the Growth of *Aspergillus* Species. MSc Dissertation., Nelson Mandela Metropolitan University

Belewa, V., Baijnath, H. and Somai, B.M. 2011. Aqueous Extracts from the Bulbs of *Tulbaghia violacea* are Antifungal against *Aspergillus flavus*. *Journal of Food Safety* **31**: 176-184.

Bernard, M. and Latge, J-P. 2001. *Aspergillus fumigatus* Cell Wall: Composition and Biosynthesis. *Medical Mycology* **39**(1): 9-17.

Bowman, S.M. and Free, S.J. 2006. The Structure and Synthesis of Fungal Cell Wall. *Bioassays* **28**: 799-808.

Bowman, J. C., Abruzzo, G.K., Flattery, A.M., Gill, C.J., Hickey, E.J., Hsu, M.J., Kahn, J.N., Liberator, P.A., Misura, A.S., Pelak, B.A., Wang, T.C. and Douglas, C.M. 2006. Efficacy of Caspofungin against *Aspergillus flavus, Aspergillus terreus* and *Aspergillus nidulans*. .*Antimicrobial Agents and Chemotherapy* **50**(12): 4202-4205.

Bradford, M.M. 1976. A Rapid and Sensitive Method for the Quantification of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry* **72**: 248-254.

Chaudhary, P.M., Tupe, S.G. and Deshpande, M.V. 2013. Chitin Synthase Inhibitors as Antifungal Agents. *Mini-Reviews in Medicinal Chemistry* **13**(2): 222-236.

Cowen, L.E. and Steinbach, W.J. 2008. Stress, Drugs and Evolution: The Role of Cellular Signalling in Fungal Drug Resistance. *Eukaryotic Cell* **7**: 747-764.

Dahiya, N., Tewari, R. and Hoondal, G.S. 2006. Biotechnological Aspects of Chinolytic Enzymes: A Review. *Applied Microbiological Biotechnology* **71**: 773-782

Emri, T., Molnar, Z., Szilagyi, M. and Posci, I. 2008. Regulation of Autolysis in *Aspergillus nidulans*. *Applied Biochemistry and Biotechnology*. **151**:211-220.

Fortwendel, J.R. Juvvadi, P.R., Pinchai, B., Perfect, Z., Alspaugh, J.A., Perfect, J.R. and Steinbach, W.J. 2009. Differential Effects of Inhibiting Chitin and 1,3-β-D-Glucan Synthesis in Ras and Calcineurin Mutants of *Aspergillus fumigatus*. *Antimicrobial Agents and Chemotherapy* **53**(2): 476-482.

Fortwendel, J.R. Juvvadi, P.R., Perfect, Z.B., Rogg, L.E., Perfect, J.R. and Steinbach, W.J. 2010. Transcriptional Regulation of Chitin Synthases by Calcineurin Controls Paradoxical Growth of *Aspergillus fumigatus* in Response to Caspofungin. *Antimicrobial Agents and Chemotherapy* **54**(4): 1555-1563.

Frost, D. J., Brandt, K., Capobianco, J and Goldman, R. 1994. Characterization of (1,3)-b-Glucan Synthase in *Candida albicans*: Microsomal Assays from the Yeast or Mycelial Forms and a Permeabilized Whole-Cell assay. *Microbiology* **140**: 2239-2246.

Fujioka, T., Mizutani, O., Furukawa, K., Sata, N., Yoshimi, A., Yamagata, Y., Nakajima, T. and Abe, K. 2007. MpkA-Dependent and -Independent Cell Wall Integrity Signalling in *Aspergillus nidulans*. *Eukaryotic Cell* **6**(8): 1497-1510.

Garcia-Effron, G., Park, S. and Perlin, D.S. 2009. Correlating Echinochandin MIC and Kinetic Inhibition of *fks1* Mutant Glucan Synthases for *Candida albicans: I*mplications for Interpretive Breakpoints. *Antimicrobial Agents and Chemotherapy* **53**(1): 112-122.

Guerriero, G., Silverstrini, L., Obersriebnig, M., Salerno, M., Pum, D. and Strauss, J. 2013. Sensitivity of *Aspergillus nidulans* to the Cellulose Synthase Inhibitor Dichlobenil: Insights from Wall-Related Genes' Expression and Ultrastructural Hyphal Morphogenesis. *PloS One* **8**(11): 1-12.

Guthrie, J.L., Khalif, S. and Castle, A.J. 2005. An Improved Method for Detection and Quantification of Chitinase Activities. *Canadian Journal of Microbiology* **51**: 491-495.

Haran, S., Schickler, H., Oppenheim, A. and Chet, I. 1995. New Components of the Chinolytic System of *Trichoderma harzianum*. *Mycology Research* **99**(4): 441-446.

Hodge, S., Alexander, I.J. and Gooday, G. 1995. Chinolytic enzymes of Pathogenic and Ectomycorrhizal Fungi. *Mycology Research* **99**(8): 935-941.

Jiminez-Ortigosa, C., Aimanianda, V., Muszekita, L., Moyna, I., Aisteen, D., Pire, S., Beau, R., Krappman, S., Beauvais, A., Dufrene, Y.F., Roncero, C. and Latge, J-P. 2012. Chitin Synthases with a Myosin-Like Domain Control the Resistance of *Aspergillus fumigatus* to Echinocandins.. *Antimicrobial Agents and Chemotherapy* **56**(12): 6121-6129.

Khan, M.S.A., Ahmad, I. and Cameotra, S.S 2013. Phenyl Aldehyde and Propanoids Exert Multiple Sites of Action Towards Cell Membrane and Cell Wall Targeting Ergosterol in *Candida Albicans*. *AMB Express* **3**(54): 2-16.

Ko, Y.-T. and Lin, Y-L. 2004. 1,3-β-Glucan Quantification by a Fluorescence Microassay and Analysis of its Distribution in Foods. *Journal of Agricultural and Food Chemistry* **52**: 3313-3318.

Larson, T.M., Kendra, D.F., Busman, M. and Brown, D.W. 2011. *Fusarium verticilliodes* Chitin Synthase CHS 5 and CHS 7 are required for Normal Growth and Pathogenicity. *Current Genetics* **57**:177-189.

Latge, J-P., Mouyna, I., Tekaia, F., Beauvais, A., Debeaupuis, J. P. and Nierman, W. 2005. Specific Molecular Features in the Organization and Biosynthesis of The Cell Wall of *Aspergillus fumigatus*. *Medical Mycology* **43**: 15-22.

Latge, J-P. 2007. The Cell Wall: A Carbohydrate Armour of the fungal Cell. *Molecular Microbiology* **66**: 279-290.

Lee, K.K., MacCallum, D.M., Jacobsen, M.D., Walker, L.A., Odds, F.C., Gow, N.A.R. and Munro, C.A. 2011. Elevated Cell Wall Chitin in *Candida albicans* Confers Echinocandin Resistance *in vivo*. *Antimicrobial Agents and Chemotherapy* **56**(1): 208-217.

Lehmann, P.F. and White, L.O. 1975. Chitin Assay Used to Demonstrate Renal Localization and Cortisone-Enhanced Growth of Aspergillus fumigatus Mycelium in Mice. *Infection and Immunity* **12**(5): 987-992.

Lenardon, M.D., Munro, C.A. and Gow, N.A.R. 2010. Chitin Synthesis and Fungal Pathogenesis. *Current Opinion in Microbiology* **13**: 416-423.

Li, D. and Li, A. 2009. Current Advances in Fungal Chitinases. *Applied Mycology*. Rai, M and Bridge, P.D. (Eds). CAB international.

Li, B., Du, Y., Li, T. and Dong, S. 2009. Investigation of 3,3' ,55'-Tetramethylbenzidine as Colorimetric Substrate for A Peroxidatic DNAzyme. *Analytica Chimica Acta* **651**: 234-240.

Lucero, H.A., Kuranda, M.J. and Bulik, D.A. 2002. A nonradioactive, High Throughput Assay for Chitin Synthase Activity. *Analytical Biochemistry* **305**: 97-105.

Magellan, H., Boccara, M., Drujon, T., Soulie, M-C., Guillou, C., Dubois, J. and Becker, H.F. 2013. Discovery of Two New Inhibitors of *Botrytis cinerea* Chitin Synthase by a Chemical Library Screening. *Bioorganic and Medicinal Chemistry* **21**: 4997-5003.

Maoz, M. and Neeman, I. 2000. Effect of *Inula viscosa* extract on chitin synthesis in dermatophytes and *Candida albicans* .*Journal of Ethnopharmacology* **2000**: 479-482.

Matsumoto, K.S. 2006. Fungal Chitinases: *Advances in Agricultural and Food Biotechnology*. 290-304. Keral, India

Mellado, E.G., Dubreucq, G., Mol, P., Sarfati, J., Paris, S., Diaquin, D.W., Holden, J., Rodriguez-Tudela, J.L. and Latge, J.P 2003. Cell Wall Biogenesis in a Double Chitin Synthase Mutant (*chsG ichsE)* of *Aspergillus fumigatus*. *Fungal Genetics and Biology* **38**: 98-109.

Motsei, M.L., Lindsey, K.L., van Staden, J. and Jager, A.K 2003. Screening of Traditionally Used South African Plants for Antifungal Activity against *Candida albicans*. *Journal of Ethnopharmacology* **86**: 235-241

Okada, H., Abe, M., Asakawa-Minemura, M., Hirata, A., Qadota, H., Morishita, K., Ohnuki, S., Nogami, S. and Ohya, Y. 2010. Multiple Functional Domains of the Yeast 1,3-β-Glucan Synthase Subunit Revealed by Quantitative Phenotypic Analysis of Temperature-Sensitive Mutants. *Genetics* **184**:1013-1024.

Plaine, A., Walker, L., da Costa, G., Mora-Montes, H.M., McKinnon, A., Gow, NAR., Gaillardin, C., Munro, C.A. and Richard, M.L. 2008. Functional Analysis of *Candida albicans* GPI-Anchored Proteins: Roles in Cell Wall Integrity and Caspofungin Sensitivity. *Fungal Genetics and Biology* **45**: 1404-1414.

Plummer, R., Bodkin, J., Power, D., Pantarat, N., Bubb, W.A., Kutchel, P.W. and Sorrell, T.C. 2007. Effect of Caspofungin on Metabolite Profiles of *Aspergillus* Species Determined by Nuclear Magnetic Resonance Spectroscopy. *Antimicrobial Agents and Chemotherapy* **51**(11):4077-4084.

Rogg, L.E., Fortwendel, J.R., Juvvadi, P.R., Lilley, A. and Steinbach, W.J. 2011. The Chitin Synthase Genes *chsA* and *chsC* are not Required for Cell Wall Stress Response in the Human Pathogen *Aspergillus fumigatus*. *Biochemical and Biophysical Research Communications* **411**: 549-554.

Rogg, L.E., Fortwendel, J.R., Juvvadi, P.R. and Steinbach, W.J. 2012. Regulation of Expression, Activity and Localization of Fungal Chitin Synthases. *Medical Mycology* **50**: 2-17.

Schuettelkopf, A.W., Gros, L., Blair, D.E., Frearson, J.A., van Aalten, D.M.F. and Gilbert, I. 2010. Acetazolamide-Based Fungal Chitinase Inhibitors. *Bioorganic and Medicinal Chemistry*. **18**(20):8334-8340.

Seidl, V. 2008. Chitinase of Filamentous Fungi: A Large Group of Diverse Proteins with Multiple Physiological Functions. *Fungal Biology Reviews*. **22**:36-42.

Shedletzky, E., Unger, C. and Delmer, D.P. 1997. A Microtiter-Based Fluorescence Assay for (1,3) -β-Glucan Synthases. *Analytical Biochemistry* **249**: 88-93.

Shin, K-W., Kwong, K-J., Kim, Y.H., Park, H-S., Kwong, G-S. and Yu, J-H. 2009. Differential Roles of the ChiB Chitinase in Autolysis and Cell Death of *Aspergillus nidulans*. *Eukaryotic Cell*. **8**(5): 738-746

Somai, B.M. and Belewa, V. 2011. Aqueous Extracts of *Tulbaghia violacea* Inhibit Germination of *Aspergillus flavus* and *Aspergillus parasiticus* Conidia. *Journal of Food Protection* **74**(6): 1007-1011.

Souza, N.A.B., de Oliveira Lima, E., Guedes, D.N., de Oliveira, F., de Souza, E.L. and de Sousa, F.B. 2010. Efficacy of *Origanum* Essential Oils for Inhibition of Potentially Pathogenic Fungi. *Brazilian Journal of Pharmaceutical Sciences* **46**(3): 500-508.

Spampinato, C. and Leonardi, D. 2013. *Candida* Infections, Causes, Targets and Resistance Mechanisms: Traditional and Alternative Antifungal Agents. *Biomed Research International* **2013**: 1-13.

Specht, C.A., Liu, Y., Robbins, P.W., Bulawa, C.F., Iartchouk, N., Winter, K.R., Riggle, P.J., Rhodes, J.C., Dodge, C.L., Culp, D.W. and Borgia, P.T. 1996. The chsD and chsE Genes of *Aspergillus nidulans* and Their Roles in Chitin Synthesis. *Fungal Genetics and Biology* **20**(153): 153-167.

Staab, J.F., Kahn, J.N. and Marr, K.A. 2010. Differential *Aspergillus lentus* Echinocandin Susceptibilities are Fksp Independent. *Antimicrobial Agents and Chemotherapy* **54**(12): 4992-4998.

Ueno, K., Namiki, Y., Mitani, H., Yamaguchi, M. and Chibana, H. 2011. Differential Cell Wall Remodeling of Two Chitin Synthase Deletants Δchs3A and Δchs3B in the Pathogenic Yeast *Candida Glabrata*. *Yeast Research* **11**: 399-407.

Verwer, P.E.B., van Duijn, M.L., Tavakol, M., Bakker-Woudenberg, I.A.J.M. and van de Sande, W.J. 2012. Reshuffling of *Aspergillus fumigatus* Cell Wall Components Chitin and β-Glucan under the Influence of Caspofungin or Nikkomycin Z Alone or in Combination. *Antimicrobial Agents and Chemotherapy* **56**(3): 1595-1598.

Walker, L.A., Munro, C.A., de Bruijn, I., Lenardon, D., McKinnon, A. and Gow, N.A.R. and 2008. Stimulation of Chitin Synthesis Rescues *Candida albicans* from Echinocandins. *PLoS Pathogens* **4**(4): 1-10.

Walker, S.S., Xu, Y., Triantafyllou, I., Waldman, M.F., Mendrick, C., Brown, N., Mann, P., Chau, A., Patel, R., Bauman, N., Norris, C., Antonacci, B., Gurnani, M., Cacciapuoti, A., McNicholas, P.M., Wainhaus, S., Herr, R.J., Kuang, R., Aslanian, R.G., Ting, P.C. and Black, T. 2011.Discovery of a Novel Class of Orally Active Antifungal β-1,3-D-Glucan Synthase Inhibitors. *Antimicrobial Agents and Chemotherapy* **55**(11): 5099-5106.

Walker, L.A., Gow, N.A.R. and Munro, C.A. 2013. Elevated Chitin Content Reduces the Susceptibility of *Candida* Species to Caspofungin. *Antimicrobial Agents and Chemotherapy* **57**(1): 146-154.

Yamazaki, H., Yamazaki, D., Takaya, N., Takagi, M., Ohta, A. and Horiuchi, H. 2007. A Chitinase Gene *chiB*, Involved in the Autolytic Process of *Aspergillus nidulans*. *Current Genetics*.**51**:89-98.

CHAPTER 6

SUMMARY AND FUTURE STUDIES

The increase in fungal infections has become a health concern, especially in immuno compromised patients (Arif *et al*., 2011). Although there are antifungal agents used to treat fungal infections, most fungal species have developed resistance towards them, while some antifungal agents are toxic to their host, which makes them not suitable for consumption (; Cabral *et al*., 2013). In commercial farming, fungal plant pathogens cause significant damage to crops resulting in losses of food products and in revenue (Vila et *al*., 2013). Most fungicides that are used to control plant pathogens are toxic to the environment with serious implications on human health and again some plant pathogens have developed resistance towards them, which makes them ineffective (Castillo *et al*., 2012).

Medicinal plants contain a wide variety of compounds and have been used either as crude extracts or pure compounds for treatment of a variety of diseases (Vila *et al*., 2013). Many compounds from medicinal plants have been shown to have antifungal activity. Their broad spectrum of antifungal activity eliminates any possibilities of emergence of resistance towards them (Cabral *et al*., 2013). Previous studies have shown that the aqueous extract of *T. violacea* had antifungal activity against *A. flavus* (Belewa 2009; Belewa et al., 2011). The identity and the type of the untifungal compounds were not known. The isolation of the active compound from the crude plant extract was carried out using TLC and agar overlay techniques and partially purified with HPLC techniques. However, identification and structure elucidation of the active compound with NMR analysis was made difficult as the yield of the purified compound was exceedingly low and at least 1mg of compound was required to obtain a conclusive result. A number of factors that contributed to this included the low yield of the active compound after HPLC fractionation and the mixture of compounds that were present in the sample indicated that the active compound may either be a complex structure or contaminated with unidentified compounds and needed further purification.

Future studies on the identification of the antifungal compound of the crude plant extract can involve the upregulation of the active compound within the plant. This would involve growth of young plantlets or seedlings in the presence of various growth stimulators and after which they will be subjected to *Agrobacterium* mediated transformation (Lee *et al*., 2009; Sivanesan and Jeong, 2009). Chromatographic fingerprinting of the various secondary metabolites that are present in the crude plant extract can be done with TLC using the appropriate solvents and detection reagents for the identification of the desired metabolites (Birk *et al*., 2013). The agar overlay technique may be used to identify the antifungal saponins and phenolics present in the crude plant extract. RP-HPLC can separate and identify the various saponins and phenolics present in the crude plant extract. Commercial standards for both phenolics and saponins can be used to identify the various phytochemicals present in the crude plant extract (Lanzotti *et al*., 2012). Possible synergistic interactions between the various phytochemical compounds of the plant extract could be investigated by a checkerboard method (Shin and Lim 2004).

Mechanism of action studies on ergosterol biosynthesis showed that the plant extract targeted ergosterol production of *A. flavus* by binding to the ergosterol in the plasma membrane and preventing ergosterol production. Investigation on the effect of the plant extract on ergosterol biosynthetic pathway showed that the plant extract caused an accumulation of the sterol intermediate 2,3-oxidosqualene and prevented the progression to lanosterol and ultimately ergosterol. Hence this indicates that the extract inhibited oxidosqualene synthase in the ergosterol biosynthetic pathway. Since saponins target ergosterol in fungi (Ahmed *et al*., 2012), the reduction in ergosterol production may have been caused by some of the saponins that were present in the plant extract. Extraction of saponins from the plant extract and investigating their effect on ergosterol production by *A. flavus* will also need to be investigated further.

The crude plant extract of *T. violacea* also targeted the cell wall of *A. flavus* by inhibiting β-(1, 3) glucan and chitin production by targeting β-(1, 3) glucan and chitin synthase respectively. Most fungal species have developed resistance towards antifungal agents targeting cell wall components by causing a compensatory increase of one cell wall component for another (Fortwendel *et al*., 2009 and 2010). A reduction of both β-(1, 3) glucan and chitin by the plant extract makes it an ideal chemotherapeutic agent and its broad range of antimicrobial activity

reduces the development of resistance by the fungus. Microscopic analysis of *A. flavus* on physiological changes in the presence of the plant extract did not show any changes in the fungal structures. Since chitin synthases of *Aspergillus* are regulated by at least seven classes of chitin synthase genes (Jiminez-Ortigosa *et al*., 2012), the plant extract may therefore be targeting the genes playing a role in cell wall chitin and not those involved in fungal growth and germination (Rogg *et al*., 2012). Further investigation on the effect of the *T. violacea* on chitin synthase genes may also be useful on the mechanism of action of the plant extract involving the regulation of chitin synthases.

Previous studies have shown that the plant extract caused excessive thickening of the cell wall and damaged the intracellular structures (Belewa 2009). The plant extract caused an increase in both the endochitinase and chitobiosidase activity of *A. flavus*. The disruption in cell membrane and cell wall component by the plant extract triggered an increase in endochitinase and chitobiosidase activity of the fungus as a survival mechanism in the presence of the plant extract. The increase in chitobiosidase and endochitinase activity is triggered by a process of autolysis, where older hyphal structures are broken down and used as a nutrient source (Yamazaki *et al*., 2007). The inhibition of chitin synthase activity by the plant extract caused a disruption in the chitin synthesis pathway of *A. flavus*. The low chitin levels in the presence of the plant extract triggered chitobiosidase activity by the fungus as a survival and defence mechanism against the plant extract and this results in accumulation of diacetylchitobiose (Chaudhary *et al*., 2013). This probably prevented the conversion of diacetylchitobiose to N-acetylglucosamine by N-acetylglucosaminidases. Possible synergistic interactions between these chitinases may be used by the fungus as survival mechanism against the plant extract.

Chitinases are divided into different classes and are associated with different genes. In *A. nidulans* autolysis is regulated by *chiB* gene which is encoded by class V chitinases (Shin *et al*., 2009). Further investigations on the genes regulating chitobiosidase and endochitinase activity of *A. flavus* will assist on the investigation of the mechanism of action of the plant extract on the chitinolytic activity of *A. flavus*.

Most fungi have different types of endochitinases, exochitinases and N-acetyl-glucosaminidases (Li and Li 2009). Purification of the crude chitinase extract will also allow for the separation and detection of these chitinases. Various chromatographic methods such as gel filtration, ion exchange and chitin affinity chromatography are useful in purification of crude chitinase extracts (Li and Li 2009). The purified extract can be separated by SDS-PAGE to determine the type of chitinase present based on the molecular weight. Zymograms would be used with the various substrates to detect various chitinases (dimer, trimer, tetramer) that may be affected by the plant extract (Guthrie *et al*., 2005).

The broad spectrum of antifungal activity of *T. violacea* aqueous extract against *A. flavus* makes it a potential chemotherapeutic agent for treatment of fungal infection. This reduces any chance of the fungus developing resistance towards it, since it targets multiple sights on its host. Further investigations on *T. violacea*'s compounds and their synergistic interactions will be useful in making it a more effective antifungal agent.

6.1 References

Ahmed, D.B., Chaieb, I., Salah, K.B., Boukamcha, H., Jannet, H.B., Mighri, Z. and Daami-Remadi, M. 2012. Antibacterial and Antifungal Activities of *Cestrum parqui* Saponins: Possible Interaction with Membrane Sterols. *International Research Journal of Plant Science* **3**(1): 001-007.

Arif, T., Mandal, T.K. and Dabur, R. 2011. Natural Products: Antifungal Agents Derived from Plants. *Opportunity, Challenge and Scope of Natural Products in Medicinal Chemistry*. 283-311. Reasearch Signpost, India

Birk, C.D., Provensi, G., Gosman, G., Reginatto, F.H. and Schenkel, E.P. 2013. TLC Fingerprint of Flavonoids and Saponins from Passiflora Species. *Journal of Lipid Chromatography and Related Technologies*. **28**(14): 2285-2291

Cabral, M.E., Figueroa, L.I.C. and Farina, J.I. 2013. Synergistic Antifungal Activity of Statin-Azole Associations as Witnessed by *Saccharomyces cerevisiae* and *Candida utilis*-Bioassay and Ergosterol Quantification. *Revista Iberoamericana de Micologia* **30**(1): 31-38.

Castillo, F., Hernandez, D., Gallegos, G., Rodriguez, R. and Aguilar, C.N. 2012. Antifungal Properties of Bioactive Compounds from Plants. *Fungicides for Plants and Animal Diseases.* Dr. Dharumadurai Dhanasekaran (Ed.) In Tech, China and Europe

Chaudhary, P.M., Tupe, S.G. and Deshpande, M.V. 2013. Chitin Synthase Inhibitors as Antifungal Agents. *Mini-Reviews in Medicinal Chemistry* **13**(2): 222-236.

Fortwendel, J.R. Juvvadi, P.R., Perfect, Z.B., Rogg, L.E., Perfect, J.R. and Steinbach, W.J. 2010. Transcriptional Regulation of Chitin Synthases by Calcineurin Controls Paradoxical Growth of *Aspergillus fumigatus* in Response to Caspofungin. *Antimicrobial Agents and Chemotherapy* **54**(4): 1555-1563

Fortwendel, J.R. Juvvadi, P.R., Pinchai, B., Perfect, Z., Alspaugh, J.A., Perfect, J.R. and Steinbach, W.J. 2009. Differential Effects of Inhibiting Chitin and 1,3-β-D-Glucan Synthesis in Ras and Calcineurin Mutants of *Aspergillus fumigatus*. *Antimicrobial Agents and Chemotherapy* **53**(2): 476-482.

Guthrie, J.L., Khalif, S. and Castle, A.J. 2005. An Improved Method for Detection and Quantification of Chitinase Activities. *Canadian Journal of Microbiology* **51**: 491-495.

Jiminez-Ortigosa, C., Aimanianda, V., Muszekita, L., Moyna, I., Aisteen, D., Pire, S., Beau, R., Krappman, S., Beauvais, A., Dufrene, Y.F., Roncero, C. and Latge, J-P. 2012. Chitin Synthases with a Myosin-Like Domain Control the Resistance of *Aspergillus fumigatus* to Echinocandins.. *Antimicrobial Agents and Chemotherapy* **56**(12): 6121-6129.

Lanzotti, V., Barile, E., Antignani, V., Bonanomi, G. and Scala, F. 2012. Antifungal Saponins from Bulbs of Garlic, *Allium sativum* L. var. *Voghiera*. *Phytochemistry* **78**: 126-134.

Lee, S.K., Kim, H.H., Kim, Y.K., Park, N.I. and Park, S.U. 2009. Plant Regeneration of Garlic (*Allium sativum* L.) *via* Somatic Embryogenesis. *Scientific Research and Essay*. **4**(13): 1569-1547.

Li, D. and Li, A. 2009. Current Advances in Fungal Chitinases. *Applied Mycology*. Rai, M and Bridge, P.D. (Eds). CAB international

Rogg, L.E., Fortwendel, J.R., Juvvadi, P.R. and Steinbach, W.J. 2012. Regulation of Expression, Activity and Localization of Fungal Chitin Synthases. *Medical Mycology* **50**: 2-17

Shin, K-W., Kwong, K-J., Kim, Y.H., Park, H-S., Kwong, G-S. and Yu, J-H. 2009. Differential Roles of the ChiB Chitinase in Autolysis and Cell Death of *Aspergillus nidulans*. *Eukaryotic Cell*. **8**(5): 738-746

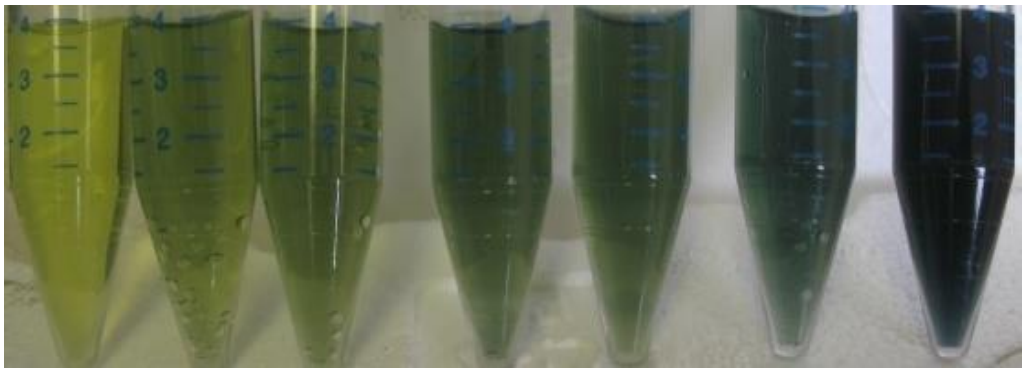
Shin, S. and Lim, S. 2004.Antifungal Effects of Essential Oils alone and in Combination with Ketaconazole against *Trichophyton* spp. *Journal of Applied Microbiology*. **97**: 1289-1296

Sivanesan, I. and Jeong, B.R. 2009. Induction and Establishment of Adventitious and Hairy Root Cultures of *Plumbago zeylanica* L. *African Journal of Biotechnology*. **8**: 5294-5300.

Vila, R., Freixa, B. and Canigueral, S. 2013. Antifungal Compounds from Plants. *Recent Advances in Pharmaceutical Sciences III*. Munoz-Torrero, D. Cortes, A. and Marino, E.L. (Eds). 23

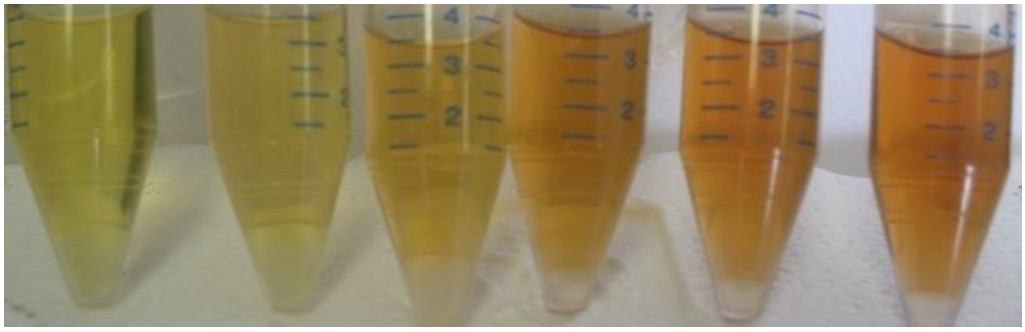
Yamazaki, H., Yamazaki, D., Takaya, N., Takagi, M., Ohta, A. and Horiuchi, H. 2007. A Chitinase Gene *chiB*, Involved in the Autolytic Process of *Aspergillus nidulans*. *Current Genetics*.**51**:89-98.

APPENDIX 1



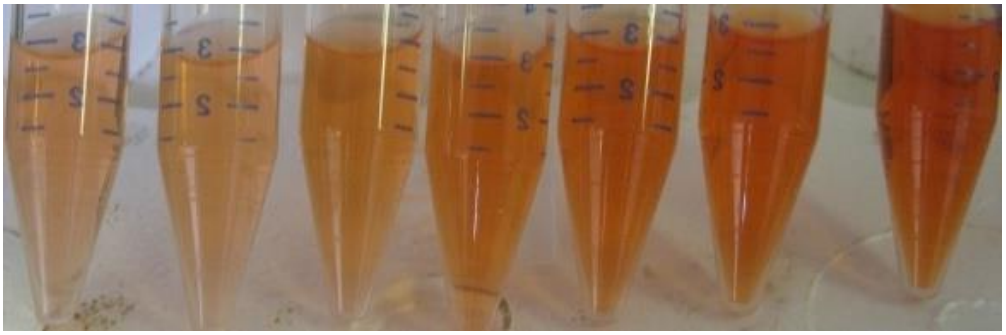
1 2 3 4 5 6 7

Figure A1: Folin C assay for total phenolic content determination. Different gallic acid concentrations were used and the results obtained were used to generate a standard curve. Total phenolic content of the crude plant extract and HEA1 was then determined from the standard curve. 1 = 0µg/ml; 2 = 7.8125µg/ml; 3 = 15.625µg/ml; 4 = 31.25 µg/ml; 5 = 62.5µg/ml; 6 = 125µg/ml and; 7 = 250µg/ml



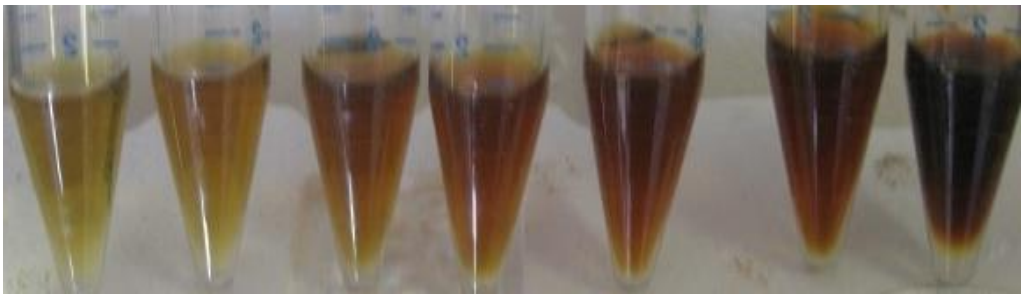
1 2 3 4 5 6

Figure A2: Tannin assay for the quantification of tannins in the crude plant exract and HEA1. Different pyrogallol concentrations were used to generate a standard curve. The presence of condensed tannins in the crude extract and HEA1 was then extrapolated from the standard curve. 1 = 0µg/ml; 2 = 390.63µg/ml; 3 = 781.25µg/ml; 4 = 15625µg/ml ; 5 = 3125µg/ml; 6 = 62500µg/ml.



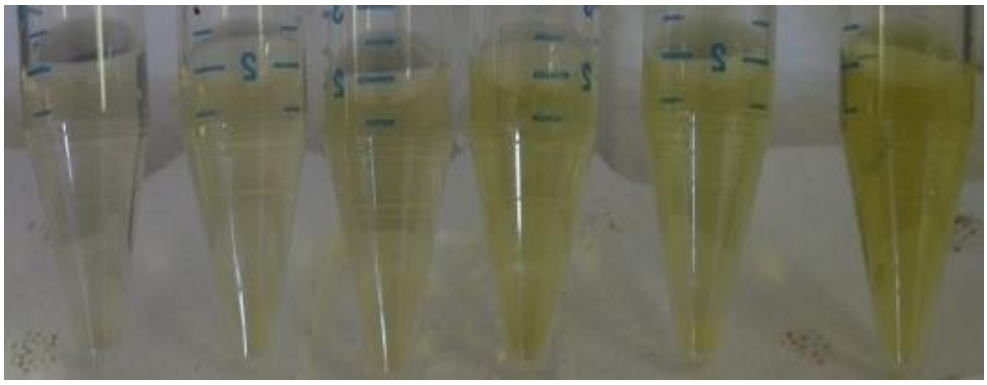
1 2 3 4 5 6 7

Figure A 3: Flavanol assay for the quantification of flavonols present within the crude extract and HEA1 using various concentrations of catechin. 1 = 0mg/ml; 2 = 10mg/ml; 3 = 20mg/ml; 4 = 40mg/ml; 5 = 60mg/ml; 6 = 80mg/ml; 7 = 100mg/ml.



1 2 3 4 5 6 7

Figure A4: Saponin assay for the quantification of total saponins present in the crude extract and HEA1. Various concentrations of disogenin were used for the construction of the standard curve. 1 = 0µg/ml; 2 = 125µg/ml; 3 = 250µg/ml; 4 = 500µg/ml; 5 = 1000µg/ml; 6 = 2000µg/ml; 7 = 4000µg/ml.



1 2 3 4 5 6

Figure A5: Steroidal saponin assay for the estimation of steroidal saponins in the crude plant extract and HEA1. Various concentrations of disogenin were used for the construction of the standard curve. 1 = 0µg/ml; 2 = 78µg/ml; 3 = 156µg/ml ; 4 = 312.5µg/ml; 5 = 625µg/ml; 6 = 1250µg/ml.

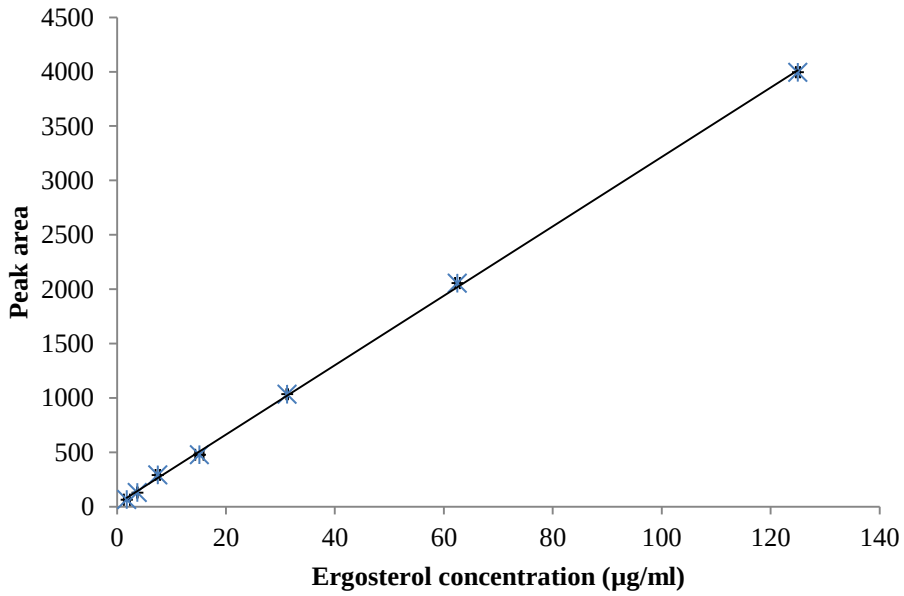


Figure A6: **Ergosterol standard curve with ergosterol standard at a range of 0µg/ml to 125µg/ml and wavelength = 282nm. Peak areas of ergosterol standard were used to construct the standard curve. y = 31.931+23.208; R^2 = 0.9997. Error bars represent standard deviation (n = 3).**

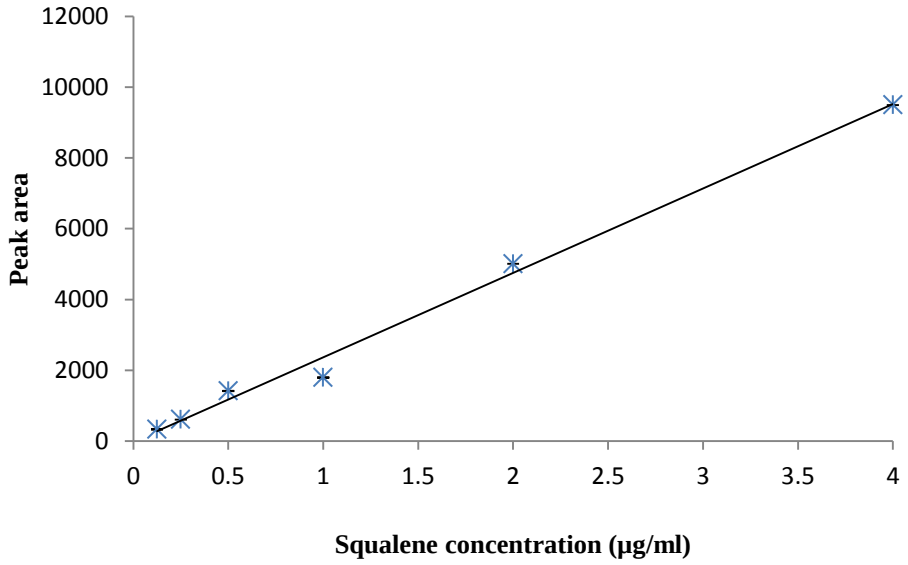


Figure A7: **Squalene standard curve with squalene standard at a range between 0µg/ml –4µg/ml and wavelength = 210nm. Peak areas of squalene standard were used to construct the standard curve. y = 2383.5x -17.637; R^2 = 0.9928. Error bars represent standard deviation (n = 3).**

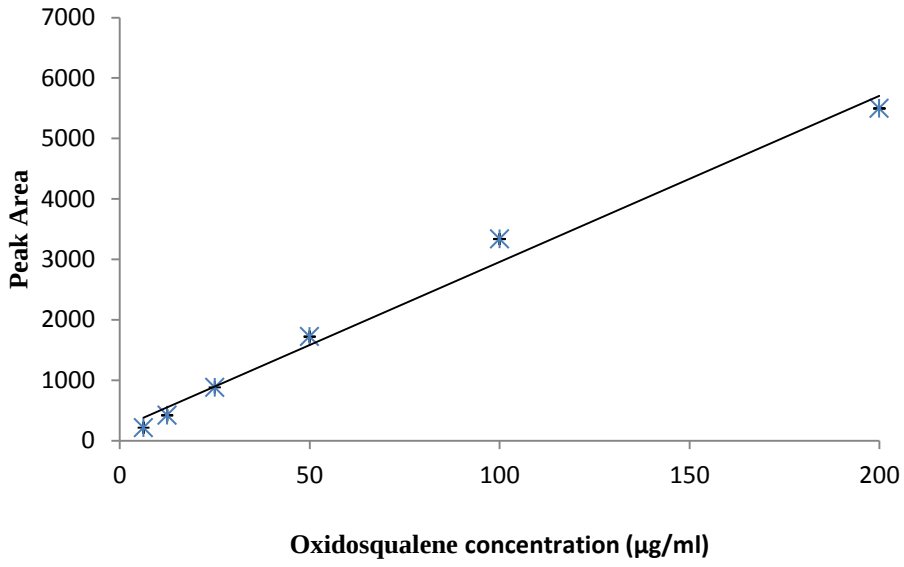


Figure A8: **Oxidosqualene standard curve with oxidosqualene standard at a range of 0µg/ml to 200µg/ml and wavelength = 210nm. Peak areas of oxidosqualene standard were used to construct the standard curve. y = 27.492x + 206.78; R^2 = 0.988. Error bars represent standard deviation (n = 3).**

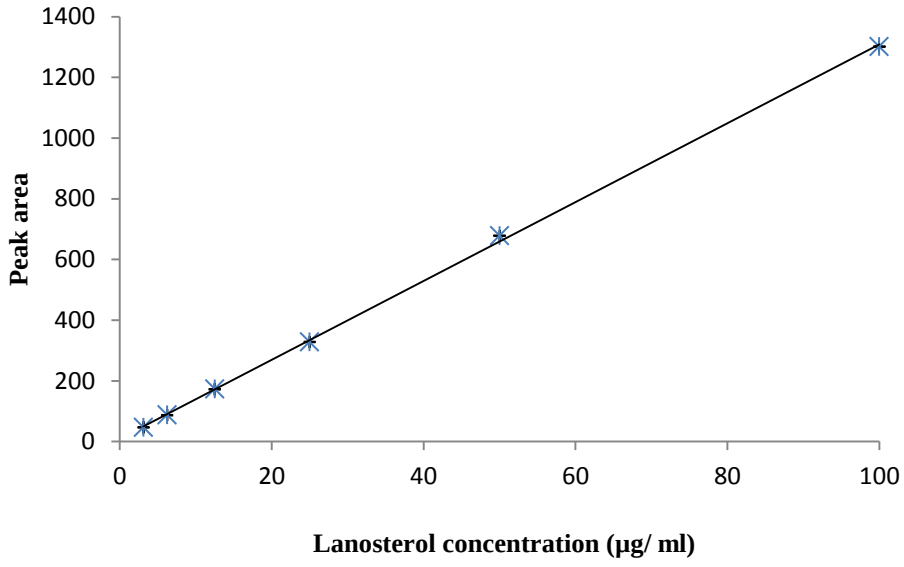


Figure A9: **Lanosterol standard curve with lanosterol standard at a range of 0µg/ml to 100µg/ml. Peak areas of lanosterol were used to construct the standard curve. y = 13.001x + 8.9905; R^2 = 0.9996. Error bars represent standard deviation (n = 3).**

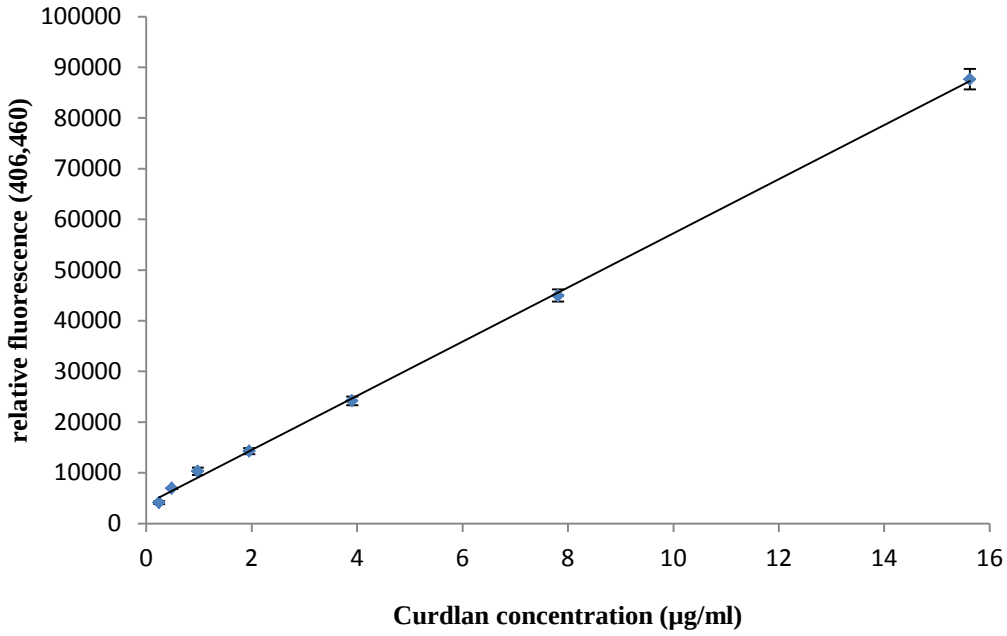


Figure A10: Standard curve of curdlan for the detection β-(1,3) glucan content at an excitation wavelength of 406nm and emmison wavelength of 460nm. y = 5466.9x + 3937; R^2 = 0.9987. Error bars represent standard deviation (n = 3).

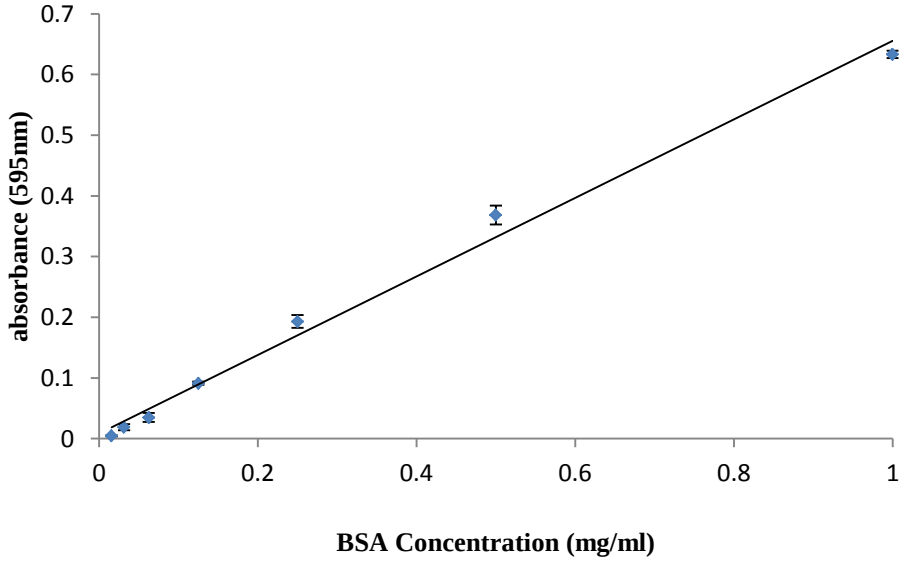


Figure A11: Standard curve of BSA for the quantification of proteins using the Bradford assay at an absorbance of 595nm. y = 0.6426x + 0.0069; R^2 = 0.9918. Error bars represent standard deviation (n = 3).

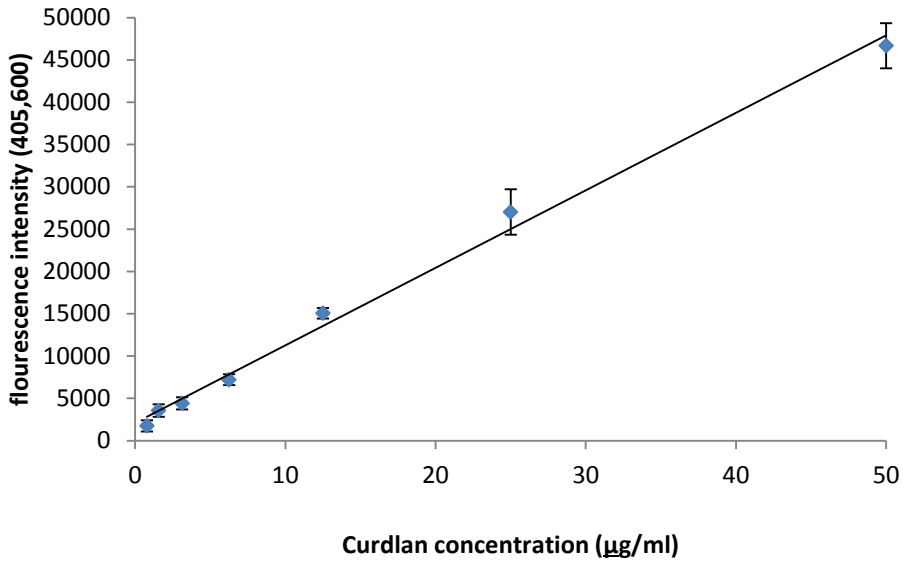


Figure A12: Standard curve of curdlan for the quantification of β-glucan synthase activity at an excitation of 405 and emission wavelength of 600nm. y = 916.05x + 2106.6; R^2 = 0.994. Error bars represent the standard deviation (n = 3).

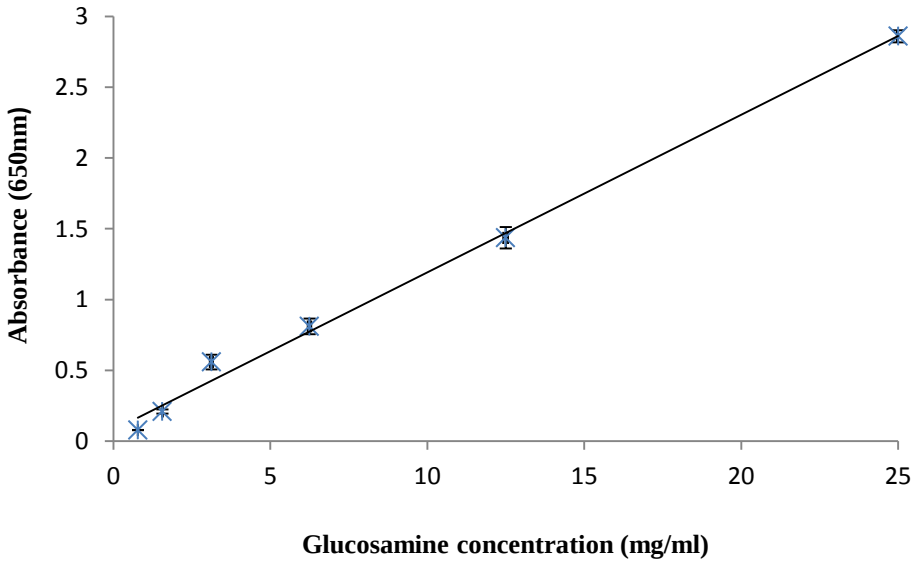


Figure A13: Standard curve of glucosamine for the quantification of total chitin content at an absorbance of 650nm. y = 0.1122x + 0.0793; R^2 = 0.995. Error bars represent standard deviation (n = 3).

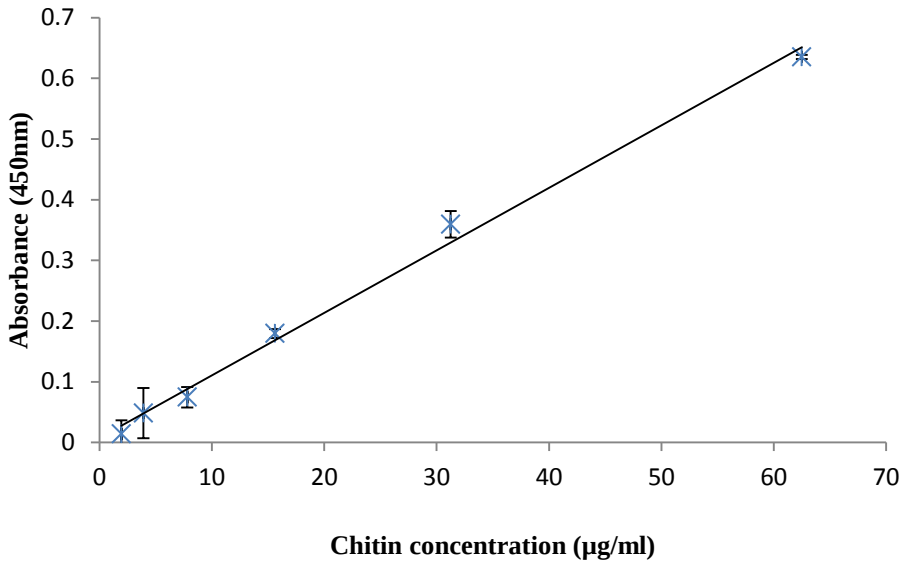


Figure A14: Standard curve of chitin for the quantification of chitin synthase of *A. flavus*. A concentration range of chitin was used for the construction of the standard curve. y = 0.0104x + 0.0051; R^2 = 0.9942. Error bars represent standard deviation (n=3).

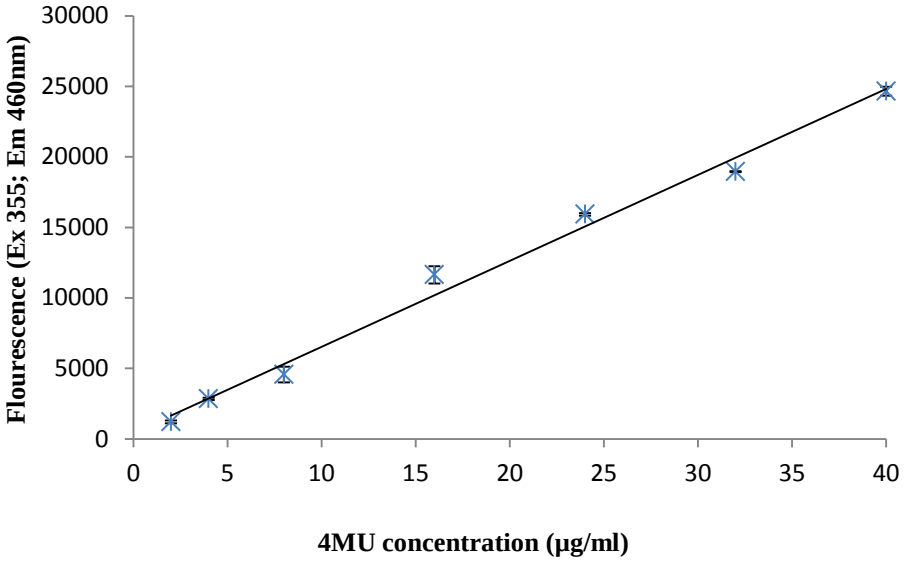


Figure A15: Standard curve 4MU for detection of chitobiosidase activity of *A. flavus*, with 4MU-$(GlcNAc)_2$ as the substrate. y = 609.56x + 423.72; R^2 = 0.9904. Error bars represent the standard deviation (n = 3).

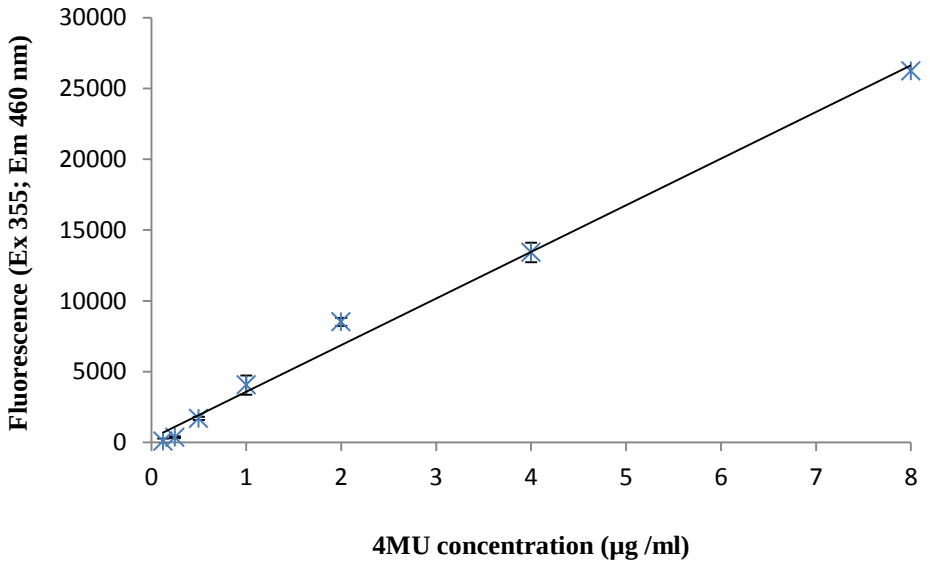


Figure A16: Standard curve of 4MU for the detection of β-N-acetylglucosaminidase activity with 4MU-GlcNAc as the substrate. y = 3290x + 308.02; R^2 = 0.9925. Error bars represent the standard deviation (n = 3).

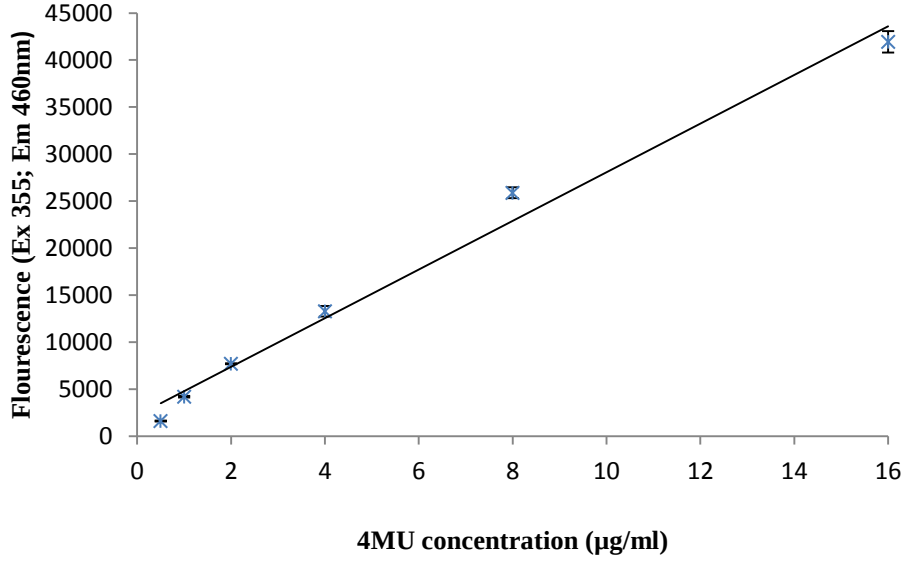
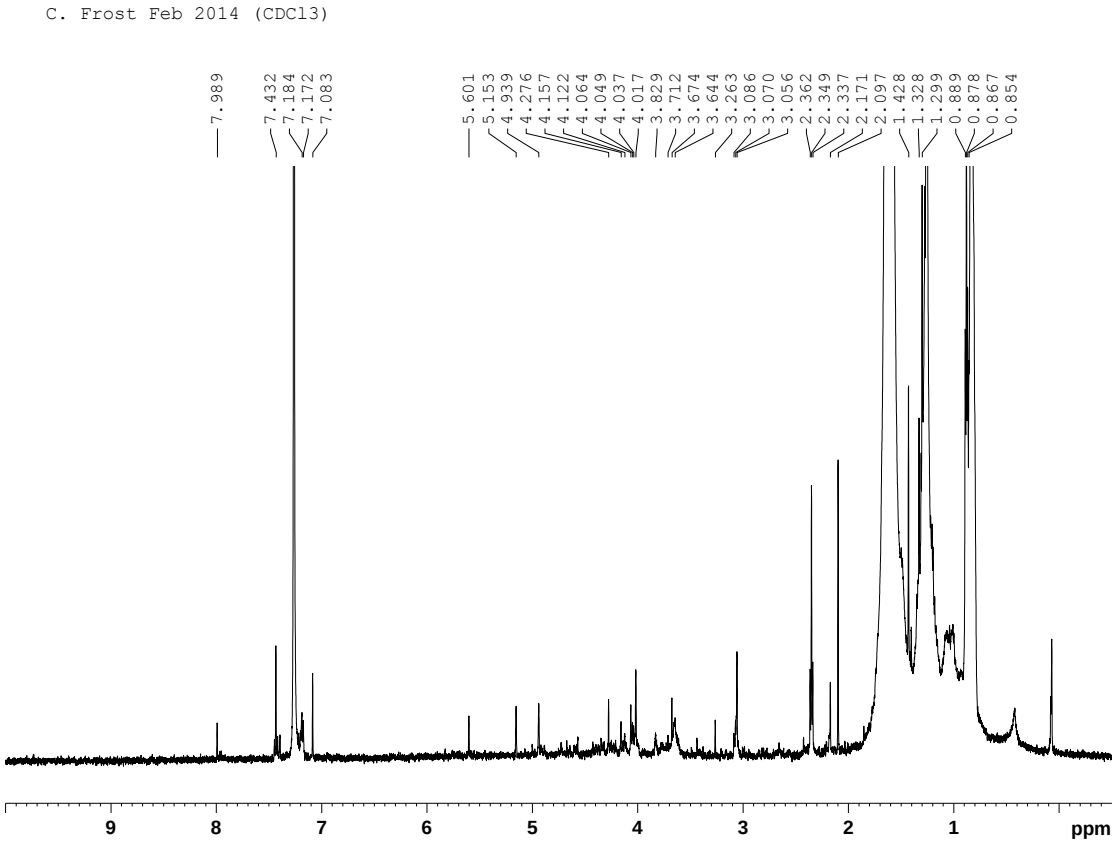


Figure A17: Standard curve of 4MU for the detection of endochitinase activity with 4MU-$(GlcNAc)_3$ as the substrate. Y = 2587x + 2181.7; R^2 = 0.9864. Error bars represent the standard deviation (n= 3).

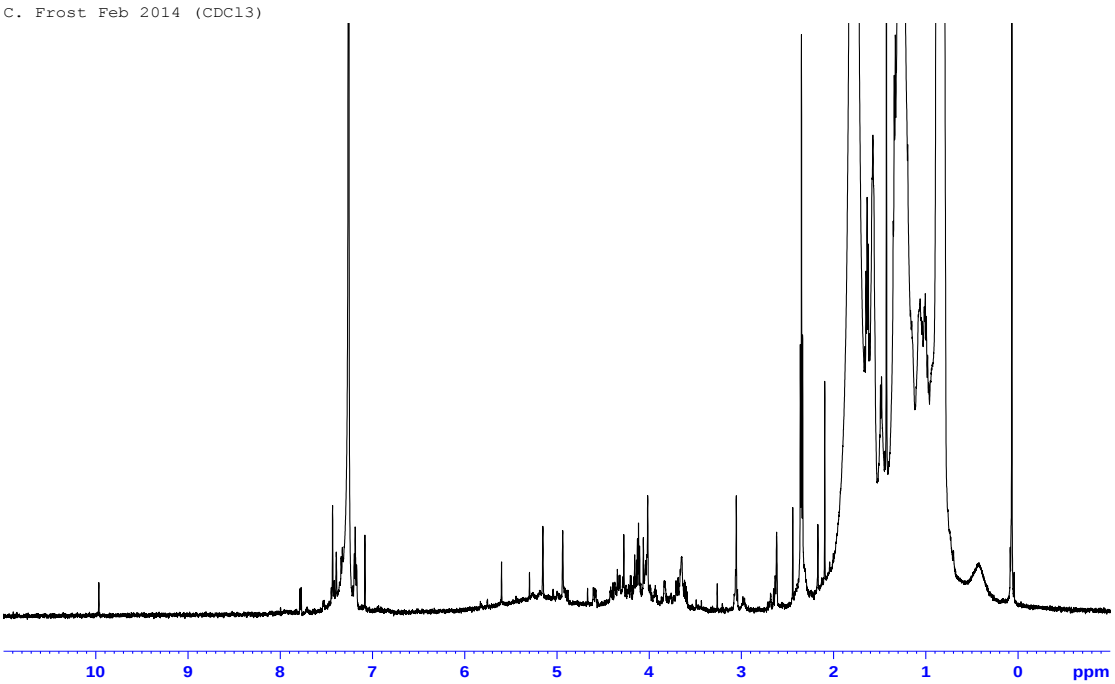
Appendix 2

NMR analysis of HEA1

HEA1 sample run on 600 NMR. ^{1}H NMR run in $CDCl_3$ showed that the amount of compound present was very small



Sample was concentrated and re-run


C. Frost Feb 2014 (CDCl3)
10
9
8
7
6
5
4
3
2
1
0
ppm