AN APPROACH TO UNDERSTANDING TOXICITY INDUCTION BY FILAMENTOUS FUNGI ON HUMAN CELL LINES

BY

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DECLARATION

I, Mary Augustina Egbuta, declare that the thesis entitled "**An approach to understanding toxicity induction by filamentous fungi and their combinations on selected human cell lines**", hereby submitted for the degree of Doctor of Philosophy in Biology, has not previously been submitted by me for a degree at this or any other university. I further declare that this is my work in design and execution and that all materials contained herein have been duly acknowledged.

Name: **Mary Augustina Egbuta**

Signature: 

Date: 30/06/2016

DEDICATION

I dedicate this study to God Almighty, for his love and faithfulness.

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GENERAL ABSTRACT

Filamentous fungi occur widely in different parts of the environment including water, soil and air. Their occurrence in the environment especially in large amounts and under certain conditions pose dangerous health risks to humans, especially immunocompromised individuals as a result of the compounds they produce during metabolism. In this regard, filamentous fungi are associated with a range of diseases including invasive and superficial infections.

In this study, species from the genera *Aspergillus*, *Fusarium* and *Penicillium* were used to investigate their combined toxic effects when exposed to two human cell lines (hepatocytes and renal epithelial cells "in vitro"). Reference isolates used were: *Aspergillus niger* (*A. niger*), *Aspergillus flavus* (*A. flavus*), *Fusarium oxysporum* (*F. oxysporum*), *Fusarium verticillioides* (*F. verticillioides*), *Penicillium chrysogenum* (*P. chrysogenum*) and *Penicillium expansum* (*P. expansum*) isolated from maize samples. Isolates were cultured on Malt Extract agar/broth and Potato dextrose agar/broth at three incubation periods (4, 9 and 14 days). Isolates were identified following deoxyribonucleotide acid (DNA) extraction, amplification and sequencing of amplified products. Fungal species were further screened for mycotoxin production after different incubation periods by high performance liquid chromatography (HPLC) analysis using specific mycotoxin standards. Production of mycotoxins varied among isolates with *F. verticilliodes* and *F. oxysporum* producing more mycotoxins compared to other species. *Aspergillus flavus* produced aflatoxins (AFs) at different stages of growth up to 11.6µg/g at 9 days whereas, *A. niger* produced ochratoxin A (OTA) between $8.63*10^{-6}$ and $5.8*10^{-4}$µg/g at 4 and 14 days of growth respectively. Production of fumonisin B_1 (FB_1), deoxynivalenol (DON) and nivalenol (NIV) by *Fusarium* species was up to 114.6µg/g at 4 days, 0.15µg/g at 14 days and 1035.27µg/g at 4 days respectively.

One characteristic of toxicity induction by microorganisms on both human and animal cells is the reduction of cell viability of the latter. A resazurin salt assay test was conducted in order to determine this effect. Human hepatocytes and renal epithelial cells were exposed to individual filamentous fungi species and their combinations for 24, 48 and 7h and cell viability determined by the ability of the cells to reduce resazurin to resofurin. Individual filamentous fungi and their combinations were able to induce a reduction in cell viability of the human cell lines at 72h of exposure with initial increase in cell proliferation at 24 and 48h. After incubation for up to 72h, there was reduction of cell viability down to 39.9 and 35.6% for hepatocytes and renal epithelial cells respectively. Filamentous fungi combinations, especially combinations of *A. niger* species and others had more deleterious cell viability reduction compared to individual species.

Cytokine secretion/production is one of the means through which the human system combats infections. Although these cytokines contribute to protecting the human system from infections, an imbalance in their secretion could help in promoting inflammation upon infection. To investigate the induction of cytokine production by the hepatocytes upon exposure to individual filamentous fungi species and their combinations, cytometric Bead Array (CBA) of Th1 and Th2 human cytokines were determined. The cells were exposed to fungal isolates individually and in combination for 3, 6, 12 and 24h and cytokine expression measured using an Accuri C6 flow cytometer. Cytokine expression was measured for some of the cells exposed to *A. flavus*, *F. verticillioides*, *F. oxysporum*, *P. chrysogenum* and *P. expansum* with the production of interleukin 2 (IL-2), interleukin 4 (IL-4) and interferon gamma (IFN-γ). Fungi combinations containing *F. verticillioides* and *F. oxysporum* induced secretion of five cytokines; IL-2, IL-4, IFN- γ, Tumour necrosis factor (TNF) and interleukin 10 (IL-10) up to 2.940pg/ml, 3.693pg/ml, 4.720pg/ml, 2.093pg/ml and 0.623pg/ml.

This study has been able to fill the knowledge gap in terms of synergistic action of some filamentous fungal species when exposed to certain cells in the human system. Furthermore, the production of DON by *F. oxysporum* in this study is a novel finding which has not been documented. The significance of this study is that the continuous exposure of humans to co-occurring filamentous fungi can be deleterious resulting in abnormal cell multiplication and reduction in cell viability as well as organ shut down.

CHAPTER ONE

GENERAL INTRODUCTION

Fungi are a large group of plant-like living organisms without chlorophyll (Ravichandra, 2013). They derive their nourishment and energy from dead organic matter and so are referred to as heterotropic eukaryotes. Fungi are mostly plant parasites and commonly found in the soil, air, water and contaminated food; they are divided into yeasts and filamentous fungi which are also referred to as moulds (Hageskal *et al.*, 2009; More *et al.*, 2010). Between the two groups of fungi, filamentous fungi, which constitutes the focus of this study, has been reported to be in existence over the last two centuries (Leslie and Summerell, 2006) and in association with human and animal health (Georgiadou and Kontoyiannis, 2012).

Genera *Aspergillus*, *Fusarium* and *Penicillium* are some of the groups of fungi classified under filamentous fungi that have been reported to be in association with humans and animals (Negedu *et al.*, 2011; Sampietro *et al.*, 2010). This group of fungi are ubiquitous and widely distributed. Their vast distribution cause them to have both positive and negative effects in our daily lives due to the metabolites they produce during their different growth phases. Positive effects associated with these micro-organisms include application in agriculture and food production as well as in the medical and pharmaceutical industry (Ward, 2012b). Negative effects include their ability to act as pathogens or produce pathogens which contribute to or aggravate disease conditions in humans and animals (Georgiadou and Kontoyiannis, 2012; Solé and Salavert, 2008).

Recent studies have shown the possibility of biotechnological implementation of some filamentous fungal species proposing their use in the biopharmaceutical industry (Chávez *et al.*, 2010; Rodríguez-Rodríguez *et al.*, 2012; Ward, 2012b). This is because they have been

studied to be excellent producers of extracellular enzymes. They are also suitable hosts for the production of recombinant proteins that could be implemented in food and pharmaceutical industries (Chávez *et al.*, 2010; Morath *et al.*, 2012). Infections with opportunistic fungal pathogens have become a major clinical problem due to an increasing number of immunocompromised patients with AIDS, organ and bone marrow transplantation or treatment with cytotoxic drugs. Also, filamentous fungi produce secondary metabolites such as mycotoxins which have negative impacts on the agricultural industry. They are also associated with a variety of human and animal diseases such as oesophageal cancer in humans, Benign Endemic Nephropathy (BEN) in humans and equine leuco-encephalo malacia (ELEM) in horses (Brown *et al.*, 2012; Richard, 2007). A wide range of these filamentous fungal species have also been reported to induce toxicity and immune suppression in humans and animals (Georgiadou and Kontoyiannis, 2012) when inhaled or ingested and as such, regarded as human and animal pathogens. Relative studies have been done with regard to understanding the health effects of these fungi when inhaled or ingested (Khan and Karuppayil, 2012; Knutsen *et al.*, 2012; Solé and Salavert, 2008) although there has been limited information on their mode of toxic activity in humans and animals. Some of these filamentous fungi with particular reference to *Aspergillus*, *Fusarium* and *Penicillium* species, have been reported to be associated with mycosis in both humans and animals.

Aspergillus species have been linked to pulmonary infections and infections of the digestive tract with the two most commonly mentioned being *A. fumigatus* and *A. flavus*. *Fusarium* species have also been reported to induce immune suppression in individuals resulting in a variety of infections of the skin, lungs, blood, sinuses and the liver. *Penicillium* species have also been reported to induce diseases in humans and animals, thus classified as pulmonary and gastro-intestinal tract (GIT) pathogens (Liu, 2011a). These fungal genera have also been mentioned in relation to immune-compromised individuals such as HIV individuals susceptible

particularly to *P. marneffei* (Woo *et al.*, 2006) and patients with haematological malignancies exposed to *A. fumigatus* and *A. flavus* (Gonçalves *et al.*, 2012; Kupfahl *et al.*, 2008).

1.1 Problem statement

Filamentous fungi commonly occur in the environment because they do not require any strict environmental conditions for survival. Furthermore, they occur mostly in combination such that it is possible to find two or all three fungal genera occurring in the same environment at the same time. Very few studies have been conducted with regard to understanding the combined negative health effects of these fungal genera in humans and animals. Considering the mode of action of these pathogenic and toxigenic fungal species, it is therefore imperative for further studies to be conducted in order to understand how these fungal species interact with one another, their modes of action (singularly and in combination) with other fungi in order to induce toxicity and pathogenesis on different organs of the body. A better understanding of the action of these fungi on humans and animals at a cellular level will contribute to intervention strategies aimed at controlling or impeding their negative health effects.

1.2 Aim of the study

Due to the different negative health effects of these group of filamentous fungal species when inhaled or ingested and the wide distribution of these fungal spores in the environment, the study investigated possible interactions of selected filamentous fungal species of the genera *Aspergillus*, *Fusarium* and *Penicillium* to induce toxicity on selected human cell lines in vitro.

1.2.1 Objectives of the study

The objectives of this study were to:

1. Determine the level of mycotoxin production by selected filamentous fungal isolates at different growth stages;

2. Evaluate the effect of the fungal isolates at different growth stages, individually and in combinations on cell viability of selected cell lines;

3. Determine and measure cytokine expression induced by the fungal isolates on selected cell lines; and

4. Evaluate the relationship between toxicity induction and cytokine expression by the isolates across different cell lines.

CHAPTER TWO

The ubiquity of filamentous fungi in relation to their importance and health risks associated with exposure to species

Abstract

Filamentous fungi are found in different habitats in the environment. They occur in mixtures and one may find many genera of filamentous fungi dominating a particular habitat or substrate. The wide distribution of filamentous fungi has resulted to its use by mankind for different purposes. Despite the economic and medical benefits of fungi, most with special reference to filamentous fungi produce metabolites that have been associated with a range of health risks in humans and animals. The association of filamentous fungi and their metabolites with different negative health conditions in humans and animals has triggered the need to investigate the different health risks induced by this family of heterotrophs. The aim of this review is to discuss the different genera of filamentous fungi and their economic relevance, extending the discussion to health risks associated with commonly occurring filamentous fungal species as well as evaluate their pathogenicity and mycotoxic properties.

Keywords: Air, soil, infections, *Aspergillus*, *Fusarium* and *Penicillium*

2.1 Introduction

Fungi, a member of a large group of eukaryotes are also classified as a kingdom and separate from plants, protists, animals and bacteria. With cell walls made up of chitin (a main disparity from plant cell walls which contain cellulose and bacterial cell walls), fungi are abundant in the environment and inconspicuous because of their small structures and their cryptic lifestyles on substrates they inhabit (Pitt *et al.*, 2000). Naturally occurring in different parts of the

environment and ecosystem, filamentous fungi are some of the most abundant known fungi (Kirk *et al.*, 2008).

Also referred to as moulds, filamentous fungi are so-called because they possess hyphae which form branches making up their mycelia. They are reported to occur naturally as well as contaminate different surfaces both indoors and outdoors. As a result of their vast occurrence in the environment, these type of fungi have been investigated over the years in terms of their positive and negative uses for mankind (Bennett, 1998; Bennett and Klich, 2009; Sauer *et al.*, 2008; Ward, 2012a). Due to the ubiquitous occurrence of filamentous fungi and their applications in different sectors of the economy, this review discusses the wide occurrence of filamentous fungi in the environment, describes the different genera of filamentous fungi in existence as well as analyse the positive and negative effects of these group of fungi to mankind.

A range of filamentous fungi species which belong to different genera have been associated with many infections affecting different organs of the human body such as the eyes, ears, nasal cavity, nails, skin, respiratory tracts and internal organs (Ahmadi *et al.*, 2012; Deshpande and Koppikar, 1999; Georgiadou and Kontoyiannis, 2012; Gugnani *et al.*, 1976; Howard, 2002). Filamentous fungal species have the ability to synthesise a variety of natural products as primary and secondary metabolites. Although some, especially those which belong to the genus *Aspergillus* (*Aspergillus niger)* and genus *Penicillium* (*Penicillium citrinum*) are used in food and pharmaceutical industries due to the metabolites they produce (Jahromi *et al.*, 2012; Laich *et al.*, 2002), these fungi have also been reported to be associated with infections and diseases (Person *et al.*, 2010; Walsh *et al.*, 2004). Some filamentous fungi have been reported to cause both superficial infections such as skin and nail infections, as well as invasive infections particularly in immune-compromised individuals (Ahmadi *et al.*, 2012; Georgiadou and

Kontoyiannis, 2012; Hedayati *et al.*, 2007c; Jain *et al.*, 2011; Oshikata *et al.*, 2013; Vonberg and Gastmeier, 2006b).

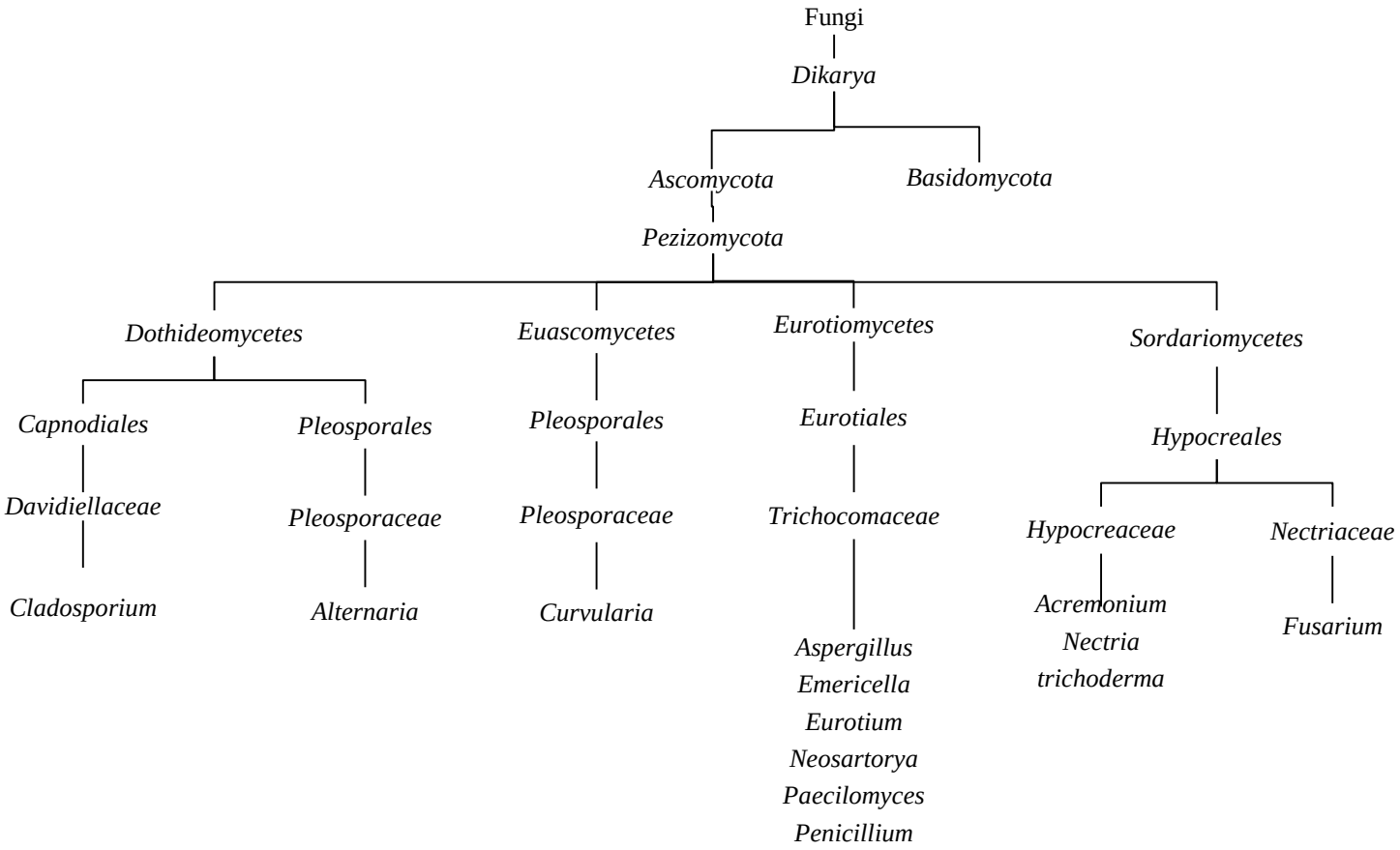
Production of mycotoxins by certain filamentous fungi, usually in response to certain conditions such as humidity and temperature (D' Mello and Macdonald, 1997.; Kuiper-Goodman, 1995) pose health risks to humans and animals. Diseases associated with mycotoxins include oesophageal cancer, liver cancer and Balkan Endemic Nephropathy (BEN) in humans, as well as equine leuco-encephalo malacia (ELEM,), hormonal disorders, immunosuppression and even deaths in animals (Brown *et al.*, 2012; Dutton, 1996; Grollman and Jelakovic, 2007; Richard, 2007).

Due to the ubiquitous occurrence of filamentous fungi and their applications in different sectors of the economy, this review discusses the wide occurrence of filamentous fungi in the environment, describes the different genera of filamentous fungi in existence as well as analyse the positive and negative effects of these group of fungi to mankind. Also, inspite of the fact that most filamentous fungi have been used and are still manipulated biotechnologically in the food and pharmaceutical/medical industry, it is of utmost importance to extensively examine the health risks of these filamentous fungi, especially those that commonly occur in the environment or in food, discussing the ability of these fungi to exert negative health effects, especially in humans taking into consideration their mycotoxic, cytotoxic, DNA damaging and immune-suppressing properties.

2.2 Filamentous fungi

A high percentage of known filamentous fungi tend to originate from the sub- Phylum "*Pezizomycotina*" (Kirk *et al.*, 2008; Moretti, 2009). Filamentous fungi encompass many genera of fungi including: *Aspergillus, Penicillium*, *Fusarium*, *Cladosporium*, *Emericella*, *Eurotium, Paecilomyces*, and *Curvularia* (Figure 2.1) with *Aspergillus*, *Penicillium*, *Fusarium*,

Alternaria and *Cladosporium* being the most investigated than the other genera (Pitt and Hocking, 1997b). In the following sections, the different genera of fungi in the group of filamentous fungi are mentioned and described taking into consideration the most and less occurring genera.



Source: (Kirk *et al.*, 2008; Moretti, 2009; Pitt *et al.*, 2000)

Figure 2.1: Distribution of filamentous fungi in phylum *Ascomycota*

2.2.1 *Aspergillus*

As a member of the *Trichocomaceae* family in the order *Eurotiales*, *Aspergillus* are reportedly the most abundant and widely distributed filamentous fungi globally although they are more frequent in warmer regions and occur more in milder zones than in warmer regions (Klich, 2002). Usually regarded as a soil fungi (Barkai-Golan, 2008), they are ubiquitous, cosmopolitan and commonly isolated from soil, plant debris and indoor environment. They have the ability to grow at reduced water activity and occur on stored foods and feed which turn mouldy. Some species of *Aspergillus* have been accepted to be mitosporic without any known sexual spore production while a teleomorphic state has been described for other species of this genus (Kirk *et al.*, 2008). With their characteristic dark colours (black, grey or green and in other cases white or milky), there are over 185 species of the genera *Aspergillus*, with *A. fumigatus* being the most commonly isolated species followed by *A. flavus* and *A. niger* (Klich, 2002d). Other species of *Aspergillus* isolated so far, though less commonly are: *A. clavatus, A. glaucus* group, *A. oryzae, A. versicolor, A. nidulans, A. terreus, A. ustus* and a host of others (Klich, 2002d).

2.2.2 *Fusarium*

First reported in 1809, these group of filamentous fungi, widely distributed in plants, the soil and known to contain a range of plant-pathogenic fungal species have been in existence for the past two centuries (Leslie and Summerell, 2006). They are primary plant pathogens, require high water activity for growth and are characterised by production of septate, fusiform to crescent-shaped macroconidia with or without microconidia (Leslie and Summerell, 2006; Pitt and Hocking, 1997c). Aside from their ability to act as plant pathogens, *Fusarium* species have been linked to a wide range of diseases and infections in humans and animals (Nucci and Anaissie, 2007b). Commonly occurring species of the genus, *Fusarium* include *Fusarium*

verticillioides, *Fusarium graminearium*, *Fusarium proliferatum*, *Fusarium sporotrichiodes*, *Fusarium solani*, *Fusarium chlamydosporum* among others (Leslie and Summerell, 2006).

2.2.3 *Penicillium*

Penicillium species are among the most common decomposers in nature. This genus of ascomycetes fungi are closely related to *Aspergillus* species but in general, are less thermo-tolerant and are most prominent ecologically in cooler areas, though they are by no means absent in the tropics (Howard, 2003). The genus *Penicillium* is characterised by production of conidia in a penicillus and it is widely distributed in the environment. Although it is certain that *Penicillia* are more diverse in terms of species and range of habitats since they have the ability to grow in almost any environment (Pitt and Hocking, 1997c), there are a wide range of *Penicillium* species in nature such as *P. citreonigum*, *P. polonicum*, *P. digitatum*, *P. chrysogenum*, *P. roqueforti*, *P. citrinum*, *P. janthinellum*, *P. simplicissimum*, *P. aurantiogriseum*, *P. camemberti*, *P. verrucosum* and *P. expansum* among others (Pitt and Hocking, 1997c).

2.2.4 *Cladosporium*

This genus is a commonly isolated saprophytes and plant pathogens which produces olive-green to brown or black colonies (Pitt and Hocking, 1997c). They occur mostly in outdoor environments and only occur indoors on moist surfaces. *Cladosporium* species occur as pathogens on fresh fruits with one of the species *Cladosporium fulvum* being the common cause of tomato leaf mould disease (Rivas and Thomas, 2005). Some common *Clasdosporium* species include *C. fulvum*, *C. cladosporioides*, *C. herbarum*, *C. salinae*, *C. spinulosum*, *C. fusiforme* and *C. arthropodii* among the over 30 known species.

2.2.5 *Alternaria, Acremonium* and *Curvularia*

Alternaria species are reported to be the major plant pathogens causing at least 20% of agricultural spoilage (Nowicki *et al.*, 2012). As a member of the *Pleosporaceae* family, this genus includes species that are found to occur almost everywhere with thick green, black or grey colonies. Some of the isolated *Alternaria* species from water, food, air and plants include: *A. alternata, A. molesta, A. solani, A. japonica, A. longipes*, and *A. infectoria*.

The genus *Acremonium* is reported to be a large and varied genus characterised by fine and hyaline hyphae produced mostly by simple phialides and single-celled conidia (Howard, 2003). Commonly isolated from dead plant materials and soil, this genus is made up of about 100 species with *Acremonium strictum* reported as one of the most common species isolated from food.

Mostly found in tropical regions and seldom in temperate zones, the genus *Curvularia* is a pathogen of many plant species and soil. This genus has the ability to withstand very high temperature up to 40^{o}C which explains its predominance in tropical regions (Pitt and Hocking, 1997c). *Curvularia* species are not as numerous as the other genera of filamentous fungi group and have been mostly isolated from soil and plant tissues/seeds. Some commonly isolated *Curvularia* species are: *C. clavata, C. penniseti, C. protuberata, C. trifolii, C. tuberculata, C. lunata, C. pallescens, C. ovoidea* with *C. lunata* and *C. pallescens* commonly isolated (Pitt and Hocking, 1997c).

2.2.6 *Emericella* and *Eurotium*

The genus *Emericella* was first mentioned in 1857 (Berkeley, 1857) and is a teleomorph of *Aspergillus* species. Referred to as the sexual state of *Aspergillus* species because of their closeness to this genus, they are likely to be present alongside their related *Aspergillus* species during long-term growth (Verweij *et al.*, 2008; Zalar *et al.*, 2008). Producing ascopores

(conidia) that are brightly coloured with smooth to roughened texture (Kirk *et al.*, 2008), species of the *Emericella* genus grow rapidly and are common in tropical and sub-tropical regions of the world (Matsuzawa *et al.*, 2010). The genus includes over thirty (30) species such as *Emericella olivicola*, *E. nidulans*, *E. stell-maris*, *E. filifera*, *E. quadrilineata* and *E. discophora* (Verweij *et al.*, 2008; Zalar *et al.*, 2008).

Closely related to the genus *Emericella* and also a member of the family *Trichocomaceae*, the genus *Eurotium* is another anamorph of *Aspergillus* species commonly foung in tropical and sub-tropical regions of the world (Kirk *et al.*, 2008) They are characterised by sperical to ellipsoidal spores that grow in chains and are rough walled. With a moderately rapid growth rate, colonies of *Eurotium* species are usually yellow or dullgreen to bluish green and have the ability to grow very well even at low water activity (Butinar *et al.*, 2005). Common *Eurotium* species include *Eurotium amstelodami*, *E. herbariorium*, *E. repens* and *E. rubrum* among others (Butinar *et al.*, 2005; Hubka *et al.*, 2013).

2.2.7 *Paecilomyces*

As a member of the similar family as *Aspergillus*, *Eurotium*, *Emericella* and *Penicillium*, the genus *Paecilomyces* is often confused with the *Penicillium* genus because of their close morphological resemblance (Kirk *et al.*, 2008). Growing rapidly, some species of this genus are regarded as thermophilic organisms due to their ability to grow well at high temperatures of up to 50^{o}C (Inglis and Tigano, 2006). Some commonly isolated *Paecilomyces* species include *Paecilomyces variotii* (Steiner *et al.*, 2013), *P. lilacinus* (Inglis and Tigano, 2006; Pastor and Guarro, 2006) and *P. fulvus* (Egbuta *et al.*, 2015b).

2.3 Distribution of filamentous fungi in the environment

Due to their ubiquitous nature, filamentous fungi are widely distributed in the environment. The vast variety of substrates on which filamentous fungi are able to grow on has also contributed to their wide distribution in the environment worldwide (Pitt and Hocking, 1997a). Although most filamentous fungi require high temperatures of up to 30^{o}C and increased water activity up to 0.97 for growth (Astoreca *et al.*, 2007), this contributes to their occurrence in mostly hot and humid regions of the world. Some fungi such as the *Penicillium* genus also have the ability to grow in temperate areas (Kirk *et al.*, 2008) causing such species to occur in colder areas of the world. The adaptive characteristics of filamentous fungi to different environmental conditions is therefore contributory to its vast occurrence in the environment. The section below evaluates their distribution (Table 2.1) in three habitats of the environment: air, soil and water.

Table 2.1: Distribution of filamentous fungi in the environment

Filamentous fungus	Environmental habitat			Reference
	Air	Soil	Water	
Acremonium				
A. strictum		✓		(Goldbeck *et al.*, 2013; Watanabe *et al.*, 2001)
A. macroclavatum		✓		
Alternaria				
A. alternata		✓	✓	(Arvanitidou *et al.*, 2000; Pastor and Guarro, 2008)
A. chartarum			✓	
A. dianthicola			✓	
A. ternuissima			✓	
Aspergillus				
A. caatingaensis		✓		(Horn and Dorner, 1998; Khan and Karuppayil, 2012; Klich, 2002a; Klich, 2002b; Nikaeen and Mirhendi, 2008; Oliveira *et al.*, 2013; Panagopoulou *et al.*, 2002; Vesper *et al.*, 2007; Warris *et al.*, 2002)
A. caespitosus		✓		
A. flavus	✓	✓	✓	
A. fumigatus	✓	✓	✓	
A. nidulans		✓		
A. niger	✓		✓	
A. nominus		✓		
A. parasiticus		✓		
A. pernambucoensis		✓		
A. restrictus		✓		
A. sydowii		✓		
A. tamari		✓		
A. terreus			✓	
A. ustus		✓		
Cladosporium				
C. cladosporioides	✓			(Ogórek *et al.*, 2012; Zalar *et al.*, 2007)
C. dominicanum			✓	
C. fusiforme			✓	
C. herbarum	✓			
C. salinae			✓	
C. sphaerospermum		✓		
C. velox			✓	
Curvularia				
C. lunata		✓		(Lucas *et al.*, 2008; Pratt, 2006; Wang *et al.*, 2014)
C. senegalensis		✓		
C. ramosa		✓		

Emericella				
E. rugulosa		✓		(Klich, 2002a)
E. quadrileanata		✓		
Eurotium				
E. amstelodami		✓		(Klich, 2002a)
E. chevalieri		✓		
E. herbariorium		✓		
E. rubrum		✓		
Fusarium				
F. acuminatum			✓	(Asan, 2011; Edel-Hermann *et al.*, 2015; Funnell-Harris and Pedersen, 2011; Gordon and Martyn, 1997; Gordon *et al.*, 2015; Palmero *et al.*, 2009; Sautour *et al.*, 2012; Scheel *et al.*, 2013; Vigier *et al.*, 1997)
F. avanaceum		✓		
F. chlamydosporum			✓	
F. concolor	✓			
F. culmorum		✓	✓	
F. equiseti	✓		✓	
F. graminearum	✓			
F. nivale		✓		
F. oxysporum		✓	✓	
F. proliferatum	✓			
F. sambucinum		✓	✓	
F. solani	✓	✓	✓	
F. subglutinans	✓			
F. sporotrichiodes		✓		
F. tricinctum		✓		
F. verticilliodes	✓		✓	
Penicillium				
P. citrinum	✓	✓		(Cruz *et al.*, 2013; Dayalan *et al.*, 2011; Jussila *et al.*, 2002; Pryce-Miller *et al.*, 2008; Sawane and Saoji, 2004; Trisuwan *et al.*, 2014)
P. commune		✓		
P. chrysogenum		✓		
P. glaber	✓			
P. lanosum		✓		
P. marneffei		✓		
P. notatum		✓		
P. oxalicum	✓			
P. sclerotiorum	✓	✓		
P. spinulosum	✓			
Paecilomyces				
P. lilicanus		✓		
P. variotii	✓			(Luangsa-ard *et al.*, 2011; Steiner *et al.*, 2013; Tarkkanen *et al.*, 2004)

2.3.1 Air

Present in both outdoor and indoor air, species of the filamentous fungi family are widely distributed in the air (Khan and Karuppayil, 2012; Ogorek *et al.,* 2014). They have been isolated from air samples collected from different areas such as hospitals, outdoor areas and households (Karwowska *et al.*, 2004; Ogorek *et al.*, 2014; Panagopoulou *et al.*, 2002). Among the many species of filamentous fungi, the *Aspergillus*, *Penicillium* and *Cladosporium* genera have been mostly isolated with lesser occurrence of *Fusarium* and other species (Viegas *et al.*, 2010). Within the genus *Aspergillus, A. flavus, A. niger* and *A. fumigatus* are the most common species isolated from air samples collected from both indoor and outdoor areas (Karwowska *et al.*, 2004; Khan and Karuppayil, 2012; Panagopoulou *et al.*, 2007).

2.3.2 Soil

The growth habit of filamentous fungi mycelia which is based upon hyphelial extension and branching, has contributed to the wide range of filamentous fungi species found naturally in soils from different regions of the world (Ritz and Young, 2004). The occurrence of filamentous fungi such as *Penicillium*, *Aspergillus*, *Trichoderma*, *Curvularia* and *Paecilomyces* species have been reported in soil from semi-arid areas characterised by low rainfall (Oliveira *et al.*, 2013). The presence of a variety of filamentous fungi species have also been reported in soil from colder regions such as the Antarctica (Kurek *et al.* (2007); Hughes *et al.* (2007). Although humidity is one condition which favours growth of filamentous fungi, some species of this family of fungi such as *Aspergillus*, *Cladosporium*, *Penicillium* and *Alternaria* are able to thrive in soil from desert areas including places that have not recorded rainfall in decades (Sterflinger *et al.*, 2012).

2.3.3 Water

The presence of fungi in water has been reported to contribute to odour and taste (Gonçalves *et al.,* 2006; Paterson *et al.*, 2009). Fungal biomass have been reported in water systems from

different parts of the world (Hayette *et al.*, 2010; Sonigo *et al.*, 2011). A range of genera of filamentous fungi have been isolated from different sources of water such as rivers, underground water, dead sea, tap and bottled water. Yamaguchi *et al.* (2007), Warris *et al.* (2001) and Okpako *et al.* (2009) reported isolation of *Penicillium*, *Aspergillus*, *Cladosporium*, *Alternaria* and other genera of filamentous fungi form tap water and drinking water. Occurrence of filamentous fungi genera with the dominance of *Penicillium*, *Cladosporium* and *Alternaria* species have also been isolated from bottled and processed water (Okpako *et al.*, 2009). Filamentous fungi isolated from rivers and underground water include *Penicillium* species and other genera of this family (Mohamed *et al.*, 2014). They also occur in water high in salts and minerals (Mbata, 2008). Among the commonly occurring filamentous fungi genera, *Fusarium* species have been seldom isolated from water (Gonçalves *et al.*, 2006; Varo *et al.*, 2007; Warris *et al.*, 2001) with little or nothing reported with regard to the occurrence of *Fusarium* in water.

2.4 Economic importance of filamentous fungi

Filamentous fungi are currently being used in the manufacturing and agricultural sectors all over the world. They are a source of raw materials for food, chemical, pharmaceutical and cosmetic industries (Michelson, 2010; Schuster *et al.*, 2002; van der Straat *et al.*, 2014). Apart from their positive impacts, filamentous fungi can have negative economic impacts, thus being beneficial or detrimental economically. There is therefore a need to evaluate the economic advantages and disadvantages of filamentous fungi as presented in Table 2.2.

Table 2.2: Economic applications of filamentous fungi

Fungi	Agriculture	Industry	Medical	References
A. flavus	Bioremediation			(Kurniati *et al.*, 2014; Romero *et al.*, 2010)
A. niger		Production of citric acid in food, cosmetics and adhesives; source of enzymes and production of gluconic acid		(Schuster *et al.*, 2002)
A. oryzae		Production of kojic acid used in the cosmetics and food industries		(Ogawa *et al.*, 1995)
A.terreus		Production of itaconic acid, a synthetic polymer	Source of antibiotics (Lovostatin)	(van der Straat *et al.*, 2014)
F. venenatum		Industrially produced as food		(Jahromi *et al.*, 2012) (Katona, 2002)
F. oxysporum	Biocontrol agent			(Kaur *et al.*, 2010)
P. adametzioides	Biocontrol agent			(Ahmed *et al.*, 2015)
P. aethiopicum			Production of antibiotic (griseofulvin)	(Frisvad *et al.*, 2004)
P. brevicompactum		Confectionary production		(Barthomeuf *et al.*, 1991)
P. camamberti		Used in cheese production		(Michelson, 2010)
P.chrysogenum			Production of antibiotic (penicillin)	(Laich *et al.*, 2002)
P.citrinum			Production of antibiotic (mevastatin)	(Jahromi *et al.*, 2012)
P. expansum			Production of antibiotic (patulum)	Frisvad *et al.*, 2004)
P. funiculosum	Used in animal feed processing			(Sahasrabudhe *et al.*, 1987)
P. glaucum			Production of immunosuppressant drug.	(Frisvad *et al.*, 2004)
P. griseofulvum			Production of antibiotics(griseofulvin, patulin and penicillin)	(Laich *et al.*, 2002)
P. janezewski			Production of antibiotics(griseofulvin)	Frisvad *et al.*, 2004)
P. nalgiovense			Production of antibiotic penicillin	(Laich *et al.*, 2002
P. patulum			Production of antibiotics(griseofulvin and patulin)	Frisvad *et al.*, 2004)
P. purpurogenum		Confectionary production		(Barthomeuf *et al.*, 1991
P. roqueforti		Cheese production, flavours and fragrances		(Nelson, 1970)

2.4.1 Agriculture

Filamentous fungi have been implemented as bioremediation agents (D'Annibale *et al.*, 2006; Mancera-López *et al.*, 2008), degrading the contents of high chemically contaminated soil and thereby reducing toxicity of the soil. Species such as *A. flavus* and *Paecilomyces farinosus* have the ability to degrade Benzo [a] pyrene in soil (Romero *et al.* (2010), whereas, *Fusarium* species can also bioremediate soils high in polycyclic aromatic hydrocarbons (Potin *et al.*, 2004). The discovery of filamentous fungi activity in bioremediation of soil has prompted more studies of other naturally occurring soil filamentous fungi for bioremediation properties. One example of such is the study by Kurniati *et al.* (2014) whereby filamentous fungi were investigated for reducing mercury in soil. These micro-organisms have also made positive impacts in their use as biocontrol agents against microbes and harmful compounds in plants and crops (Abbas *et al.*, 2011; Ahmed *et al.*, 2015; Kaur *et al.*, 2010). The potential of using filamentous fungi in biofuel production has been investigated by Zheng *et al.* (2012) and found to be feasible.

Some genera of filamentous fungi are reported in association with plant diseases and food spoilage in agriculture, contaminating crops at different stages of production (Dutton, 2009). *Aspergillus* species such as *A. niger*, *A. flavus*, *A. fumigatus*, *A. alliaceus*, *A. carbonarius* and *A. ochraceus*, as well as *Fusarium*, *Penicillium* and *Alternaria* genera are some examples that cause infections and contamination in plants and plant products respectively (Dutton, 2009; Egbuta *et al.*, 2015b; Perrone *et al.*, 2007). Fungal Infection/ contamination of food crops and food products results to a reduced nutritional value and quality of food crops (Perrone *et al.*, 2007) as well as subsequent economical losses (Zain, 2011).

2.4.2 Manufacturing industry

In the paper manufacturing industry, filamentous fungi are implemented in the manufacture of high quality paper suitable for writing and printing (Jerusik, 2010), with reports that fungal

mycelium make up about 10% of good paper quality content. The use of filamentous fungi in industries to compost industrial waste has also been reported by Mohammad *et al.* (2012) indicating the contribution of these micro-organisms in disposal of waste generated from processing palm produce.

2.4.3 Food industry

As a source of different enzymes (Archer, 2000; Guimarães *et al.*, 2006; Khokhar *et al.*, 2012), filamentous fungi are currently being used in different areas of the food manufacturing industry (Kirk *et al.*, 2008) . The activity of filamentous fungi during fermentation has contributed to its use in food manufacturing. An example of such is the use of *A. niger* for fermentation to produce citric acid (Majumder *et al.*, 2010; Max *et al.*, 2010), which is one of the main sources of industrially produced citric acid. Also, the ability of filamentous fungi to produce enzymes, vitamins, lipids, proteins, flavours and other valuable compounds which are implemented in food production (Sahasrabudhe and Sankpal, 2001).

2.4.4 Pharmaceutical/Medical

Chemical compounds produced by filamentous fungi are important to the medical and pharmaceutical industry. This importance can be beneficial or detrimental properties of the compounds and their effects on both humans and animals. Filamentous fungi produce different metabolites that have proven to have different inhibitory effects in metabolic pathways. An example of such compounds are the Statins which include Lovostatin produced by *A. terreus* (Goswami *et al.*, 2012b), Mevastatin produced by *P. citrinum* (Manzoni and Rollini, 2002) and Pravastatin produced by *P. chrysogenum* (McLean *et al.*, 2015). The function of Statins is to inhibit the enzyme hydroxymethyl glutaryl-Coenzyme A (HMG-CoA) reductase which is the first enzyme in cholesterol biosynthesis (Manzoni and Rollini, 2002), thereby lowering blood cholesterol levels in individuals who have high cholesterol levels. Some other filamentous fungus (*Fusarium oxysporum*) have been investigated and found to produce Cyclosporin-A(an

immunosuppressant) currently used in the treatment of cancer, organ transplant patients and in the treatment of auto-immune diseases including AIDS (Sharmila *et al.*, 2012).

The ability of filamentous fungi to inhibit microbial growth has also been investigated. He *et al.* (2002) found that filamentous fungi species could produce Pyrrocidines A and B, which are effective antibiotics against gram-positive bacteria including resistant strains. Echinocandins produced by *Aspergillus* species have been reported by Goswami *et al.* (2012a) to inhibit an enzyme that facilitates fungal cell wall formation in fungal species. Other anti-microbial activities of filamentous fungi reported include inhibition of *Escherichia coli, Staphylococcus aureus* and *Candida albicans* (Svahn *et al.*, 2012) and anti-oxidant activities (Smith, 2014). Filamentous fungi biofilm are currently used as biocatalysts for the production of human drug metabolites since they have been proven to have longer effective time (Amadio *et al.*, 2013). This is a process required for drug development which contributes to assessing toxicity of a drug in pharmacokinetic studies.

2.5 Production of toxins by filamentous fungi

Previously mentioned in the text, many species belonging to the filamentous fungi group produce secondary metabolites known as mycotoxins. In most cases, these substances have toxic effects on humans and animals (Bennett and Klich, 2003). These mycotoxins include the aflatoxins, ochratoxins, fumonisins, trichothecenes, deoxynivalenol, zearalenone, gliotoxin, etc. There are over 300 mycotoxins synthesised by filamentous fungi (Hussein and Brasel, 2001) and production of mycotoxin is common with species of genus *Aspergillus, Penicillium, Fusarium, Alternaria* and *Cladosporium* (Sweeney and Dobson, 1998b). It is usually common to find one mycotoxin being synthesised by more than one fungal species and genera as is the case with ochratoxin A produced by *A.niger, A.ochraceus* and *P.viridicatum*. There is also another situation where one fungal species has the ability to produce more than one mycotoxin

as is the case with *F.verticilliodes* and *F.culmorum* producing fumonisin B_1, moniliformin, nivalenol, deoxynivalenol and other mycotoxins at the same time (Lillards-Roberts, 2011) as shown in Table 2.3.

Table 2.3: Filamentous fungi species and mycotoxins produced

Fungal species	Mycotoxin produced
Aspergillus	
A. carneus	Citrinin
A. clavatus	Cytochlasin E, Patulin, Tryptoquivalene
A. flavus	Aflatoxins, Sterigmatocystin
A. fumigatus	Fumagilin, Gliotoxin, Verruculogen, viriditoxin
A. nidulans	Sterigmatocystin
A. niger	Malformin, Oxalic acid, Ochratoxin A
A. ochraceus	Ochratoxin A, Penicillic acid, Destruxin,
A. terreus	Citrinin, Citreoviridin
A. ustus	Austdiol, Austamide, Austocystin
A. versicolor	Cyclopiazonic acid, Sterigmatocystin
A. parasiticus	Aflatoxins
Fusarium	
F. avenaceum	Enniatins, Fructagenin +1, HT-2 toxin, Ipomeanine, Lateritin +1, Lycomerasmin +1, Moniliformin, Monoacetoxyscirpenol, Neosolaniol, Nivalenol, Sambucynin
F. culmorum	Deoxynivalenol,Fructagenin +1, HT-2 toxin, Ipomeanine, Lateritin +1, Lycomerasmin +1, Moniliformin, Neosolaniol
F.equiseti	Moniliformin, Nivelenol, Monoacetoxyscirpenol, Acetoxyscirpenediol, Acetyldeoxynivalenol, Acetylneosolaniol, Acetyl T-2 toxin, Avenacein +1, Beauvericin +2, Butenolide, Calonectrin, Deacetylcalonectrin, T-1 toxin, zearalenol, T-1 toxin, T-2 toxin,
F. nivale	Deoxynivalenol diacetate, HT-2 toxin, Ipomeanine, Lateritin +1, Lycomerasmin +1, Moniliformin, Monoacetoxyscirpenol, Sambucynin.
F. oxysporum	Moniliformin, Monoacetoxyscirpenol, Neosolaniol, Nivalenol, Acetoxyscirpenediol, Acetyldeoxynivalenol, Acetylneosolaniol, Acetyl T-2 toxin, Avenacein +1, Beauvericin +2, Butenolide, Calonectrin, Deacetylcalonectrin, zearalenone
F. roseum	Fructagenin +1, Moniliformin, Monoacetoxyscirpenol, Neosolaniol, NT-1 toxin, N-2 toxin
F. solani	Enniatins, T-1 toxin, T-2 toxin, Sambucynin, Scirpentriol
F. verticillioides	Fumonisins, Monoacetoxyscirpenol, Neosolaniol, Ipomeanine, Avenacein +1, Beauvericin +2, Fusaric acid, Fusarin
F, graminearum	Zearalenone, Yavanicin+1
Penicillium	
P. viridicatum	Ochratoxin A, Rubrosulphin, Viopurpurin, Viomellein
P. citrinin	Citrinin

P. verrucosum	Citrinin
P. hirsutum	Citrinin
P. citreoviride	Citreoviridin
P. islandicum	Islanditoxin
P. expansum	Patulin
P.roqueforti	Patulin
P. griseofulvum	Patulin
P. claviforme	Patulin
P. crustosum	Penitrem, Viomellein
P. rubrum	Rubratoxin
P. brunneum	Rugulosin
P.kloeckeri	Rugulosin
P. rugulosum	Sterigmatocystin, Rugulosin
P.aurantiogriseum	Viomellein

Source: (Barkai-Golan, 2008; Lillards-Roberts, 2011; Sweeney and Dobson, 1998b)

Aspergillus species are producers of a wide range of mycotoxins such as aflatoxins and sterigmatocystin (produced by *A. flavus*), ochratoxin A, malformin, oxalic acid and fumonisin B_2 (produced by *A. niger*), viriditoxin and gliotoxin (produced by *A. fumigatus*), tryptoquivalene and cytochalasin E (produced by *A. clavatus*) among others (Bennett and Klich, 2003). *Fusarium* species are known producers of mycotoxins such as fumonisins, acetoxyscirpenediol, moniliformin, nivalenol, enniatins, fusaric acid, and fusarin among others (Sweeney and Dobson, 1998a). Other mycotoxins produced by *Penicillium* species include ochratoxin A, islanditoxin, penitrem, rubratoxin, rubroskyrin, rubrosulphin, rugulosin, citrinin, citreoviridin, gliotoxin, patulin, viopurpurin and viomellein (Sweeney and Dobson, 1998a).

Richard (2007) reported that mycotoxins synthesised by filamentous fungi have been conjecturally associated with diseases. They induce powerful biological effects of which a prolonged and continuous exposure either by ingestion or inhalation could lead to harmful and negative health implications (Prelusky *et al.*, 1994; Steyn, 1995). Aflatoxins, one of the five most important occurring mycotoxins (IARC, 2002b), comprises of aflatoxin B_1, B_2, G_1 and G_2. They are primarily hepatoxic toxins that mainly target the liver. Aflatoxin B_1 (AFB_1) is the most potent and classified as a human Group 1 carcinogen by the International Agency for Research on Cancer (IARC, 1993a-b) Ochratoxin A is another major mycotoxin classified as a possible human carcinogen by the IARC. Targeting mainly the kidney, this toxin is nephrotoxic, teratogenic, carcinogenic and immuno-suppressive in many animal species (Stoev, 1998). Other major mycotoxins such as fumonisins, deoxynivalenol and zearalenone also induce carcinogenic, teratogenic, mutagenic, genotoxic and immune suppressing effects in humans and animals (Hussein and Brasel, 2001; Kumar *et al.*, 2008; Richard, 2007).

There are severe negative health conditions associated with mycotoxin poisoning in humans and animals. One of such cases is the Balkan Endemic Nephropathy, where it was reported that OTA was associated with this disorder in the Balkan areas of south-eastern Europe (Grollman

and Jelakovic, 2007; Pfohl-Leszkowicz *et al.*, 2002; Richard, 2007). Hussein and Brasel (2001) also reported acute aflatoxin exposures associated with epidemics of acute hepatitis in China and Africa which resulted in deaths. Some of the metabolites are classified as carcinogens by the International Agency for Research on Cancer because of the negative health effects (Table 2.4) they exert on different organs of the body (IARC, 1993a-a; IARC, 1993b-b; IARC, 2002b; IARC, 2012a).

Table 2.4: Health effects of common mycotoxins and target organs

Mycotoxin	Health effect	Target organ	Reference
Aflatoxins	Hepatotoxic and immune-suppressive	Liver	(Steyn, 1995)
Ochratoxin A	Carcinogenic, genotoxic, Immuno-suppressive, nephrotoxic and causing upper urinary tract disease	Kidney, liver	(Mally, 2012; Pfohl-Leszkowicz and Manderville, 2012; Sorrenti *et al.*, 2013)
Fumonisins	Carcinogenic, hepatotoxic, nephrotoxic, immunosuppressive	Gastro-intestinal tract (GIT), liver, kidney	(Chu and Li, 1994; Marasas *et al.*, 1988; Soriano and Dragacci, 2004)
Deoxynivalenol	Nausea, vomiting, diarrhea, reproductive effects and toxicosis	Reproductive organs, GIT	(Kuiper-Goodman, 1994; Prelusky *et al.*, 1994; Richard, 2007)
T-2 toxin	Hepatotoxic, genotoxic and immune-suppressive	GIT, Immune system	(Hymery *et al.*, 2009; Li *et al.*, 2006)
Zearalenone	Carcinogenic, hormonal imbalance and reproductive effects	Reproductive organs	(D' Mello and Macdonald, 1997.; Miller and Trenholm, 1994.)
Nivalenol	Anorexic, immunotoxic, haematotoxic and genotoxic	GIT, immune system	(Bony *et al.*, 2007; Kubosaki *et al.*, 2008; Wu *et al.*, 2012)
Sterigmatocystin	Genotoxic, cytotoxic, immunotoxic and carcinogenic	Liver, immune system, kidney	(Huang *et al.*, 2014; Terao *et al.*, 1978)
Cyclopiazonic acid	Immunotoxic and hepatotoxic	Muscle, hepatic tissue and spleen	(Antony *et al.*, 2003; Burdock and Flamm, 2000; Morrissey *et al.*, 1985)
Moniliformin	Cardiotoxic, muscular disorders, immunotoxic	Heart, kidney, and muscles	(Peltonen *et al.*, 2010; Zhang *et al.*, 2010)

Enniatins	Immunotoxic, cytotoxic	Immune system	(Gammelsrud *et al.*, 2012 ; Juan-García *et al.*, 2013; Prosperini *et al.*, 2014)
Gliotoxin	Immunotoxic, nephrotoxic, hepatotoxic and genotoxic	Kidney, liver, immune system	(DeWitte-Orr and Bols, 2005; Mueller *et al.*, 2013; Nieminen *et al.*, 2002; Niide *et al.*, 2006)
Citreoviridin	Teratogenic and immunotoxic	Not specific	(Hou *et al.*, 2014; Morrissey and Vesonder, 1986)
Citrinin	Nephrotoxic	Kidney	(Bennett and Klich, 2003)

2.6 Infections induced by filamentous fungi

Filamentous fungal species are widespread in the environment and have been reported in association with some human and animal infections and diseases (Howard, 2002; Howard, 2003). A host of fungal infections have been reported in association with *Aspergillus*, *Fusarium* and *Penicillium* (Table 2.5). These fungal genera induce infections in a specific manner attacking specific organs and parts of the body (Vonberg and Gastmeier, 2006a).

Table 2.5: Infections induced by fungi species and organs they target

Fungi specie	Target organ	Disease induced	Reference
Aspergillus candidus	Respiratory tract, brain, ear and nails	Respiratory disease, otomycosis, onychomycosis, brain granuloma	(Ahmadi *et al.*, 2012; Ribeiro *et al.*, 2005)
Aspergillus flavus	Nails, respiratory tract, bone and eye	Sinusitis, keratitis, aspergillosis, osteomyelitis	(Hedayati *et al.*, 2007a; Zhang *et al.*, 2005)
Aspergillus fumigatus	Respiratory tract	Pulomonary infections	(LatgÉ, 2003)
Aspergillus niger	Ears, throat and respiratory tract	Otomycosis, pulmonary aspergillosis	(Georgiadou and Kontoyiannis, 2012)
Aspergilus versicolor	Nose, eyes, throat, nails,	Invasive aspergillosis, onychomycosis	(Benndorf *et al.*, 2008; Charles *et al.*, 2011)
Fusarium oxysporum	Eyes and Nails	Keratitis, onychomycosis	(Jain *et al.*, 2011)
Fusarium solani	Eyes, respiratory tract, nails, skin and bone	Keratitis, sinusitis, endophtalmitis, onychomycosis, cutenous infections, mycetoma and arthritis	(Esnakula *et al.*, 2013; Jain *et al.*, 2011)
Fusarium verticillioides	Eyes, skin, internal organs such as lungs, etc.	Keratomycosis, skin lesions, proliferation of internal organs	(Cocchi *et al.*, 2011; Georgiadou *et al.*, 2014; Mochizuki *et al.*, 2012)
Penicillium citrinum	Eyes and respiratory tract	Keratitis, asthma, pneumonia	(Mok *et al.*, 1997; Walsh *et al.*, 2004)
Penicillium marneffei	Blood, skin and respiratory tract	Fungemia, skin lesions, anaemia	(Walsh *et al.*, 2004)

2.6.1 *Aspergillus* species

Within the genus *Aspergillus*, over 20 species have been reported as causative agents of opportunistic infections in man. Apart from the production of mycotoxins, *Aspergillus* species are well-known to play a role in three different clinical settings in man: opportunistic infections, allergic states and toxicoses (Howard, 2003) with the most route of transmission of spores being through inhalation (Vonberg and Gastmeier, 2006a). *Aspergillus* species have been mentioned in association with a range of infections such as allergic bronchopulmonary aspergillosis (ABPA), aspergilloma, chronic necrotizing aspergillosis (CNA) and invasive pulmonary aspergillosis (IPA) generally referred to as aspergillosis. They usually affect the lungs and could also spread to other parts of the body. Aspergillosis usually affects people with existing health conditions, especially those with damaged lungs and suppressed immunity (Khan and Karuppayil, 2012) and the use of drugs and medications have contributed to reducing immunity even in healthy individuals. Common signs associated with aspergillosis include difficulty in breathing, increased thirst, diarrhoea and fever (Pattron, 2006). Out of all the species of genus *Aspergillus, A. niger, A. flavus, A. fumigatus, A. versicolor* and *A. nidulans* are reported to have the ability to induce disease and infection (Galimberti *et al.*, 2012). Even though *A. ochraceus* has the ability to produce mycotoxins, there is no report of this species inducing any form of infection in humans or animals.

2.6.1.1 *Aspergillus fumigatus*

Aspergillus fumigatus is the most commonly occurring aerial pathogen with life-threatening properties (Chaudhuri *et al.*, 2011). It is commonly isolated from blood and tissues of individuals exhibiting symptoms associated with *A. fumigatus* infection (Galimberti *et al.*, 2012; LatgÉ, 2003). *Aspergillus fumigatus* has been reported in association with a range of pulmonary infections. It has been reported to produce metabolites such as polypeptide allergens responsible for asthma and rhinitis (LatgÉ, 2003). It also produces mycotoxins such as

gliotoxins that have negative health effects on humans and animals as well as β−1,3 glucans that are known modulators of the immune system (Fisher *et al.*, 2000; Kupfahl *et al.*, 2008). *Aspergillus fumigatus* has been reported as a source of increased immune-suppression and possibly mortality in immuno-compromised individuals, with the characteristic of immune-suppression attributed to the production of the toxic metabolite gliotoxin during hyphal growth under specific favourable conditions (Kupfahl *et al.*, 2008). A recent study by Wartenberg *et al.* (2011) revealed the production of proteins and other allergens by *A. fumigatus*. It also has the ability to induce infections and diseases.

2.6.1.2 *Aspergillus flavus*

Aspergillus flavus, a commonly isolated *Aspergillus* species from soil and contaminated food commodities, is also a common fungal pathogen associated with a range of fungal infections. Aa a common causative agent in invasive aspergillosis and superficial infections, *A. flavus* has been associated with clinical syndromes such as chronic granulomatous sinusitis, keratitis, cutenous aspergillosis, wound infections and osteomyelitis (Hedayati *et al.*, 2007a; Zhang *et al.*, 2005). This fungal species has been reported to cause adverse health conditions in immuno-compromised individuals which could sometimes be fatal (Vonberg and Gastmeier, 2006a) and can be virulent in healthy individuals exposed to the spores. Li *et al.* (2009) reported a virulent case of severe infection at Huashan hospital, Fadan University, China which started as a rash and was consequently followed by swelling, purulent secretions, skin erosion and bleeding on the glans. This severe cutaneous aspergillosis had to be treated with an antifungal agent and subsequent plastic surgery to close the wound.

2.6.1.3 *Aspergillus versicolor*

This slow growing *Aspergillus* species is commonly found in damp indoor environments and food products (Engelhart *et al.*, 2002). Apart from the production of hepatoxic and carcinogenic

mycotoxin sterigmatocystin (Fog Nielsen, 2003), this opportunistic pathogen has been reported to contain more than 20 allergens and it irritates the nose, eyes and throat (Benndorf *et al.*, 2008). *Aspergillus versicolor* has been reported to be another causative agent of aspergillosis with the fungi being a major cause of onychomycosis ,a fungal infection of the nails (Torres-Rodriguez" *et al.*, 1998). *Aspergillus versicolor* is also a causative agent of invasive aspergillosis as shown in a case study by ,Charles *et al.* (2011). An immuno-competent patient on mechanical ventilation support was reportedly diagnosed to have invasive pulmonary aspergillosis due to *A. versicolor* which eventually culminated in the death of the patient due to treatment failure.

2.6.1.4 *Aspergillus candidus*

Aspergillus candidus, a common contaminant of grain dust and common producer of potent cytotoxic substances, p-terphenyl metabolites and terpenins (Krysinska-Traczyk and Dutkiewicz, 2000; Shahan *et al.*, 1998) is reported to be one of the causes of respiratory diseases in humans. This fungus has been identified as a potential respiratory hazard for individuals who are constantly exposed to it, even in immune competent individuals. A range of infections have been attributed to this *Aspergillus* species which includes invasive aspergillosis, otomycosis, brain granuloma, onychomycosis, allergic alveolitis and mycotoxicosis (Ahmadi *et al.*, 2012; Ribeiro *et al.*, 2005)

2.6.1.5 *Aspergillus niger*

With the ability to grow on a wide variety of substances, *A.niger* is a common contaminant of food, soil and indoor environment. Although its spores are widespread, the fungus has been reported to be a less likely cause of human diseases compared to other *Aspergillus* species (Person *et al.*, 2010). *Aspergillus niger* normally invades tissues which have already been rendered susceptible by bacterial infections, physical injury or accumulation of cerumen in the

external auditory canal. Along with other *Aspergillus* species, *A. niger* is another causative agent of otomycosis, a superficial fungal infection in the ear, throat or nose that can be sub-acute or chronic. As is the case with most filamentous fungal species, immune compromised individuals are also susceptible to *A. niger* infections. In most cases, they cause invasive pulmonary aspergillosis (Georgiadou and Kontoyiannis, 2012; Person *et al.*, 2010) characterised by chronic productive cough and coughing up blood.

2.6.2 *Fusarium* species

This genus of filamentous fungi which contains over fifty species commonly found in the soil and in association with plants such as cereals and grains, is a causative agent of superficial and systemic infections. The fungi can infect humans and animals and infection can be through inhalation of air-borne conidia and through cuts in the skin (Georgiadou *et al.*, 2014). In addition to the fact that it causes a range of opportunistic infections, some *Fusarium* species produce mycotoxins which affect human and animal health if they enter the food chain. Infections caused by *Fusarium* species are generally referred to as fusariosis which is largely dependent on the immune status of the host and the route of entry of the infection (Nucci and Anaissie, 2002; Nucci and Anaissie, 2007b).

Among immunocompetent hosts, the common *Fusarium* infections are keratitis and onychomycosis with other less common conditions such as sinusitis, pneumonia, thrombophlebitis and fungemia (Nucci and Anaissie, 2007b). With immune-compromised individuals, those at high risk of fusariosis are those with prolonged and profound neutropenia as well as T-cell immunodeficiency (Consigny *et al.*, 2003). It is not all species of the genus that possess the ability to induce disease or infection with only a few causing infections such as *F. verticilliodes*, *F. solani* complex, *F. oxysporum* and *F. proliferatum*, with *F.solani* being the most frequent (Nucci and Anaissie, 2007b).

2.6.2.1 *Fusarium verticilliodes*

Commonly contaminating maize, rice, other cereals and grains, *F. verticillioides* is one of the producers of the mycotoxin fumonisin which has been reported to have adverse negative health effects including oesophageal cancer (Chu and Li, 1994; Craddock, 1992; Sydenham *et al.*, 1990). This human pathogen is a common causative agent of infection in immune-compromised individuals than in immune-competent individuals. This is because one of the important predisposing factors to *F. verticillioides* infection is severe prolonged neutropenia (Cocchi *et al.*, 2011; Georgiadou *et al.*, 2014). Infection by *F.verticilliodes* has been reported to occur in immune-compromised individuals who have undergone major organ re-transplant due to rejections by their bodies (Cocchi *et al.*, 2011; Consigny *et al.*, 2003; Tezcan *et al.*, 2009).

Another infection induced by *F. verticillioides* is *Fusarium* keratomycosis, a fungal infection of the cornea characterised by red and painful eyes as well signs of ulcers (Mochizuki *et al.*, 2012). Symptoms associated with *F. verticillioides* infections include necrotic lesions on the skin, mostly on the legs, fever and endothelial proliferation of internal organs (Cocchi *et al.*, 2011; Georgiadou *et al.*, 2014; Kriek *et al.*, 1981; Tezcan *et al.*, 2009).

2.6.2.2 *Fusarium solani*

The most virulent of all *Fusarium* species and often reported in relation to fusariosis, *F. solani* is a *Fusarium* species that commonly occurs in the environment (O'Donnell *et al.*, 2004; Zhang *et al.*, 2006). *Fusarium solani*, which also acts as a plant pathogen, has been reported to induce a range of diseases and infections in both immune-competent and immune-compromised individuals. These diseases include keratitis, endophtalmitis, onychomycosis, cutaneous infections, sub-cutaneous infections, arthritis, mycetoma, sinusitis and disseminated infections in immune-compromised individuals (Esnakula *et al.*, 2013; Jain *et al.*, 2011). Although a

variety of *Fusarium* species are causative agents of mycotic keratitis, *F. solani* is frequently reported compared to other species (Howard, 2003; Zhang *et al.*, 2006). Cases of fungal infections such as mycotic keratitis due to *F. solani* were reported as far back as the 1970s (Gugnani *et al.*, 1976) with individuals developing corneal ulcers. Symptoms of *F. solani* infections include ulcers, fevers, skin lesions and organ membrane disruption.

2.6.2.3 *Fusarium oxysporum*

Fusarium oxysporum, which comprises all the species, varieties and forms within a group called section *Elegans*, is a highly ubiquitous *Fusarium* species. It has the ability to survive in different environments such as deserts, tropical and temperate forests as well as grasslands (Gordon, 1997). Strains of this fungi species have been classified as non-pathogenic, human pathogenic and plant pathogenic (Howard, 2002; Roncero *et al.*, 2003). The human pathogenic strains of *F. oxysporum* are known to cause infections in both humans and animals whether immune-competent or immune-compromised (Jain *et al.*, 2011). Infections such as keratitis, onychomycosis are some of the infections associated with *F.oxysporum* as well as localised necrotic diseases (Chithra *et al.*, 2008; Jain *et al.*, 2011).

2.6.3 *Penicillium* species

Penicillium species are diverse and widely distributed in the environment. Despite their abundance and diversity in the environment, they are not commonly associated with human and animal infections (Mok *et al.*, 1997). Some of the few pathogenic species affecting plants, *P. citrinum*, *P. chrysogenum*, *P. digitatum*, *P. expansum* and *P. marneffei* are commonly associated with humans and animals with mode of infection mostly through inhalation and sometimes ingestion (Imwidthaya *et al.*, 2001; Walsh *et al.*, 2004). Diseases resulting from *Penicillium* infection are generally referred to as Penicilliosis. Species of this genus have been

mentioned in association with infections such as keratitis, endophtalmitis, otomycosis, pneumonia, endocarditis and urinary tract infections (Deshpande and Koppikar, 1999).

2.6.3.1 *Penicillium citrinum*

Considered as one of the commonly occurring *Penicillium* species, this fungus is a plant pathogen as well as a human and animal pathogen. The fungus also produces the mycotoxins, ochratoxin and citrinin, which has been reported in association with some fungal infections and other diseases such as keratitis, asthma and pneumonia (Mok *et al.*, 1997; Walsh *et al.*, 2004) which in some cases, may be fatal.

2.6.3.2 *Penicillium marneffei*

This fungus is the third most common opportunistic pathogen and attacks individuals suffering from acquired immune deficiency syndrome (AIDS) especially in areas where it is endemic (Imwidthaya *et al.*, 2001; Mok *et al.*, 1997). *Penicillium marneffei* is also a common fungus that affects individuals with haematological malignancies as well as individuals receiving immune-suppressive therapy when exposed to it. In areas such as Southeast Asia, this fungus has continued to cause morbidity and mortality in human immunodeficiency virus (HIV) positive patients who are either unaware of their status or unable to take anti- retroviral therapy (Walsh *et al.*, 2004). Individuals exposed to *P. marneffei* infections exhibit clinical manifestations such as weight loss, skin lesions, fungemia, pulmonary lesions, anaemia, cough and low-grade fever (Walsh *et al.*, 2004).

2.6.3.3 Other less common pathogenic *Penicillium* species

Penicillium digitatum, *P. expansum* and *P. chrysogenum* are some of the other less common pathogenic fungi belonging to the genus *Penicillium*. Although they are not frequently mentioned in relation to penicilliosis, these fungal species cause infections in human that could be fatal. *Penicillin digitatum*, which is a post-harvest plant pathogen, was reported in

association with fatal pneumonia (Oshikata *et al.*, 2013). *Penicillium chrysogenum* and *P. expansum* have been reported to be causative agents of necrotising esophagitis, endophthalmitis, keratitis and asthma (Walsh *et al.*, 2004).

2.7 Cytotoxicity induction by filamentous fungi

Cytotoxicity is the ability of a compound or substance to induce toxicity on cells. Many compounds are known to be cytotoxic to both human and animal cells thereby inducing reduction in cell viabilities, apoptosis or complete cell death (necrosis). Fungi and their metabolites are not left out in the group of cytotoxic compounds, especially to humans and animals. Cytotoxic actions of fungi isolates are reported to be either beneficial or a health risk. The beneficial cytotoxic effects of fungi isolates can be considered when these fungi act as endophytes, inhibiting the pathogenic effects of other fungi species or bacteria in plants or food crops (Suryanarayanan *et al.*, 2000). Another beneficial cytotoxic effect of fungi is the ability of some of these species to act as anti-tumour and anti-cancer agents thereby inhibiting the uncontrolled proliferation of cancer cells (Arivudainambi *et al.*, 2014; Gangadevi and Muthumary, 2007; Sudarmono *et al.*, 2006). Some species belonging to the genus *Fusarium* have been reported to induce cytotoxicity in various cancer cell lines including HCT-116 cells (Human colon carcinoma), MCF-7 cells (breast cancer cell), PC-3 cells (prostate cancer cell), A-549 cells (human alveolar basal epithelial cells), HeLa cells and HepG2 cells (Chakravarthi *et al.*, 2013; Katoch *et al.*, 2014).

The negative cytotoxic effects of fungal species have been recorded in some studies involving normal human and animal cell lines. Species belonging to the *Aspergillus* genus such as *A. fumigatus, A. niger, A. flavus* and *A. ochraceus* induce reduced cell viability and cell death in cell lines exposed to them (Gniadek *et al.*, 2011; Kamei *et al.*, 2002). Aflatoxins, ochratoxins, gliotoxin and other mycotoxins which are produced by these *Aspergillus* fungi, induce reduced

cell viabilities in both human and animal cells at varying concentrations (Heussner *et al.*, 2006; Hussein and Brasel, 2001; Lei *et al.*, 2013; Stoev *et al.*, 2009). *Fusarium* species and their metabolites also induce cytotoxicity on both human and animal cell lines. Studies conducted by Abeywickrama and Bean (1992), Hameed *et al.* (2009) and Langseth *et al.* (1999) reveal the cytotoxic effects of *F. culmorum*, *F. acuminatum*, *F. graminearum*, *F. solani*, *F. equiseti*, *F. poae*, *F. sporotrichioides* and *F. tricinctum* on different mammalian cell lines inhibiting cell viability and even causing cell death. The cytotoxic effects of *Fusarium* species could be attributed to the secondary metabolites they produce during the course of their growth in response to stimuli. Mycotoxins such as FB_1, zearalenone, enniatins, T-2 toxin and trichothecenes produced by *Fusarium* species exert cytotoxic effects on human and animal cells at varying concentrations and duration of exposure (Abbas *et al.*, 2013; Cetin and Bullerman, 2005; Gutleb *et al.*, 2002; Juan-García *et al.*, 2013; Wan *et al.*, 2013). The cytotoxic effects of *Penicillium* species are not as common as other genera of fungi mentioned although a few *Penicillium* species have shown in past that they have the tendency to inhibit cell proliferation and cell viability. Shah *et al.* (2014) examined the cytotoxic effects of *P. verrucosum* on normal and cancer cells, resulting in reduced cell viabilities of normal cells and inhibiting cell proliferation in cancer cells. A study conducted by Geiger *et al.* (2013) revealed that *Penicillium* species isolated from shell-fish were able to exert cytotoxic effects on cell lines when exposed thus posing a health concern for consumers of the product. *Penicillium* species also produce the mycotoxins: ochratoxin A, citrinin, patulin and penicillinic acid, which have cytotoxic effects on mammalian cell lines resulting in reduced cell viability depending on the concentration and duration of exposure (Mwanza *et al.*, 2009; Oh *et al.*, 2012; Stoev *et al.*, 2009).

2.8 Immunosuppression by filamentous fungi

Immunosuppression, which is an act that brings about a reduction of the efficacy of the immune system, occurs in two ways; deliberate immunosuppression where the activity of the immune system is reduced by use of immunosuppressive drugs or immunosuppressant and in deliberate immunosuppression whereby the efficacy of the immune system is reduced due to factors such as aging, disease, malnutrition and infection (Abbas and Lichtman, 2010). Due to the different health effects of these filamentous fungi species and the metabolites they produce, this group of micro-organisms contribute in reducing the efficacy of the immune system of mammals upon continuous exposure.

The subsequent effect of cytotoxicity induction by fungal species on cells and its components can result to immunosuppression (Kamei *et al.*, 2002). Corrier (1991) maintains that filamentous fungal metabolites achieve reduced immune activity in cells by suppressing T and B lymphocyte activity, immunoglobulin production and anti-body production. They also reduce interferon activity and impair macrophage effect or cell functions. Furthermore, a study by Pahl *et al.* (1996) revealed that gliotoxin produced by *A. fumigatus* could promote immunosuppression by interfering with the activation of transcription factors involved in T cell activation. Fontaine *et al.* (2011) reported that *A. fumigatus* secretes a polysaccharide Galactosaminogalactan (GG) which favours aspergillosis by inducing neutrophil apoptosis. The neutrophils which act as pathogen destroyers, are killed by the secretion of this polysaccharide thus resulting in immunosuppression in the host.

2.9 DNA damage by filamentous fungi species

The DNA is the molecule which encodes all the genetic information necessary for proper development and functioning of a living organism. It can be damaged due to alterations in the

chemical structure of the DNA in the form of a break in the strand, a chemically changed base or a missing base in the backbone. Alterations in the DNA of a living organism can result in mutations in the cell and genomic instability (altered gene functions and expressions) which in the long run, can contribute to progression of cancer in cells (Tudek *et al.*, 2010). Filamentous fungi are responsible for DNA damage alongside chemical carcinogens, metabolites, ultraviolet radiation and polycyclic aromatic carbons. Mycotoxin production by filamentous fungi is a possible indication that these group of micro-organisms are causative agents of DNA damage in living organisms. This assumption is supported with previous studies showing the ability of mycotoxins produced by filamentous fungi such as *Aspergillus*, *Fusarium* and *Penicillium* to induce DNA damage both in human and animal cells (Fehr *et al.*, 2010; McLean and Dutton, 1995; Oh *et al.*, 2012; Wang and Groopman, 1999).

A major reason for DNA susceptibility to damage by mycotoxins is due to the nucleophilic hetero-atoms in the organic-bases of nucleic acids (such as nitrogen and oxygen atoms) which are susceptible to attacks by mycotoxins forming covalent bonds with them (McLean and Dutton, 1995). The association between DNA and mycotoxins result to the formation of DNA adducts which impair DNA synthesis and eventually increase the activation of oncogenes (McLean and Dutton, 1995; Pfohl-Leszkowicz *et al.*, 2009; Wang and Groopman, 1999). Aflatoxin B_1, ochratoxin A, sterigmatocystin and zearalenone, among other mycotoxins produced by species of *Aspergillus*, *Fusarium* and *Penicillium* genera have been reported to bind to DNA in the cells forming DNA adducts thus resulting in the activation of oncogene formation, inhibition of DNA synthesis, disruption of normal DNA replication and DNA polyploidy in cells (Egner *et al.*, 2001; Wang and Groopman, 1999). A less commonly occurring species, *Alternaria alternata* has also been reported to be associated with human DNA damage. It does this by producing the mycotoxin alternariol, which interferes with the human DNA topoisomerase (enzymes that regulate excessive winding or insufficient winding

of DNA) resulting to instability in DNA and subsequently, DNA double strand break (Fehr *et al.*, 2010).

2.10 Presumed synergistic effects of fungi

Filamentous fungi commonly occur in the environment and it is always possible to find more than one species in a particular place at a time. The possibility of synergistic health effects has become important considering the fact that these fungi species exert acute and chronic health effects individually. Several studies have reported the synergistic effects of metabolites of filamentous fungi on human and animal cell lines. Studies by Mwanza *et al.* (2009), Stoev *et al.* (2009) and Creppy *et al.* (2004) revealed that a combination of FB_1 produced by *F. verticillioides* and OTA produced mainly by *A. ochraceus* and *P. viridicatum* induced a great drop in cell viability of phytohaemagglutinin-p (PHA) and stimulated human and swine blood lymphocytes compared to individual effects of the metabolites. A mixture of AFB_1 and DON as well as AFB_1 and ZEA resulted in synergistic cytotoxic effects on porcine kidney cells on a dose-dependent ratio (Lei *et al.*, 2013). Another study by Wan *et al.* (2013) and Ruiz *et al.* (2011) revealed synergistic cytotoxic effects of *Fusarium* toxins: DON, ZEA, FB1, NIV, T-2 toxin and BEA) mixtures on normal swine jejunal epithelial cells and hamster ovarian cells resulting in loss of cell viability.

2.11 Future prospects

It is imperative that research on infections caused by filamentous fungi species be conducted in order to understand the molecular nature of the micro-organisms. This is because most fungal infections are treated using antibiotics and sometimes, in the long run, develop resistance to drugs and probably lead to death of the host (Charles *et al.*, 2011). There is limited knowledge on the molecular pathway of infections caused by most pathogenic fungi even though much is

known about the metabolites they produce. Further knowledge on the molecular pathway of infections, including genes expressed and enzymes activated by these fungi could contribute to the design and manufacture of drugs. Furthermore, the presence of more than one species occurring at a particular environment at a time, could lead to co-infections by different species and cause more adverse effects since they are already pathogenic individually. Knowledge of the genes expressed due to interaction of these fungi species and enzymes expressed could contribute to combating the scourge of fungal infections. It is therefore recommended that further research be conducted on co-infections by filamentous fungi species in order to examine the molecular nature of the fungi at time of infection and to determine which genes or enzymes to target when manufacturing drugs to combat fungal infections.

2.12 Conclusion

Considering the wide distribution and economic importance of filamentous fungi discussed in this review, it is evident that filamentous fungi are both beneficial and detrimental to the human race. Therefore, research work should be centred on exploiting the different uses of this group of diverse organisms. Also, there is a need to address their negative health effects as well as carry out studies to investigate various ways of reducing their harmful impacts. The impression that infections caused by this group of micro-organisms seldom occur should be re-considered because these filamentous fungi cause an alarming number of detrimental infections that should not be overlooked.

CHAPTER THREE

Production of mycotoxins by filamentous fungi at different growth stages

Abstract

The fungal species used to determine synergistic toxic effects of filamentous fungi on human cells were screened for production of mycotoxin as part of the study. *Aspergillus flavus*, *A. niger*, *Fusarium oxysporum*, *F. verticillioides*, *Penicillium chrysogenum* and *P. expansum* were used. In other to investigate production of metabolites by the micro-organisms when cultured at different incubation periods, using PDA (potato dextrose agar) and MEA (malt extract agar) were used. Isolates were cultured for 4, 9 and 14 days at 25 ^{o}C in the dark and extracted with methanol and methanol/formic acid (25:1, v/v) solution. High performance liquid chromatography (HPLC) and thin layer chromatography were used to quantify and identify mycotoxins present in the extracts. Mycotoxins detected in the different fungal extracts included aflatoxin B_1 (0.3-11.6 µg/g), aflatoxin B_2 (0.01-4.2 µg/g), aflatoxin G_1 (0.07-0.7 µg/g), aflatoxin G_2 (0-1.4 µg/g) produced by *A. flavus*; ochratoxin A ($8.63*10^{-6}$ µg/g) produced by *A. niger* and fumonisin B_1 (5.0-114.62 µg/g), deoxynivalenol (0-0.15 µg/g) and nivalenol (358.15-1035.27 µg/g) produced by *Fusarium* species. Fungi species did not produce patulin, ZEA and T-2 toxin. The production of deoxynivalenol by *F. oxysporum* isolates emerged as a novel finding of the study. This might be explained by possible mutations in the isolate due to several factors influenced by environmental conditions.

Keywords: *Fusarium*, *Penicillium*, *Aspergillus*, Fumonisins and HPLC

3.1 Introduction

Secondary metabolism in micro-organisms which normally occur at the stationary or resting phase of growth, a means by which the organism adapts to its environment (Schmidt-Heydt *et al.*, 2008). The production of secondary metabolites by micro-organisms (bacteria and fungi) which is associated with developmental processes (Calvo *et al.*, 2002) normally occur at sporulation stages of microbial growth. Filamentous fungi, produce secondary metabolites such as flavonoids (Araújo *et al.*, 2013), terpenes (Kramer and Abraham, 2012), antibiotics (Funa *et al.*, 2007) and mycotoxins (Mounjouenpou *et al.*, 2008; Taniwaki *et al.*, 2009) which are usually produced during or after sporulation stage of growth. (Calvo *et al.*, 2002). Filamentous fungi begin to sporulate at 2 to 4 days of incubation, it can be assumed that production of metabolites by this group of fungi begins from this period and continues for as long as conditions are favourable.

Mycotoxins, produced by filamentous fungi are chemical compounds that have more negative effects in nature affecting humans, animals and plants/crops (Richard, 2007), thus they are reported to be associated with immune-suppression, inflammation, cancers and hormonal imbalance (Bennett and Klich, 2003; Bennett and Klich, 2009; Marin *et al.*, 2013; Mwanza *et al.*, 2009; Shephard, 2008). One of such mycotoxins is aflatoxin produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxins, especially aflatoxin B_1 shown in Figure 3.1 is classified as a group 1 human carcinogen by the International Agency for Research on Cancer (IARC, 1993a-b) because of its association with liver and kidney abnormalities as well hepatitis in both humans and animals .

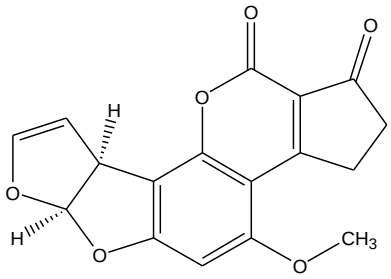


Figure 3.1: Chemical structure of aflatoxin B_1

Production of mycotoxins by filamentous fungi in most cases, is reported to be influenced by climatic conditions such as temperature and humidity as well as other factors such as pests, improper handling of crops/processed foods, improper storage and transportation (Picot *et al.*, 2010; Wagacha and Muthomi, 2008). Also, mycotoxin production by filamentous fungi is specific to certain species. Regardless of the specificity of the mycotoxin produced, it is common to find one mycotoxin being produced by two or more species, as well as one filamentous fungi species producing more than one mycotoxin. For example, *A. flavus* and *A. parasiticus* produce aflatoxins while *A. niger* can produce two mycotoxins citrinin (Vahidnia and Chaichi-Nosrati, 2014) and ochratoxin A (Figure 3.2) (Abarca *et al.*, 1994; Ostry *et al.*, 2013).

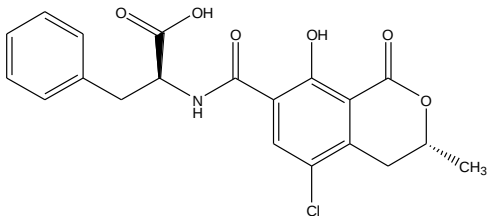


Figure 3.2: Chemical structure of ochratoxin A

Laboratory culture of filamentous fungi on different biological growth media have also shown that the degree of mycotoxin production varies with the media (Fakruddin *et al.*, 2015; Marino *et al.*, 2014; Vismer *et al.*, 2004). Based on the aforementioned, the aim of this study was thus

to determine the degree of production of mycotoxins by some selected filamentous fungi in order to determine their degree of toxicity.

3.2 Materials and methods

3.2.1 Fungal isolates

Three filamentous fungal genera (*Aspergillus*, *Fusarium* and *Penicillium*) isolated from food samples (maize) were used for the analysis. The fungal isolates were obtained from the culture collection centre of Food, Environment and Health Research Group (FEHRG), Faculty of Health Sciences, University of Johannesburg.

3.2.2 Mycotoxin standards

Mycotoxin standards: aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) & G_2 (AFG_2); ochratoxin A (OTA); Fumonisin B_1 (FB_1); Patulin (PAT); Zearalenone (ZEA); Nivalenol (NIV); T-2 toxin and Deoxynivalenol (DON) were procured from Sigma Aldrich, South Africa.

3.2.3 Chemical solvents

All solvents used were of analytical grade and procured from Sigma Aldrich, South Africa and Merck Pty Ltd, South Africa.

3.2.4 Culture of filamentous fungi

For Molecular identification of filamentous fungi species, two types of growth media were used- Potato dextrose broth (PDA) and malt extract broth (MEA) for 4, 9 and 14 days at 30^{o}C. Fungal species were also cultured for mycotoxin production on Potato dextrose Agar (PDA) and malt extract agar (MEA) at 30^{o}C for the same three periods of 4, 9 and 14 days. Fungal spores were harvested for metabolite extraction and mycelium collected for DNA extraction. All treatments and analyses were done in triplicates.

3.2.5 DNA extraction and PCR analysis

To confirm the species of reference isolates, Deoxyribose Nucleic Acid (DNA) was extracted from mycelia of fungal isolates with a Zymo DNA extraction kit (Inqaba Biotec, South Africa). Mycelia of fungal species were lysed in tubes containing beads with lysing solution. Fungal DNA was subsequently extracted and amplified using a Polymerase chain reaction (PCR) mastermix and primer pair FF2/FRI. The FF2 forward primer (5′-GGTTCTATTTTGTTGGTTTCTA-3) and FR1 reverse primer (5′-CTCTCAATCTGTCAATCCTTATT-3′) (Zhou *et al.*, 2000) were procured from Inqaba Biotec, South Africa. Polymerase chain reaction was done with a C-1000 Bio-Rad thermal cycler. Initial denaturation of fungal DNA at 95^{o}C for 3 mins was followed by 39 cycles of a three-step denaturation at 94^{o}C for 1 min, primer reannealing at 52^{o}C for I min and extension at 72^{o}C for 2mins with further extension for 10mins at 72^{o}C. PCR products were sequenced at Inqaba Biotec, South Africa.

3.2.6 Extraction of mycotoxins from fungal isolates

This was done following modified method of Marino *et al.* (2014). Agar plugs of spores weighing between 0.8g and 1.2g of PDA and MEA cultured isolates from the six reference species (*A. niger*, *A. flavus*, *F. oxysporum*, *F. verticilliodes*, *P. chrysogenum* and *P. expansum*) were collected at 4, 9 and 14 days of growth. The plugs were collected from triplicate cultures per isolate and transferred to 2ml pre-weighed amber vials. In total, for each period of growth, 18 samples were collected per culture media used. Two (2) millilitres of methanol (CH_4O) was added to the fungal spores of all isolates with the exception of the vial containing *A. niger*, to which 2 ml of methanol/formic acid ((25:1) (v/v)) was added. Fungal plugs were allowed to stand in methanol for 2 to 3 h, after which the fungal spores in solvent were filtered with a sterile 0.22µm syringe filter. The filtrates were allowed to dry and fungal extracts re-dissolved

in 1ml methanol were stored for quantification by high performance liquid chromatography (HPLC).

3.2.7 High performance liquid chromatography (HPLC)

Quantification of mycotoxins was done with a Shimadzu HPLC equipment fitted with a DGU-20A 5R degassing unit, a SIL-20A HT prominence autosampler and a CTO-20A. Aflatoxins, ochratoxin A, zearalenone and fumonisin were detected using a fluorescence detector (Egbuta *et al.*, 2013) and the other mycotoxins, deoxynivalenol, citrinin, T-2 toxin, patulin and nivalenol) (Aktaş *et al.*, 2004; Makun *et al.*, 2011) were detected using a photo diode array detector. Following the method described by Abdulkadar *et al.* (2004) with some modifications, samples were post-derivatised using a kobra cell for the detection of aflatoxins Detection of fumonisin was done following the method described by Phoku *et al.* (2012) and (Shepherd *et al.*, 1994)with pre-column derivatisation using OPA (ortho-phtalaldehyde). Ortho-pthaladehyde was prepared by dissolving 40mg of OPA in 1ml methanol, and diluted with 5ml 0.1 mol/L disodium tetraborate ($Na_2B_4O_7$) and 50µl of 2-mercaptoethanol added to the solution. Peak areas of mycotoxins detected were recorded and concentration of detected mycotoxins calculated, using the equation generated from calibration curve of standard mycotoxins used in the analysis.

3.2.8 Thin layer chromatography (TLC)

Further TLC analysis was performed using aluminium backed silica gel plates in specific mobile phases for mycotoxins. Following the methods described by Egbuta *et al.* (2015a), 20µl of fungal extracts were spotted on evenly cut TLC plates (20cm by 20cm) and placed in TLC tanks containing mobile phases and run. After each run, plates were dried and viewed at between 250 and 365 nm using a Spectroline CM-10A fluorescence analysis cabinet (UV instrument) manufactured by Spectronics Corporation, Westbury, New York, USA.

Compounds such as deoxynivalenol, nivalenol and fumonisins which did not fluorescence under UV were further derivatised to enhance fluorescence. Deoxynivalenol and nivalenol were derivatised using 20% Aluminium chloride in methanol whereas, fumonisins were derivatised with prepared anisaldehyde solution. Derivatization was followed by heating plates in an oven between 120^{o}C and 180^{o}C. Plates were viewed under UV light for deoxynivalenol and nivalenol detection, while fumonisins were visible after heating in the oven.

3.2.9 Statistical analysis

Data generated from hplc was analysed using Microsoft Excel, 2010. Mean mycotoxin concentrations were calculated and results presented.

3.3 Results

Analysis using High Performance Liquid Chromatography revealed that some filamentous fungi species used in this study produced mycotoxins. Table 3.1 summarises mycotoxins production by fungal species at varying concentrations when cultured on PDA and MEA for the different periods. *Aspergillus flavus* species cultured on MEA produced highest concentrations of AFB1 after 9 days of growth which was the same as cultures on PDA. Figure 3.3 presents a graphical explanation of the degree of AFB1, AFB2, AFG1 and AFG2 by *A. flavus* cultured on both PDA and MEA.

Table 3.1: Mycotoxin production by fungi species cultured on PDA and MEA determined by HPLC

Mycotoxins	**Filamentous fungi**					
	A. flavus	***A. niger***	***F. oxysporum***	***F. verticillioides***	***P. chrysogenum***	***P. expansum***
Aflatoxins	+	-	-	-	-	-
Ochratoxin A	-	+	-	-	-	-
Fumonisins	-	-	+	+	-	-
Zearalenone	-	-	-	-	-	-
Deoxynivalenol	-	-	+	+	-	-
Nivalenol	-	-	+	-	-	-
T-2 toxin	-	-	-	-	-	-
Patulin	-	-	-	-	-	-

+: positive, -: negative

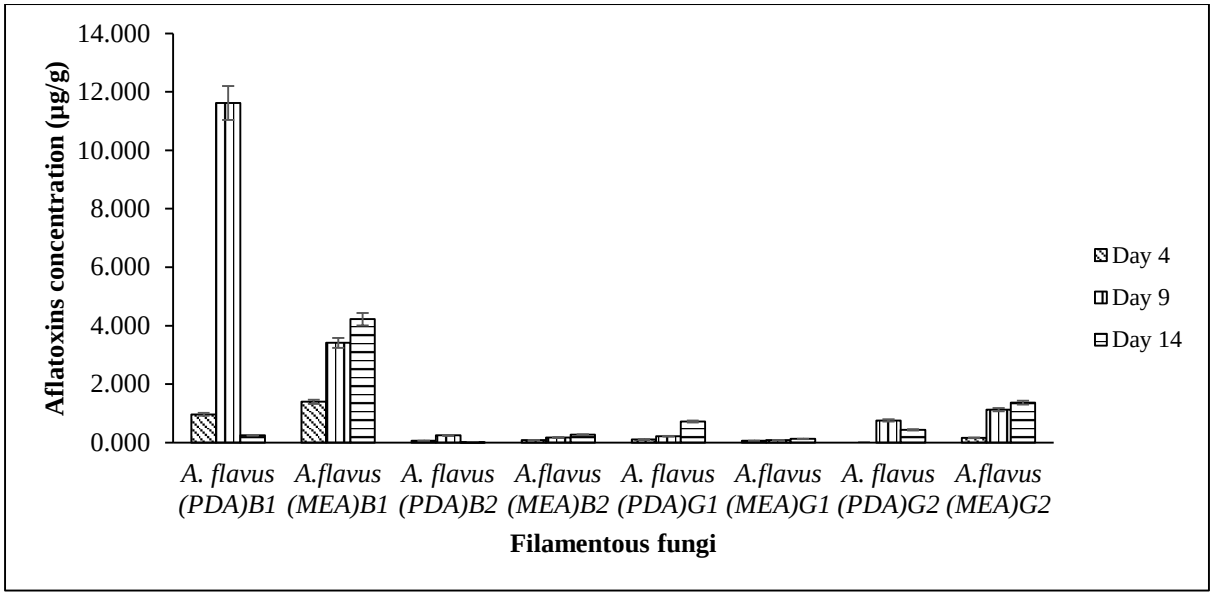


Fig. 3.3: Production of aflatoxins by *Aspergillus flavus* cultured for 4, 9 and 14 days at 30°C

Extracts from *F. verticilliodes* and *F. oxysporum* contained varying concentrations of deoxynivalenol (Fig 3.4) and fumonisin B_1 (Fig. 3.5) and extracts from *F. verticilliodes* isolates showing production of nivalenol (Fig. 3.6). There was an increase in FB_1 production by *F. verticillioides* after 9 days of growth on PDA and 14 days of growth on MEA. *Fusarium oxysporum* also produced high concentration of FB_1 (114µg/g) after 4 days of incubation on MEA. Production of DON by *F. verticillioides* on MEA increased on the 14th day of growth but was not the same for isolates cultured on PDA which showed more production of DON after 9 days of growth with a decline in production at 14 days of growth. Production of nivalenol was detected in fungal extracts from *F. verticillioides* isolates cultured on both growth medium with production of NIV high at 4 and 9 days of growth on both MEA and PDA respectively.

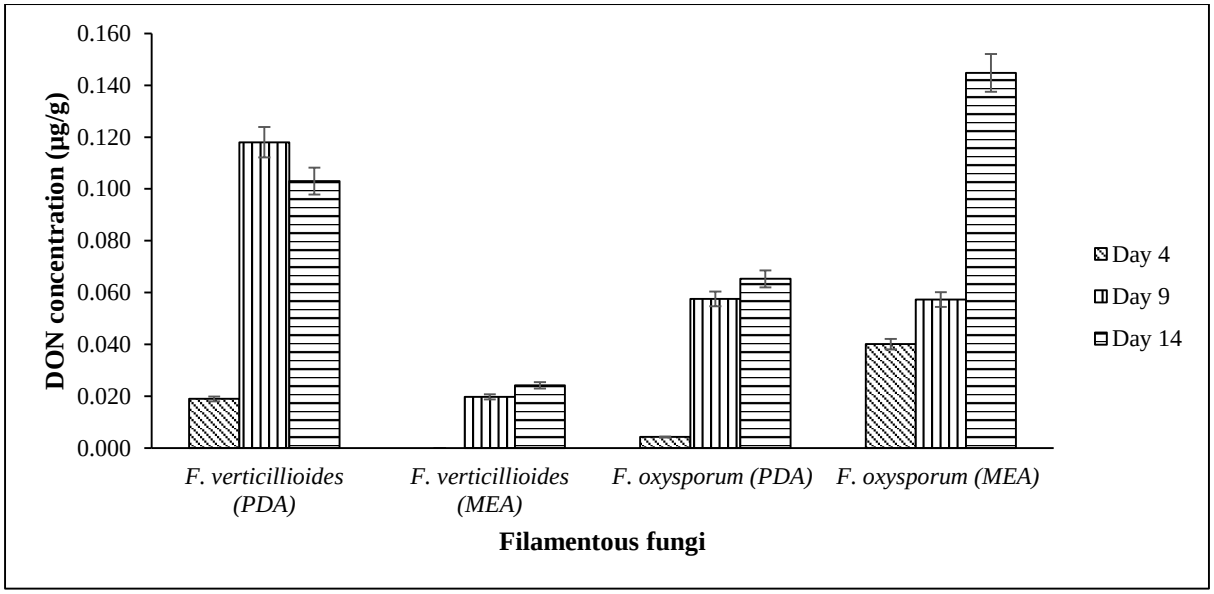


Figure 3.4: Influence of media on production of deoxynivalenol by *Fusarium verticillioides* and *Fusarium oxysporum* cultured for 4, 9 and 14 days at 30ºC

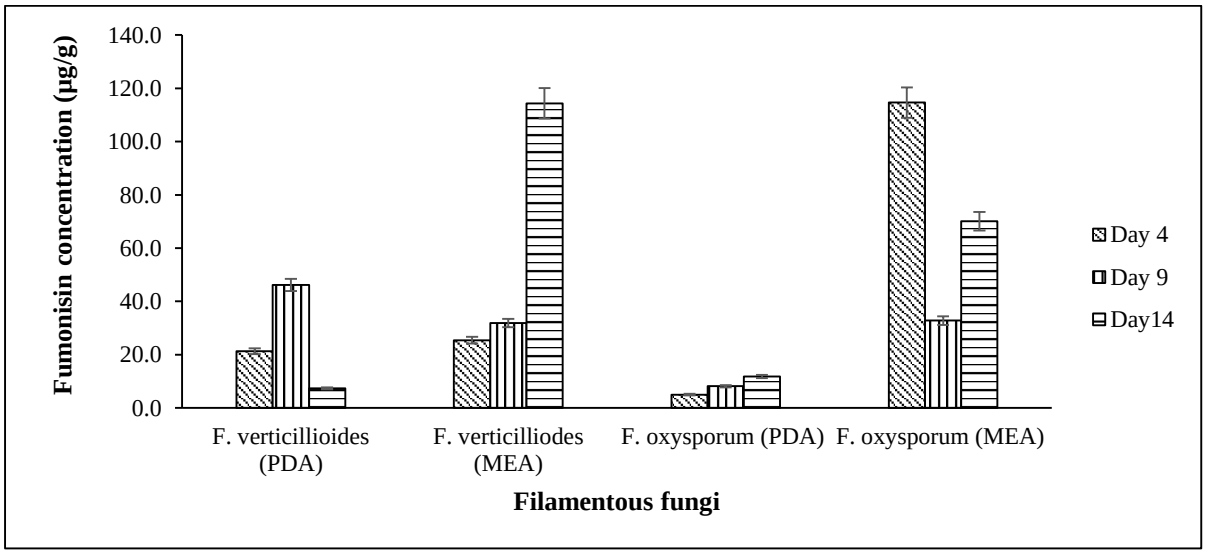


Figure 3.5: Influence of media on production of fumonisin B1 by *Fusarium verticillioides* and *Fusarium oxysporum* cultured for 4, 9 and 14 days at 30ºC

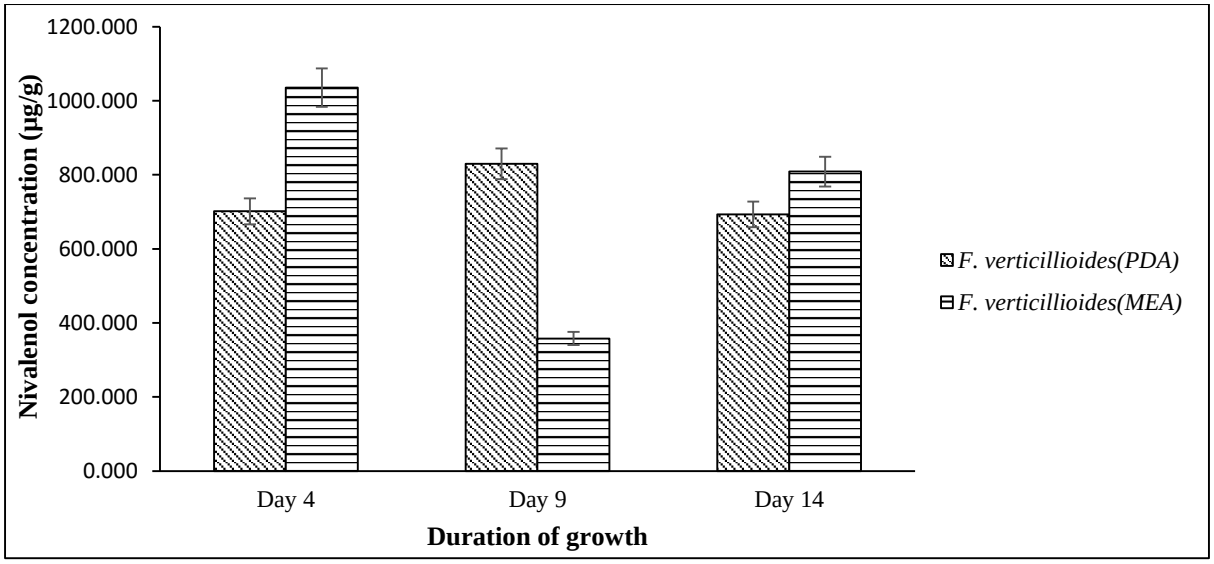


Figure 3.6: Influence of media on production of nivalenol by *Fusarium verticillioides* cultured for 4, 9 and 14 days at 30°C

Methanol extracts from reference *Fusarium* isolates were analysed for zearalenone but none was positive on both growth media. Extracts of *A. niger* were screened for ochratoxin A (OTA) and citrinin production. There was very little production of OTA by *A. niger* on both PDA and MEA (Fig 3.7), although more concentration of OTA was produced by isolates cultured on the latter growth media after 14 days of growth. There was no production of citrinin by cultures of *A. niger* at the different growth periods. Furthermore, *Penicillium* isolates did not produce any mycotoxin screened for.

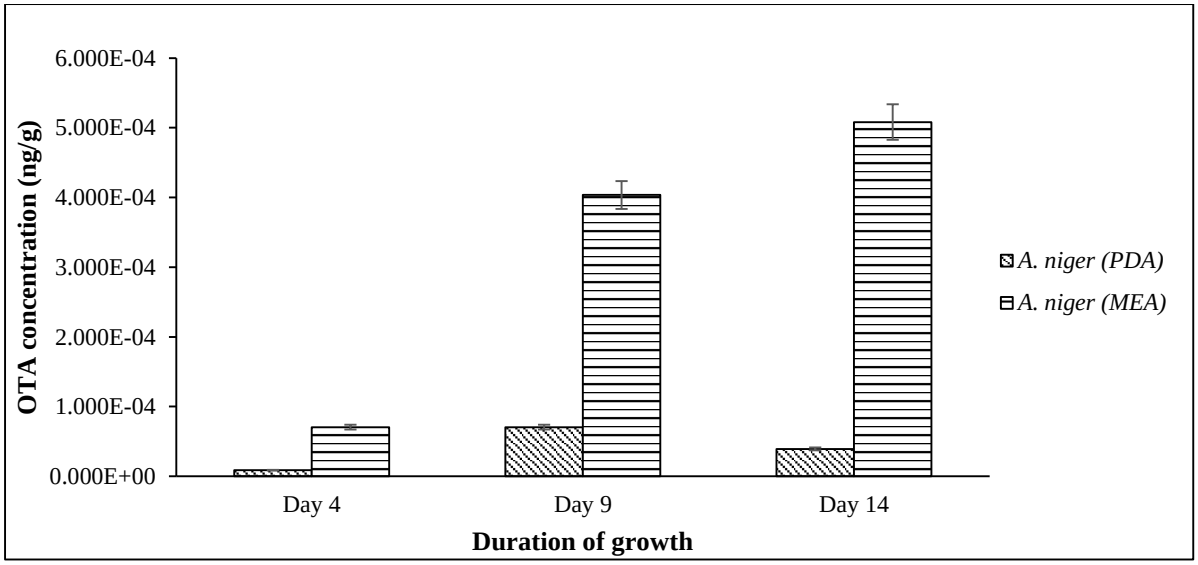


Figure 3.7: Influence of media on the production of ochratoxin A by *Aspergillus niger* cultured for 4, 9 and 14 days at 30ºC

3.3.1 Thin layer chromatography (TLC)

Analysis of extracts using TLC technique confirmed the presence of the mycotoxins detected via HPLC. Some of the fungal extracts had mycotoxin concentration levels below detection limit for TLC whereas others were above the detection limit. Mycotoxins detected by TLC in some fungal extracts are represented in figures 3.8 – 3.12. Extracts positive on TLC paper corresponded with higher mycotoxin concentrated extracts analysed by HPLC.

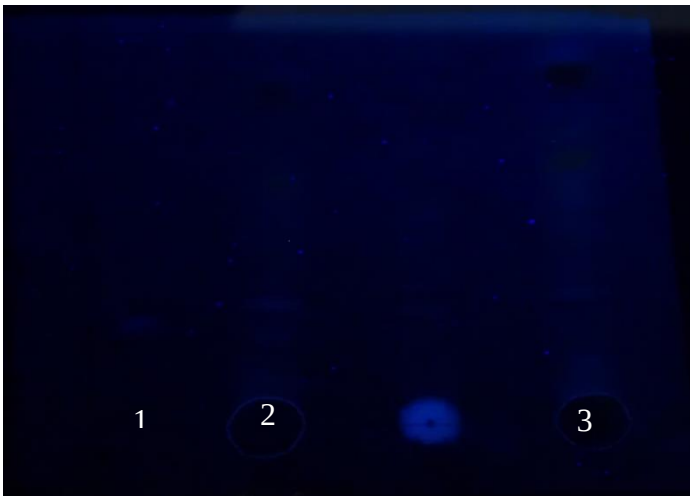


Figure 3.8: Thin layer chromatography spots showing extracts positive for the production of deoxynivalenol.

1- standard, 2 & 3- positive *Fusarium* extracts

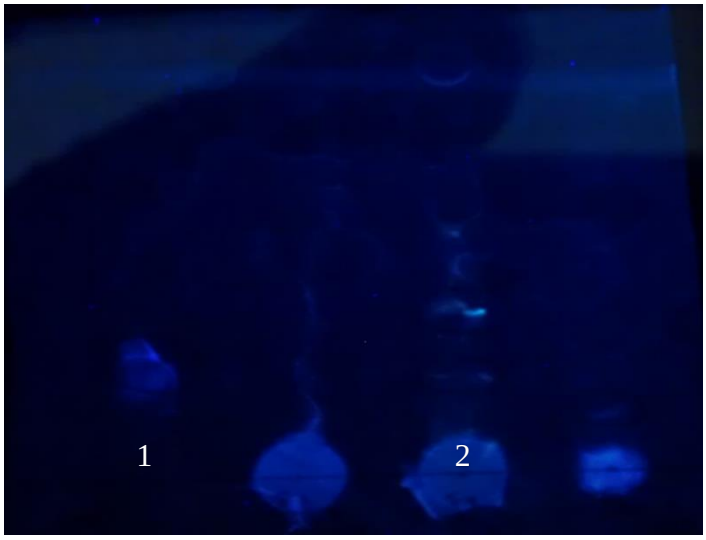


Figure 3.9: Thin layer chromatography spots showing extracts positive for the production of nivalenol.

1- standard, 2- positive *Fusarium* extracts

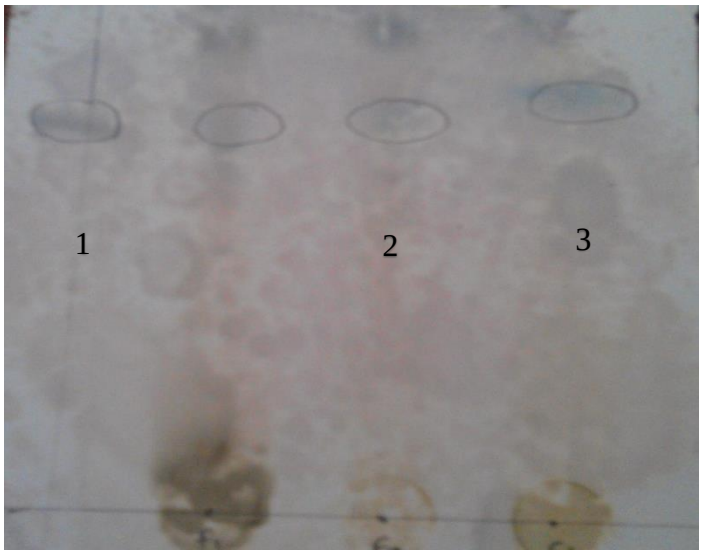


Figure 3.10: Thin layer chromatography spots showing extracts positive for the production of fumonisin B1.

1- standard, 2 & 3- positive *Fusarium* extracts

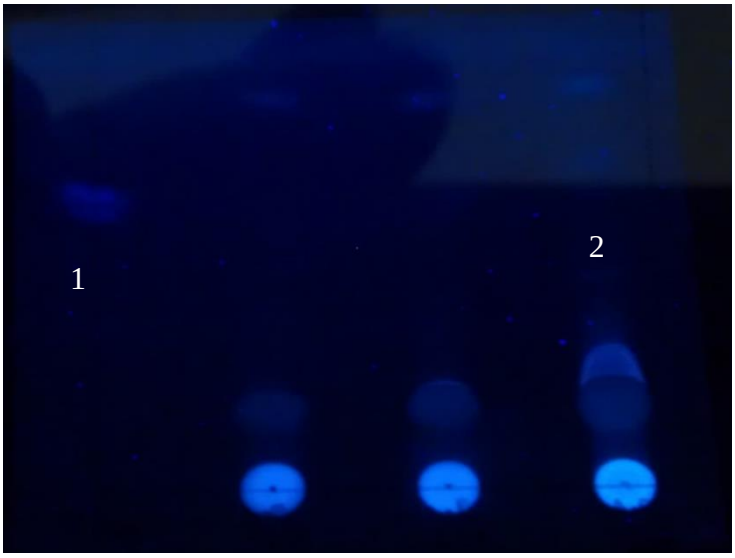


Figure 3.11: Thin layer chromatography spots showing extracts positive for the production of aflatoxins.

1- aflatoxin B_1 and aflatoxin B_2 standard, 2- positive Aspergillus flavus extracts

3.4 Discussion

Production of mycotoxin in filamentous fungi is to enable this group of micro-organisms to adapt to stress in the environment (Schmidt-Heydt *et al.*, 2008). Stress can be abiotic (water activity, temperature and pH) as well as biotic. Fungal species used in this study produced different concentration of mycotoxins at the different periods of culture. *Aspergillus flavus* produced different aflatoxins at different concentrations (Figure 3.4) on both PDA and MEA when cultured for 4, 9 and 14 days. The level of aflatoxin production by *A. flavus* isolates used in this study varied between the two culture media. Also, quantification of HPLC with post-column derivatisation using a kobra cell indicated that there was variation in production of aflatoxins at different durations of culture/growth of the fungus. Production of aflatoxin B_1 on PDA was highest after 9 days of culture (11.6µg/g) with subsequent reduction at 14^{th} day of culture (0.3 µg/g), whereas cultures on MEA produced highest AFB_1 concentration on the 14^{th} day of culture. The production of the other aflatoxins (B_2 and G_2) followed the same pattern of production on PDA and MEA as AFB_1. Production of aflatoxin G_2 on PDA and MEA increased

as the duration of culture incubation increased with highest concentration of 1.4µg/g on MEA at 14 days of growth. The production of Afs on the two culture media as well the variation in concentration at different durations of growth, conform with other studies regarding the production of aflatoxins in different culture media such as Czapek dox agar, Yeast extract sucrose broth and PDA (Fakruddin *et al.*, 2015; Ritter *et al.*, 2011; Schindler *et al.*, 1967) as well as other substrates (Klich, 2007; Lai *et al.*, 2015). These studies corroborate with reports that water activity and temperature contribute to aflatoxin production.

Extracts from *F. verticillioides* and *F. oxysporum* were also positive for deoxynivalenol with increased production as incubation time increased on both culture media. Although production of FB_1 by both fungal species was minimal on both growth media (Figure 3.5), *F. verticillioides* produced more deoxynivalenol on PDA (0.02µg/g -0.12µg/g) compared to MEA (0- 0.02µg/g) whereas, *F. oxysporum* produced more deoxynivalenol on MEA (0.04µg/g -0.15µg/g) compared to PDA (0.004µg/g - 0.065µg/g). The findings of this study are in agreement with results obtained by Matny (2013) who found production of deoxynivalenol by *F. verticillioide*s in millet, corn, straw and wheat cultures. However, no study has so far been documented on the production of DON by *F. oxysporum*.

Fumonisins are mycotoxins known to be produced by *Fusarium* species, particularly *F. verticillioides* and *F. proliferatum* (Rheeder *et al.*, 2002). This study revealed that *F. verticillioides* isolates produced FB_1 on both PDA and MEA at 4, 9 and 14 days of incubation. Production of FB_1 (Figure 3.6) was more on MEA compared to PDA and the highest concentration of FB_1 was up to 114357.5µg/g at 14 days of incubation on MEA and the least concentration of 7363.3µg/g at 14 days of culture on PDA. Picot *et al.* (2010), Samapundo *et al.* (2005) and (Sanchis *et al.*, 2006) stated that water activity, nutritional content of culture media and temperature are ideal factors that influence the production of fumonisin by its producers. This could be the reason for the decrease in production of FB_1 by cultures on PDA.

Fusarium oxysporum also produced FB_1 on both PDA and MEA as confirmed by previous studies (Abbas *et al.* (1995) Seo *et al.* (1996) Irzykowska *et al.* (2012) Waskiewicz *et al.* (2010) Waśkiewicz *et al.* (2009). *Fusarium oxysporum* isolates produced very high concentration of FB_1 (114617µg/g) more than the amount produced by *F. verticilliodes* isolates. Other studies have reported the production of FB_1 by *F. oxysporum* from 300ng/g (Abbas *et al.*, 1995) to 0.9µg/g (Irzykowska *et al.*, 2012) and 4.8µg/g (Waskiewicz *et al.*, 2010). A major reason for the production of FBs is the presence of FUM gene clusters in *F. oxysporum* responsible for the production of fumonisins (Proctor *et al.*, 2008).

Production of nivalenol by *F. oxysporum* (Figure 3.7) showed highest production by the 9^{th} day of incubation on PDA (829.83µg/g) and on the 4^{th} day of incubation on MEA. Although there are very few reports on the production of nivalenol by *F. oxysporum,* Lee *et al.* (1986) reported on the production of nivalenol by some strains of *F. oxysporum* up to 77.4 µg/g. The report by this study on the production of DON by *F. oxysporum* is a novelty and could be explained by several factors among which is possibly a mutation of genes in the strains due to environmental conditions and climate change. These assumptions are in correlation with the report of Paterson and Lima (2010) who confirmed that in vitro studies have shown that fungal mutations can occur when environmental conditions are changed, also, the increase of mycotoxins and UV radiation may cause fungi to mutate on crops and produce different mycotoxins.

The concentration of OTA produced by *A. niger* isolates was not very much but there was a variation in OTA production in the two cultures at different stages or duration of growth. Production of Ochratoxin A on MEA was higher than its production on the other culture medium (PDA) (Figure 3.8) with the highest concentration of OTA at $5.1*10^{-4}$ ng/g. Also, there was a reduction in the production of OTA by isolates on PDA after the 9^{th} day of culture whereas production of OTA increased on MEA as culture duration increased. The production of OTA using the *A. niger* strain correlates with other reports of *A. niger* producing OTA in

different types of cultures (Abarca *et al.*, 1994; Marino *et al.*, 2014). The reason for the drop in production of OTA on PDA after 9 days of incubation could be attributed to the ability of *A. niger* to detoxify OTA produced in cultures (Marino *et al.*, 2014; Varga *et al.*, 2000) which is enabled by the assimilation of phenylalanine moiety in the OTA molecule by *A. niger* when there is insufficient nitrogen in the culture (Varga *et al.*, 2002). It is assumed that nutritional composition of the medium could have contributed to more production of OTA on MEA in comparison with PDA (Pitt and Hocking, 2012).

The other mycotoxins, T-2 toxin, patulin and zearalenone investigated in this study were not identified in the fungal isolates used. This could be explained by the inability of these fungal species to produce the mycotoxins, and there is no published literature confirming their ability to produce them. *Penicillium* species used in this study did not produce any of the mycotoxins screened for. The factors responsible for the production of these target mycotoxins could be attributed primarily to the presence of genes responsible for synthesis of the mycotoxins (Bennett and Klich, 2009) in filamentous fungi, and enhanced by other factors such as type of media, temperature and water activity.

Generally, concentration of mycotoxins was more on MEA than PDA (Fig. 3.5 to 3.8). Malt extra agar is rich in a variety of components including fats, proteins, calcium, vitamin A and sodium which is lacking in PDA and these are some nutritional components of a growth media including carbohydrates, fibre and iron that are essential for fungal growth and metabolite production (Gebala and Sandle, 2013). Studies by Polizzi *et al.* (2012), Gebala and Sandle (2013) indicated that MEA alongside Saboraud dextrose agar (SDA) were very effective in the growth of a variety of filamentous fungi which produced different volatile compounds including mycotoxins. This therefore implies that the degree of production of metabolites by these fungi in the experiment is dependent on the nutritional composition of the growth media. This is because increased concentration of nutritional factors in growth media such as proteins

can result in the production of enzymes which will facilitate the biosynthesis of metabolites in the micro-organisms. Although cells of living organisms have the ability to produce these nutritional components, additional components in the growth media as in the case of MEA can contribute to the production of metabolites. Also, the presence of vitamins in MEA could have contributed to the production of more metabolites in comparison to PDA because Deacon (2006) in his study, indicated that vitamins and amino acids enable fungi to utilize nitrate or ammonium in growth media to access nitrogen which it requires for proper growth and production of metabolites. Analysis using Thin Layer Chromatography revealed some mycotoxins present in fungal extracts slightly above limits of the TLC detection, after extracts were concentrated.

3.5 Conclusion

The chapter has demonstrated that filamentous fungi isolates can produce mycotoxins which constitute a health threat. The fact that some species which are not usually associated with certain mycotoxins such as *F. oxysporum* producing deoxynivalenol is a new discovery. Further (LC-MS/ NMR) work is needed to support the findings. The presence of these fungal species in the environment should be a cause for concern because of their ability to produce metabolites that are most times harmful to both humans and animals.

CHAPTER FOUR

"*In vitro*" studies on the proliferation of human hepatocytes and renal epithelial cells induced by filamentous fungi

Abstract

In this study, the effect of individual fungal species and their combinations on cell viability of healthy human hepatocytes and renal epithelial cells was investigated in vitro. Fungal species were cultured on Malt Extract Agar (MEA) and Potato Dextrose Agar (PDA). Resazurin salt assay was used to determine the degree of increase or decrease in cell viability upon exposure to filamentous fungi individually and in combinations at a duration of 24 to 72 h. Individual fungal species induced an increase in hepatocyte cell proliferation over 100% by extra 152 after 24 h and fungi combinations induced cell proliferation exceeded 100% by 255.5 after 48 h. Renal epithelial cells also proliferated above 100% by an extra 330.1 value after 48 h of exposure to filamentous fungi in combination. Subsequently, fungi induced a reduction in cell viability of hepatocytes and renal epithelial cells up to 39.9% and 35.6% respectively after 72 h of exposure. The study showed that continued exposure of human hepatocytes and renal epithelial cells to filamentous fungi, particularly in combinations could result in drastic reductions in cell viability thus resulting in immune suppression and consequently complete cell death.

Keywords: Hepatocytes, filamentous fungi, renal epithelial cells, resazurin and viability

4.1 Introduction

Filamentous fungi have been mentioned in relation to diseases and infections in humans causing both superficial and invasive infections (Ahmadi *et al.*, 2012; Cocchi *et al.*, 2011) that can be detrimental. Human exposure to filamentous fungi is mainly through inhalation, ingestion and penetration (Hedayati *et al.*, 2007b) and has been attributed to the ubiquitous nature of the micro-organisms (Pitt and Hocking, 1997c). It is always common to find mycotoxins contaminating a particular substrate which has certain toxin producing fungi present.

The liver is a vital organ in the human digestive system and plays a major role in metabolism, protein synthesis, storage of proteins, synthesis of cholesterol, bile products synthesis, detoxification and excretion of exogenous and endogenous substances. Hepatocytes (which are one of the two main epithelial cells of the liver) make up about 70-85% of the liver's cytoplasmic mass and are mainly responsible for the different functions of the liver in the human body (Fausto, 2000; Fausto and Campbell, 2003) as well as meeting replacement needs of the liver during normal or mild cellular loss (Oh *et al.*, 2002). The liver is a primary target of some filamentous fungi, especially species belonging to *Aspergillus* genus. It has been reported that aflatoxins and ochratoxin A produced mainly by *Aspergillus* species primarily target the liver thus resulting in liver cancer and other liver-related diseases.

The kidney is another major organ in the human and animal body which contributes in maintaining balance in the body through the removal of waste products of metabolism. The kidney is involved in a number of essential regulatory activities such as regulation of electrocytes, acid-base balance maintenance and regulation of blood pressure (McCampbell and Wingert, 2012). The kidney is made up of different compartments, including the nephrons which help the kidney perform its waste removal function. Epithelial cells located in the

epithelia of nephrons assist in converting glomerular filtrate into concentrated urine thus adjusting it to maintain a steady state in the body (Baud, 2003). These cells are very important to the survival of the kidney because many reports of kidney diseases have been attributed to damage of the nephron which is a functional part of the kidney (McCampbell and Wingert, 2012). Epithelial cells also have the ability to dedifferentiate and proliferate when there is injury to the kidney, migrating to exposed parts of the membranes of the nephrons to bring about regeneration in the kidney (Bonventre, 2003).

The occurrence of different filamentous fungi species which belong to different genera in a particular environment, or on a substrate has been confirmed by some studies reporting the co-occurrence of different filamentous fungi (Egbuta *et al.*, 2015b; Klich, 2002c; Pitt and Hocking, 1997c) at a time. Although a lot has been reported about the different infections and diseases caused by filamentous fungi (Chithra *et al.*, 2008; Georgiadou *et al.*, 2014; Hedayati *et al.*, 2007b; Jain *et al.*, 2011; Nucci and Anaissie, 2007a), there is inadequate knowledge on the effects of these fungi on internal organs of the human body (including liver and the kidney), especially when they act in synergy. This study was conducted in order to investigate the synergistic action of different filamentous fungi on human hepatocytes and renal epithelial cells in vitro. The target cell lines are very important to the stability and proper functioning of organisms. The ability of filamentous fungi individually and in combinations to alter cell viability of human hepatocytes and renal epithelial cells was determined.

4.2 Materials and methods

4.2.1 Ethical considerations

Prior to commencement of the study, ethical approval was sought for experiment using human cell lines and it was issued by the North-West University, Mafikeng Campus Ethical Committee. The ethical approval is attached in the appendix of this thesis.

4.2.2 Chemical solvents

All chemical solvents used were of analytical grade and procured from Sigma-Aldrich South Africa and Merck Pty ltd South Africa.

4.2.3 Sampling

Fungal species mentioned in the previous chapters were used in the study. Healthy human hepatocytes and renal epithelial cells were procured from Inqaba Biotech, South Africa, transported on dry ice to North-West university, Mafikeng campus and stored liquid nitrogen (-196^{o}C) vapour prior to use for cytotoxicity studies.

4.2.4 Reagents and equipments

4.2.4.1 Fungal analysis

Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), sterile 1X Phosphate Buffered Saline (PBS), sterile Petri dishes, 2ml eppendorf tubes and micro centrifuge, 15% glycerol were used.

4.2.4.2 Cell culture

Tissue culture treated flasks (TC-75) manufactured by Corning life Sciences; Complete culture medium (Dubelccos Modified Eagles Medium (DMEM), 10% Fetal Bovine Serum (FBS), 2mM L-alanyl glutamine and 0.1% penicillin/streptomycin), CO_2 incubator (Shel lab), trypsin/EDTA solution, trypsin neutralising solution, dubelccos phosphate buffered saline (DPBS), poly-L-lysine, sterile 15ml centrifuge tubes, sterile pipettes (200µl, 1ml and 10ml),

neuber counting chamber, 0.4% tryphan blue, cover slips and sterile 50ml centrifuge tubes, centrifuge (Tomos Multistar).

4.2.4.3 Cytotoxicity analysis

Resazurin salt, tissue culture 96-well plates, 5% methanol, sterile 1X PBS, sterile pipettes, neuber counting chamber, 0.4% tryphan blue, cover slips and multi plate reader (HEALES MB-580) were used in the study.

4.2.5 Fungal analysis

Fungi isolates were cultured in triplicates on PDA and MEA for 4, 9 and 14 days. Agar plugs of fungal spores were collected into sterile 2ml eppendorf tubes containing 1ml of sterile 1X PBS (pH 7.4). Spores were centrifuged at 7,500 rpm for 2 minutes and the supernatant collected leaving behind mycelium and agar. Supernatant was made up to 1ml with 1X PBS and spore concentration determined using a Neubauer counting chamber. Fungal spore solution was stored at -20 ^{o}C for further use.

4.2.6 Cell culture procedure

Cells were cultured under sterile conditions following a modified method of Runge *et al.* (2000). Tissue culture treated flasks (TC-75) were coated with poly-L-lysine and put in the incubator overnight. Frozen hepatocytes and renal epithelial cells were thawed in a 37^{o}C water bath differently and transferred to sterile 50ml centrifuge containing 20ml of complete cell culture media. This was gently mixed, poured into a poly-L-lysine treated culture flask and put in a 5% CO_2 incubator set at 37^{o}C. Cells were cultured for one week with culture media changed every 24 hours and replaced with fresh media. Cell viability was determined at the beginning of cell culture with a Neubauer counting chamber imploring the tryphan blue stain exclusion method.

Attachment cells were harvested with DPBS and trypsin/EDTA solution. A trypsin neutralising solution was used to stop trypsination and detached cells collected into a 50ml centrifuge tube. Cells were centrifuged at 7,500 rpm for 3 mins and sediment cells collected and transferred into cell culture media for cytotoxicity analysis. Cell viability was determined with the tryphan blue stain exclusion method.

4.2.7 Cytotoxicity analysis (Resazurin salt assay)

Cytotoxicity analysis with resazurin salt was carried out following the method described by Rasmussen *et al.* (2011) with slight modifications. Harvested hepatocytes and renal epithelial cells were seeded into 96-well TC-treated plates at approximately 5,000 cells per well and placed in a 5% CO_2 incubator at 37^{o}C for 24h to allow cells to attach. Cells were exposed to equal volumes of 1X PBS, 5% methanol, individual fungal spore concentrations of fungi species and their combinations (Table 4.1), and returned to the incubator for 24 to 72h. Control cells which were used for the experiment were not exposed to anything and left to incubate for 24 to 72h along with exposed cells. After the required duration of exposure, 0.4mg/ml of resazurin salt in sterilised distilled/deionised water was added to each well at a proportion of 10µl per 100µl of culture solution and returned to the incubator for 2 to 3h. Optical density (OD) readings were taken at wavelengths of 680nm and 600nm in order to determine reduction of resazurin salt to the fluorescence resofurin by viable cells in culture. Final OD values were determined by subtracting OD values at 600nm from OD values of 680nm. The cell viabilty of exposed cells was thus calculated:

$$\frac{\text{OD values of exposed cells}}{\text{OD values of PBS control cells}} \times 100$$

Table 4.1: Filamentous fungi combinations

Filamentous fungi	Combinations	Filamentous fungi	Combinations
A. flavus+ *A. niger*+ *F. oxysporum* + *F.verticillioides*	C1	*A. niger* + *P. expansum*	C11
A. flavus+ *A. niger*+ *P. chrysogenum* + *P. expansum*	C2	*F. oxysporum* + *F. verticillioides*	C12
A. flavus+ *A. niger*	C3	*F. oxysporum* + *P. chrysogenum*	C13
A. flavus + *F. oxysporum*	C4	*F. oxysporum* + *P. expansum*	C14
A. flavus + *F. verticillioides*	C5	*F. verticillioides* + *P. chrysogenum*	C15
A. flavus + *P. chrysogenum*	C6	*F. verticillioides* + *P. expansum*	C16
A. flavus + *P. expansum*	C7	*P. chrysogenum*+ *P. expansum*	C17
A. niger + *F. oxysporum*	C8	*F. oxysporum* + *F. verticillioides* + *P. chrysogenum*+ *P. expansum*	C18
A. niger + *F.verticillioides*	C9	All isolates	C19
A. niger + *P. chrysogenum*	C10		

4.2.8 Statistical analysis

Statistical analysis was done using SPSS (version 21). Statistical analysis software and MS Excel (2010) were used to determine descriptive statistics of different cell viabilities and to prepare charts of cell viability. Minimum and maximum cell viabilities, mean viabilities, and standard deviation values as well as standard error values were also calculated.

4.3 Results

Cytotoxicity analysis showed the effects of filamentous fungi spores on both human cell lines *in vitro*. Spore concentration of species (per ml) cultured on MEA varied from $6.0 * 10^3$ to $7.2 * 10^4$ and $10.7 * 10^5$ to $13.2 * 10^5$ for *F. verticillioides* and *A. niger* respectively (Table 4.2).

Table 4.2: Spore concentrations of filamentous fungi species cultured on malt extract agar in different days

Filamentous fungal species	Spore concentration per ml		
	Day 4	Day 9	Day 14
A. flavus	$5.0 * 10^4$	$7.2 * 10^4$	$8.6 * 10^4$
A. niger	$10.7 * 10^5$	$11.7 * 10^5$	$13.2 * 10^5$
F. oxysporum	$7.9 * 10^4$	$4.0 * 10^5$	$5.0 * 10^5$
F. verticillioides	$6.0 * 10^3$	$7.6 * 10^3$	$7.2 * 10^4$
P. chrysogenum	$10.1 * 10^4$	$11.8 * 10^4$	$10.4 * 10^5$
P. expansum	$9.8 * 10^3$	$5.3 * 10^4$	$6.1 * 10^4$

Table 4.3: Spore concentrations of filamentous fungi species cultured on potato dextrose agar in different days

Filamentous fungal species	Spore concentration per ml		
	Day 4	Day 9	Day 14
A. flavus	$4.7 *10^4$	$6.3 * 10^4$	$9.2 * 10^4$
A. niger	$8.6 * 10^5$	$10.9 * 10^5$	$13.1 * 10^5$
F. oxysporum	$8.2 * 10^4$	$5.1 * 10^5$	$5.4 * 10^5$
F. verticillioides	$4.2 * 10^3$	$6.1 * 10^3$	$7.4 * 10^4$
P. chrysogenum	$6.0 * 10^4$	$8.2 * 10^4$	$8.7 * 10^4$
P. expansum	$5.2 * 10^3$	$8.2 * 10^4$	$2.3 * 10^4$

4.3.1 Human hepatocytes

Individual fungal species were able to alter cell viability of hepatocytes in different degrees due to duration of exposure. Generally, cells proliferated after 24h incubation with fungal species with reduction in viability when cells were exposed further for 72h. Four days old culture of *A. niger* was able to induce cell viability up to 250% after 24h and less than 80% after 72h of exposure. Results from 9 and 14 days old cultures showed almost similar effects on hepatocytes after 24, 48 and 72h. *Aspergillus flavus* and *Fusarium* species induced reduction in cell viability of hepatocytes which was somewhat different with other species which induced cell proliferation at 24h and reduced cell viability at 72h. The only exceptions were 14-day old cultures which induced cell proliferation over 100% after 48h of exposure as shown in Figures 4.1-4.6.

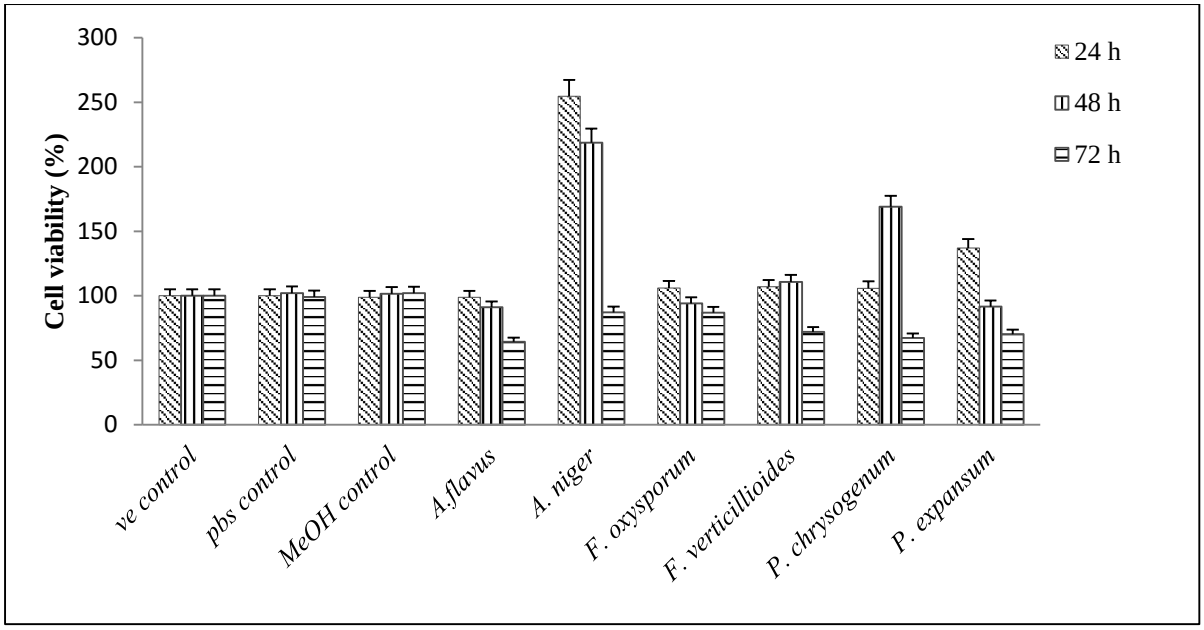


Figure 4.1: Alterations in cell viability of hepatocytes after exposure to four-day old potato dextrose agar cultures of individual filamentous fungi species for 24 to 72h. Pbs- phosphate buffered saline, MeOH- methanol.

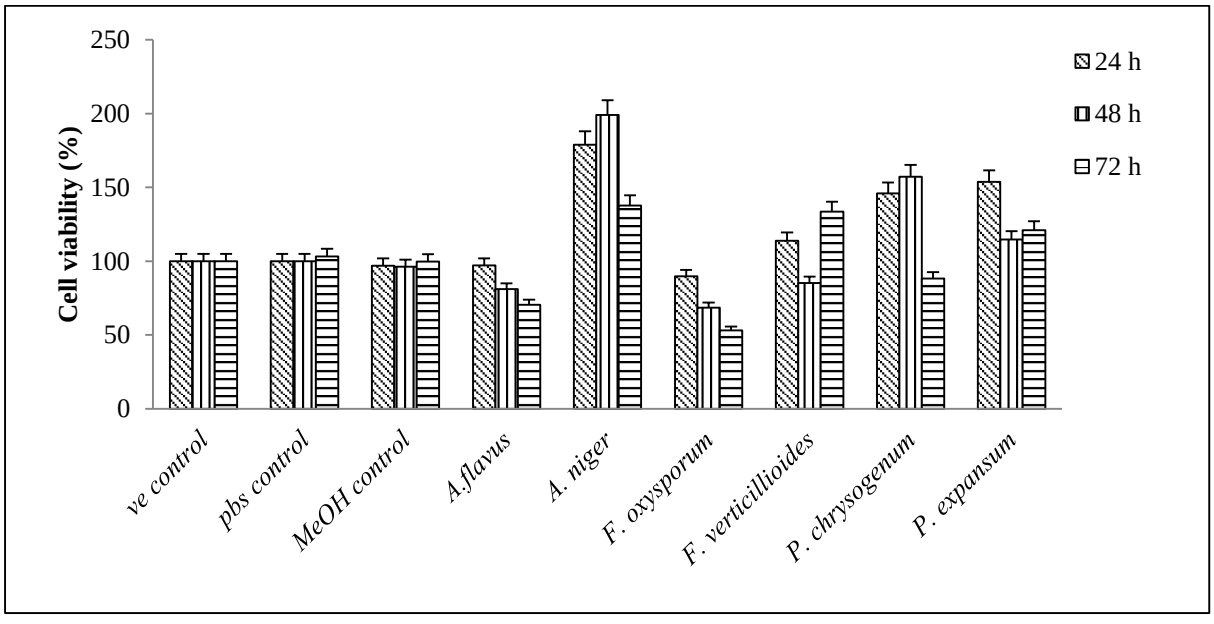


Figure 4.2: Alterations in cell viability of hepatocytes after exposure to nine-day old potato dextrose agar cultures of individual filamentous fungi species for 24 to 72h. Pbs- phosphate buffered saline, MeOH- methanol.

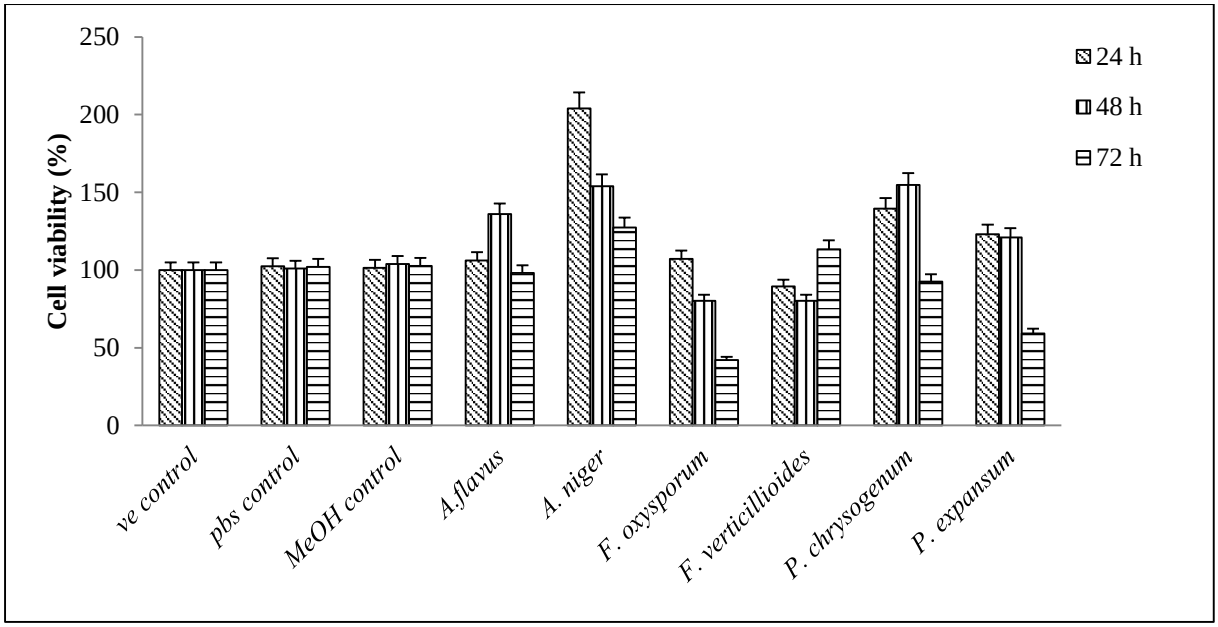


Figure 4.3: Alterations in cell viability of hepatocytes after exposure to 14-day old potato dextrose agar cultures of individual filamentous fungi species for 24 to 72h. Pbs- phosphate buffered saline, MeOH- methanol.

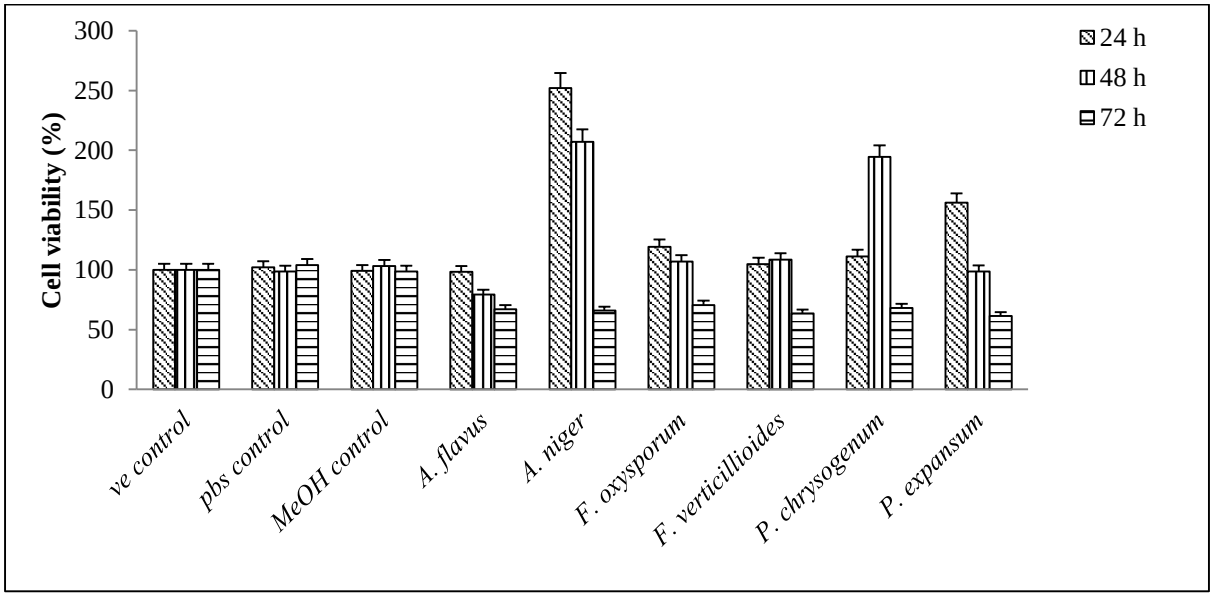


Figure 4.4: Alterations in cell viability of hepatocytes after exposure to 4-day old malt extract agar cultures of filamentous fungi for 24, 48 and 72h. Pbs- phosphate buffered saline, MeOH- methanol.

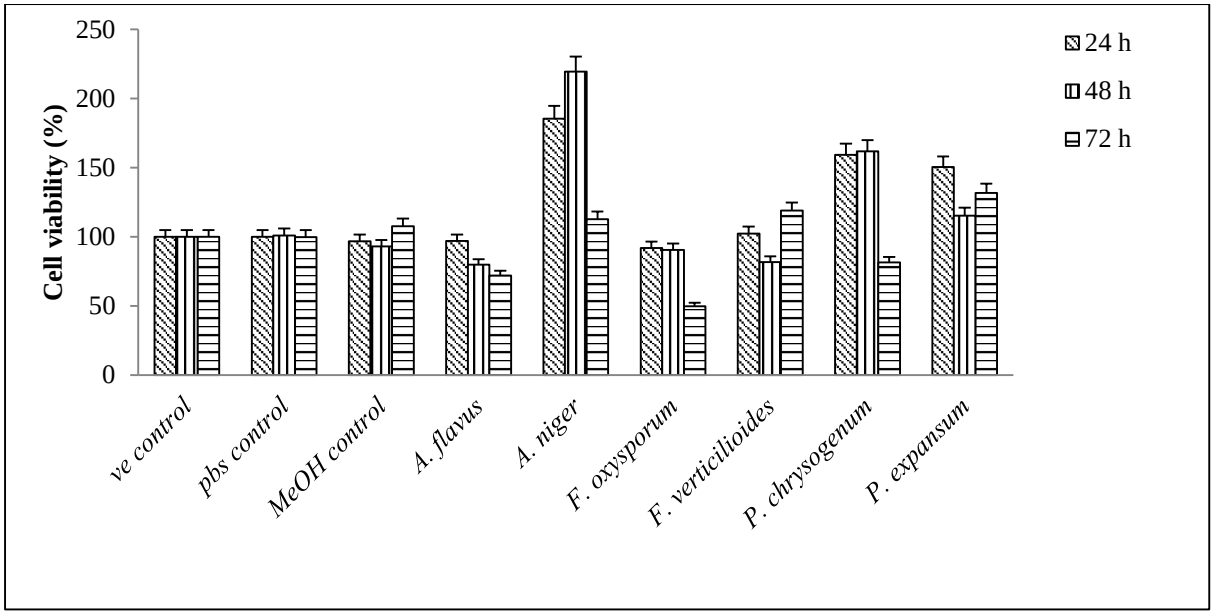


Figure 4.5: Alterations in cell viability of hepatocytes after exposure to 9-day old malt extract agar cultures of filamentous fungi for 24, 48 and 72h. Pbs- phosphate buffered saline, MeOH- methanol.

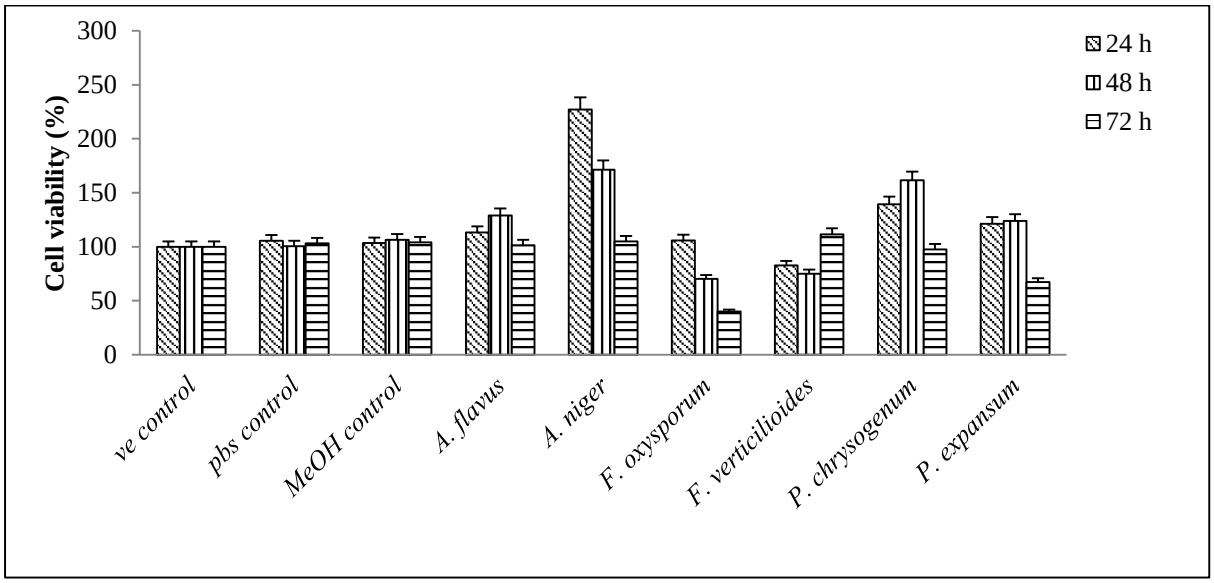


Figure 4.6: Alterations in cell viability of hepatocytes after exposure to 14-day old malt extract agar cultures of individual filamentous fungi for 24, 48 and 72h. Pbs- phosphate buffered saline, MeOH- methanol

Filamentous fungi combinations (Table 4.1) from 4 to 14 days old cultures induced increased cell viabilities on hepatocytes after 24 and 48h of exposure exceeding 100% by over a value of 100 followed by resultant decrease in cell viability below 100% in most cases at 72h. Four days

old fungi combinations showed excessive increase in cell viability after 48h and reduced drastically at 72h of exposure. Nine (9) and 14 days old fungi combinations were able to induce increase in cell viability after 24 and 48h and later reduced, however, in most cases, not below 100%. Figures 4.7 to 4.12 show column description of cell viability alterations induced by filamentous fungi combinations. *Aspergillus niger* combinations (C1 to C3 and C8 to C11) induced very high degrees of cell proliferation of hepatocytes after 48 h of exposure.

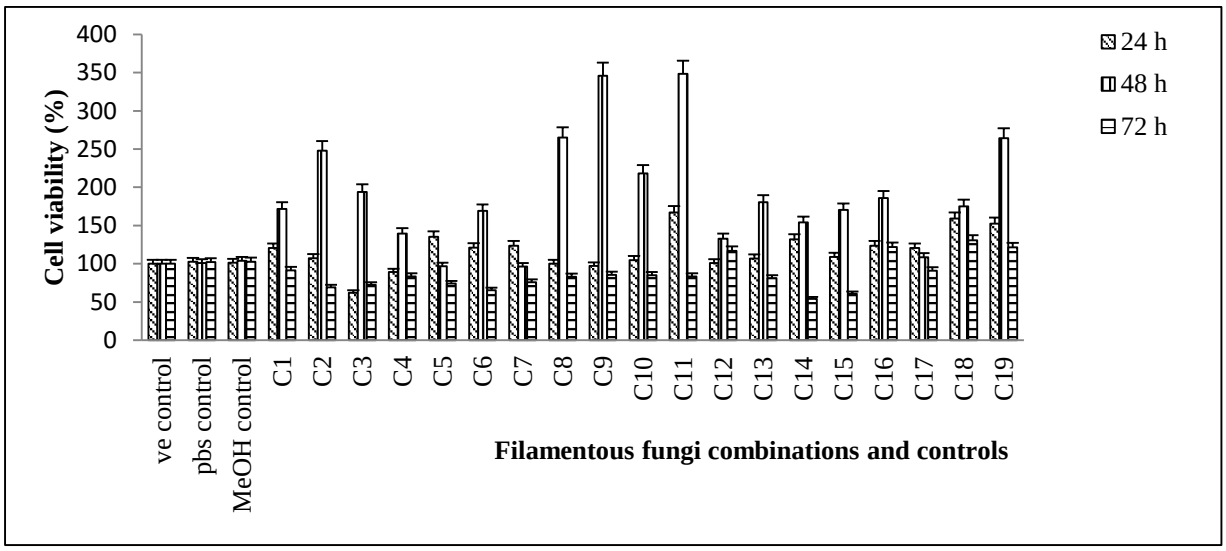


Figure 4.7: Alterations in cell viability of hepatocytes after exposure to four-day old potato dextrose agar cultures of filamentous fungi combinations for 24 to 72h. Pbs- phosphate buffered saline, MeOH- methanol.

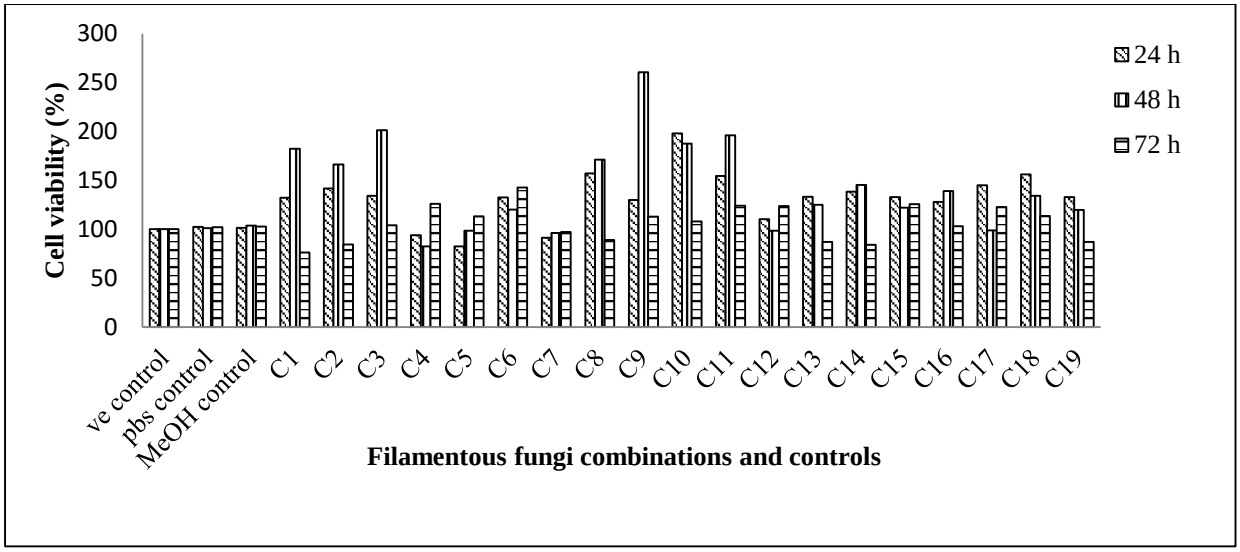


Figure 4.8: Alterations in cell viability of hepatocytes after exposure to nine-day old potato dextrose agar cultures of filamentous fungi combinations for 24 to 72h. Pbs- phosphate buffered saline, MeOH- methanol.

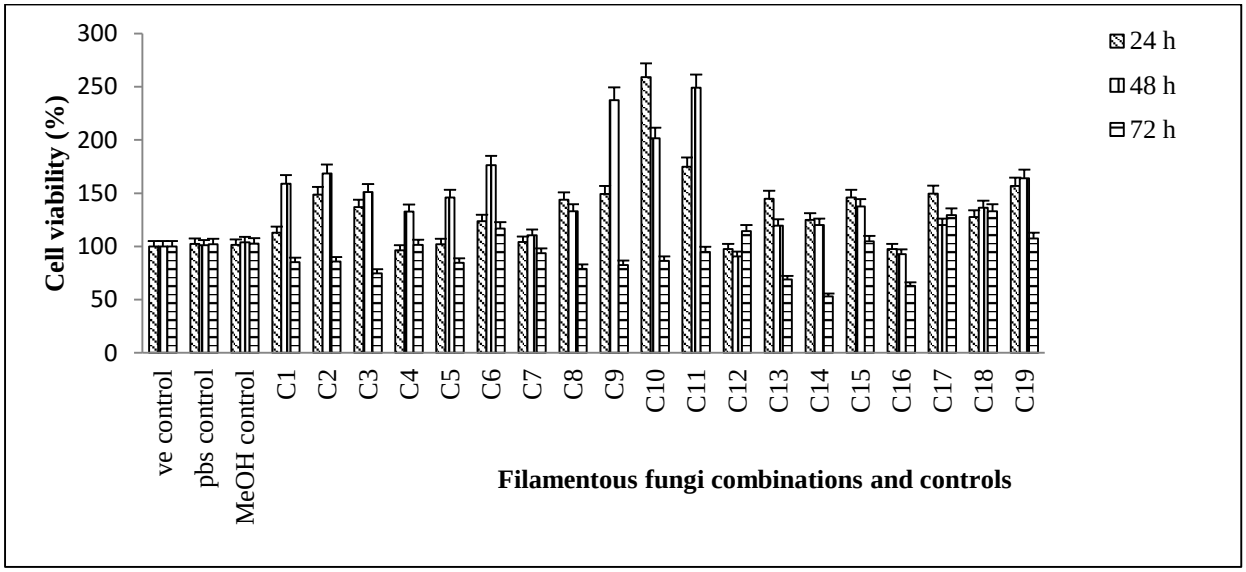


Figure 4.9: Alterations in cell viability of hepatocytes after exposure to 14-day old potato dextrose agar cultures of filamentous fungi combinations for 24 to 72h. Pbs- phosphate buffered saline, MeOH- methanol.

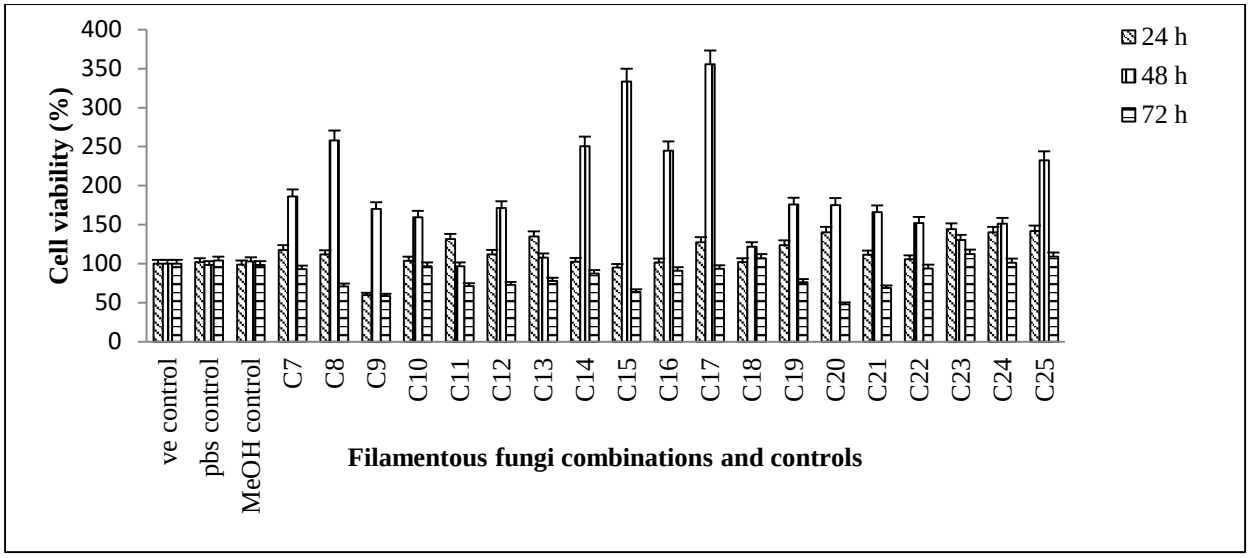


Figure 4.10: Alterations in cell viability of hepatocytes after exposure to 4-day old malt extract agar cultures of filamentous fungi combinations for 24 to 72h. Pbs- phosphate buffered saline, MeOH- methanol

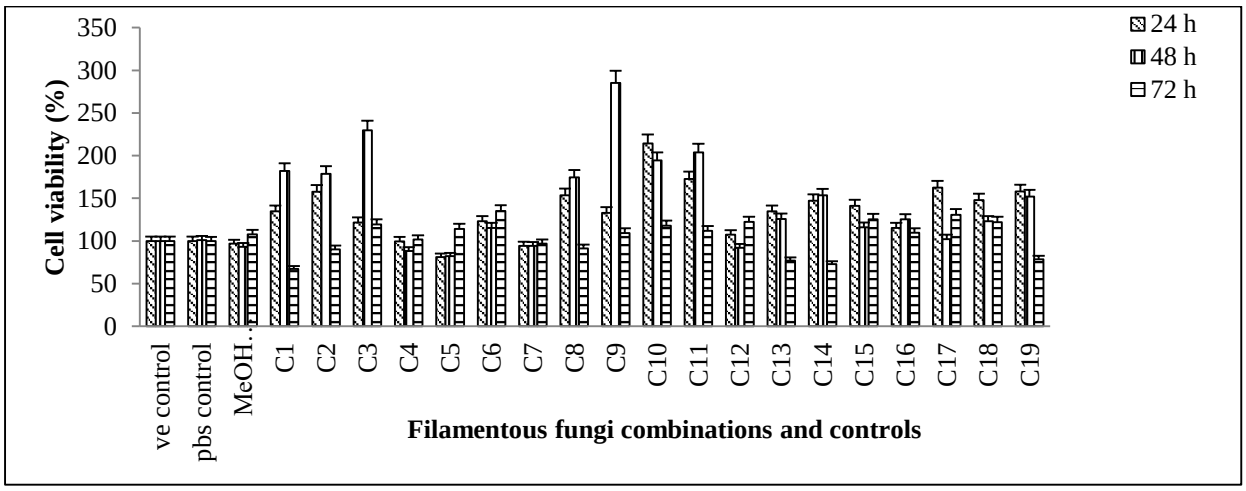


Figure 4.11: Alterations in cell viability of hepatocytes after exposure to 9-day old malt extract agar cultures of filamentous fungi combinations for 24 to 72h. Pbs- phosphate buffered saline, MeOH- methanol

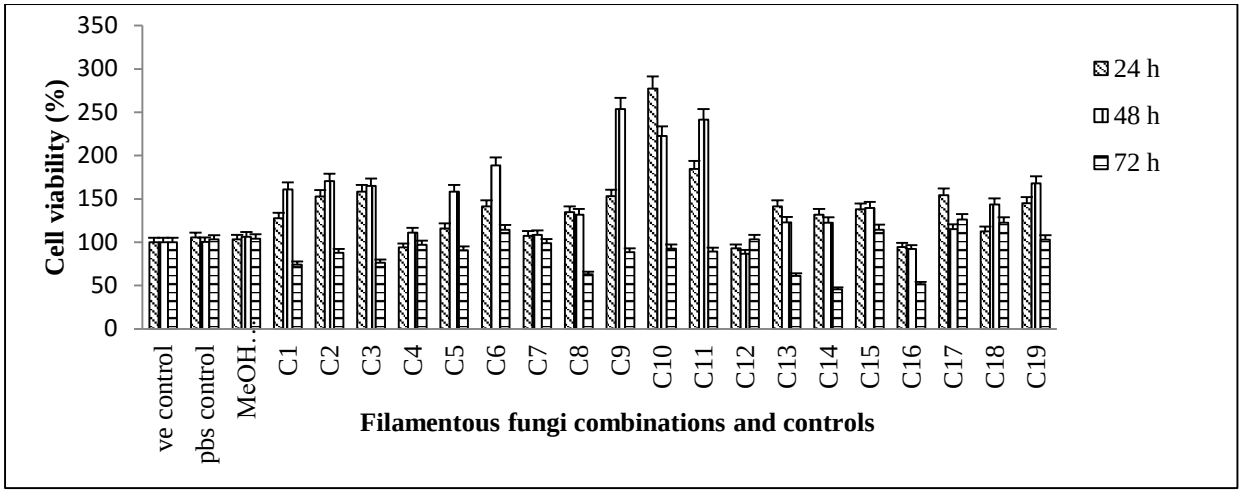


Figure 4.12: Alterations in cell viability of hepatocytes after exposure to 14-day old malt extract agar cultures of filamentous fungi combinations for 24 to 72h. Pbs- phosphate buffered saline, MeOH- methanol.

Descriptive statistics for response of hepatocytes to individual fungi species cultured on MEA revealed that cell viability ranged from 39.9% (induced by 14-day old *F. oxysporum* after 72h exposure) to over 100% by 152.0 (induced by *A. niger* (4 days culture) after 24h exposure). Individual fungi species cultured on PDA (Table 5.3) show cell viabilities ranging from 42.0% by *F. oxysporum* (14 days old) after 72h to a value of 154.6 above 100% by *A. niger* (4 days old) after 24 hours. Fungal combinations induced cell viabilities between 46.3% (*A. niger* and *P. expansum*) and 255.5 above 100% (*F.oxysporum* and *P. expansum*) for MEA cultured fungi. Potato dextrose agar (PDA) cultures of fungi combinations induced cell viabilities between 53.1% (*P. expansum* and *F. oxysporum*) and 248.3 above 100% (*A. niger* and *P. expansum*).

4.3.2 Human renal epithelial cells

Human renal epithelial cells exposed to individual filamentous fungi isolates cultured on PDA proliferated at 24h of exposure and cell viability reduced in most wells as duration of exposure to the fungal isolates increased. This reaction was in contrast to the proliferation of control cells which was closely similar to proliferation of PBS control cells. *Aspergillus flavus* and *A. niger* spores triggered proliferation of cells at 24h which reduced as exposure increased to 72h. The

pattern was the same for all filamentous fungi cultures harvested at 4, 9 and 14 days (Figures 4.13 to 4.15). The highest proliferation of above 100% by 96.9% was induced by *P. chrysogenum* at 24h of exposure. Filamentous fungal Isolates harvested from MEA at 4 days of culture triggered similar responses in cells above 100% by extra 100%. Nine days old *Aspergillus niger* isolates triggered the highest proliferation of cells at 48h of exposure which reduced at 72h (Figures 5.16-5.18). Fourteen days old isolates of other fungi species induced same responses in renal cells with *A. niger* inducing cell viability as low as 42.2%.

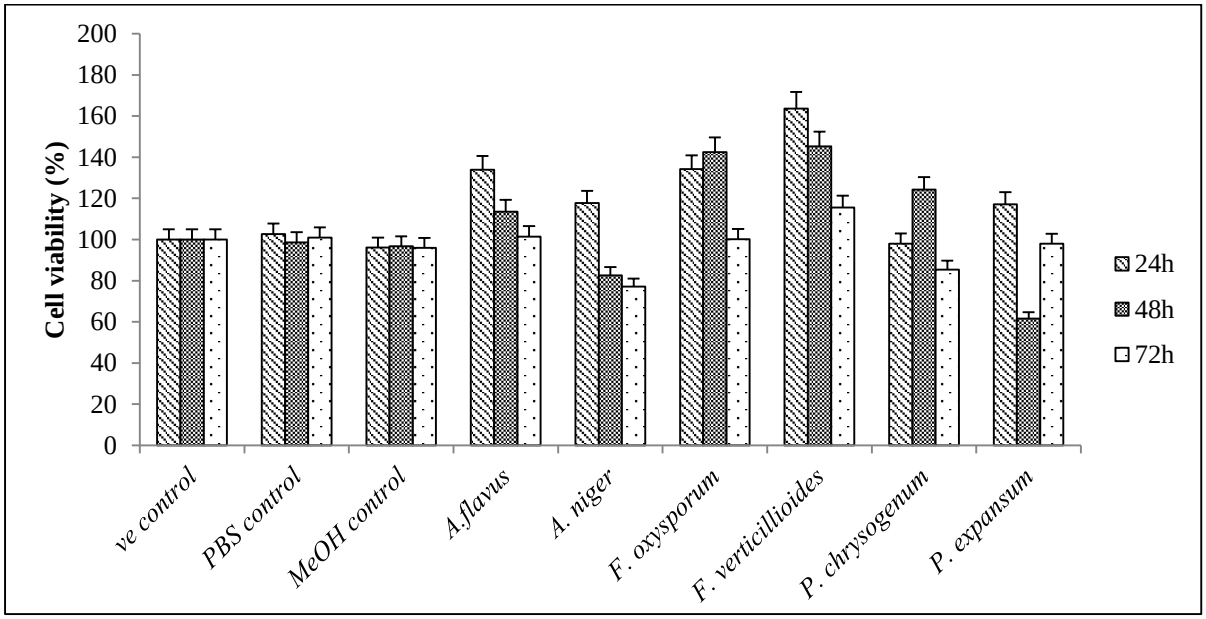


Figure 4.13: Proliferation of renal epithelial cells after exposure to 4-day old PDA cultures of filamentous fungi. PBS- phosphate buffered saline, MeOH- methanol.

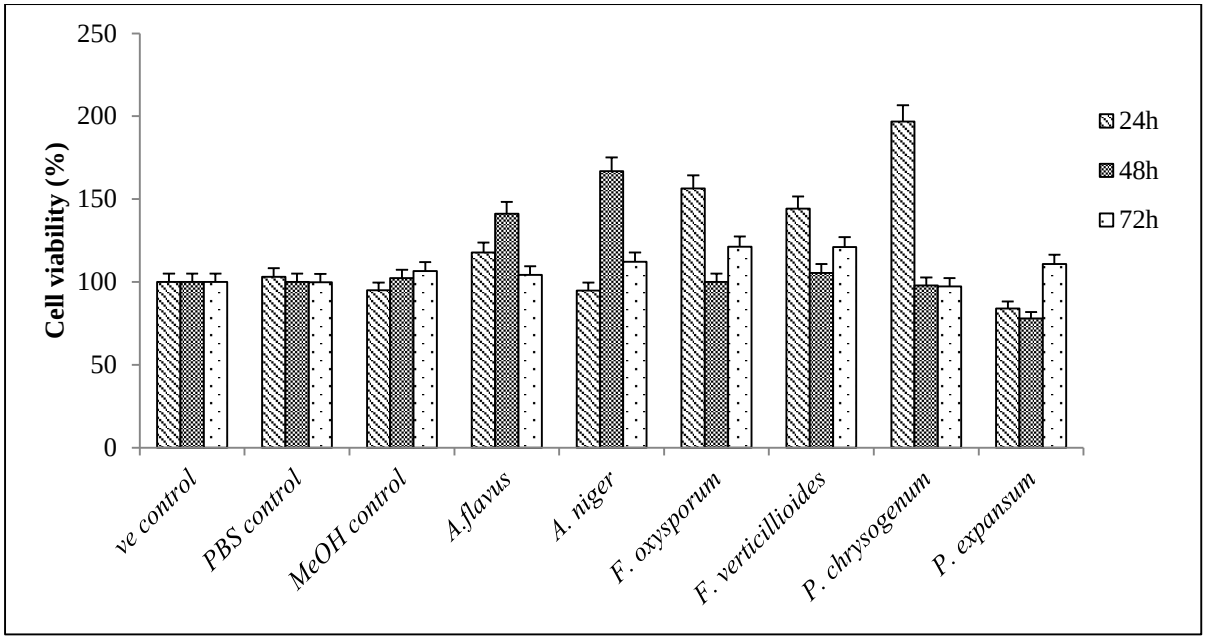


Figure 4.14: Proliferation of renal epithelial cells after exposure to 9-day old PDA cultures of filamentous fungi. PBS- phosphate buffered saline, MeOH- methanol.

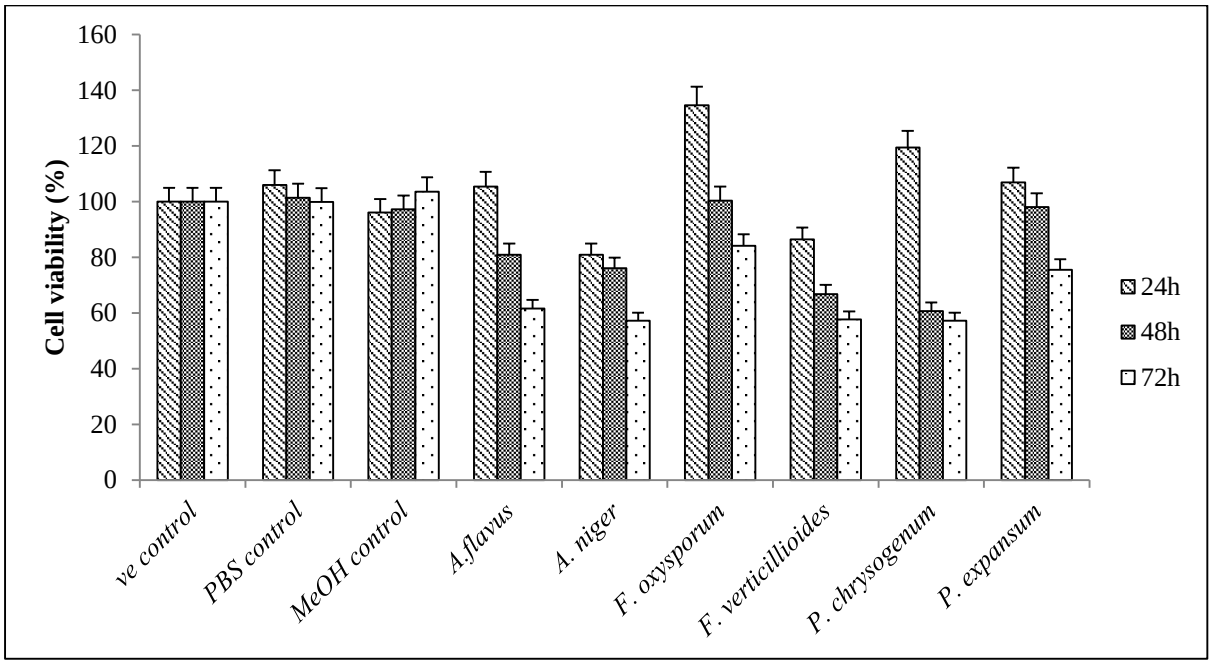


Figure 4.15: Proliferation of renal epithelial cells after exposure to 14-day old PDA cultures of filamentous fungi. PBS- phosphate buffered saline, MeOH- methanol.

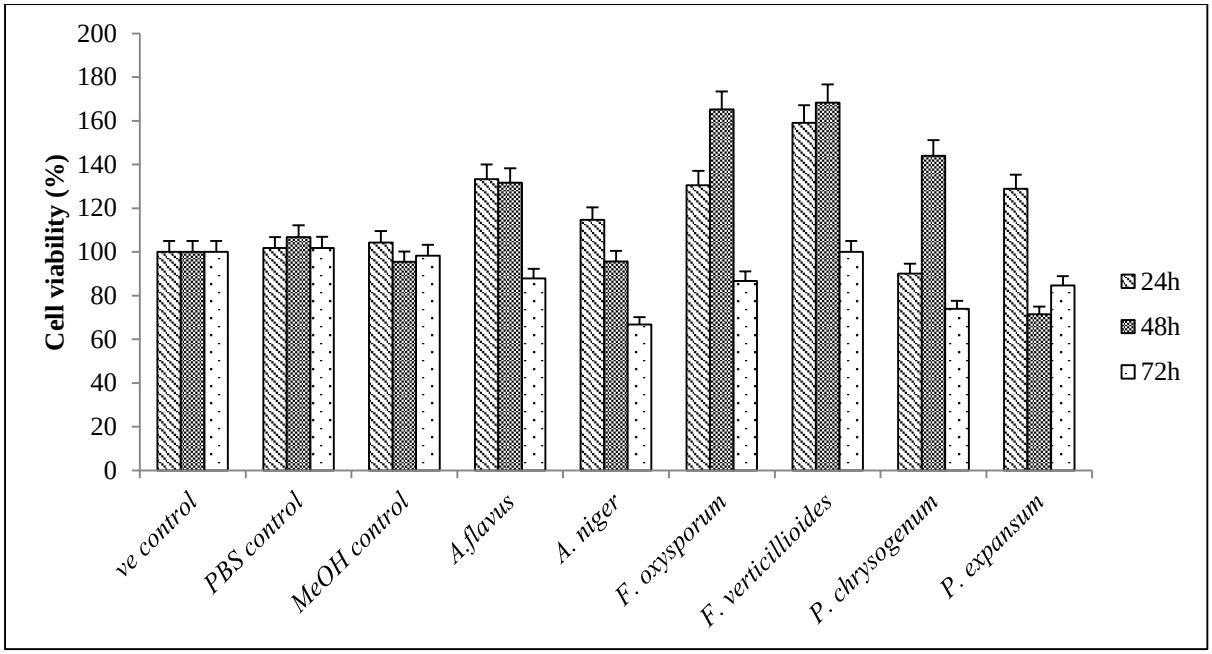


Figure 4.16: Proliferation of renal epithelial cells after exposure to 4-day old MEA cultures of filamentous fungi. PBS- phosphate buffered saline, MeOH- methanol.

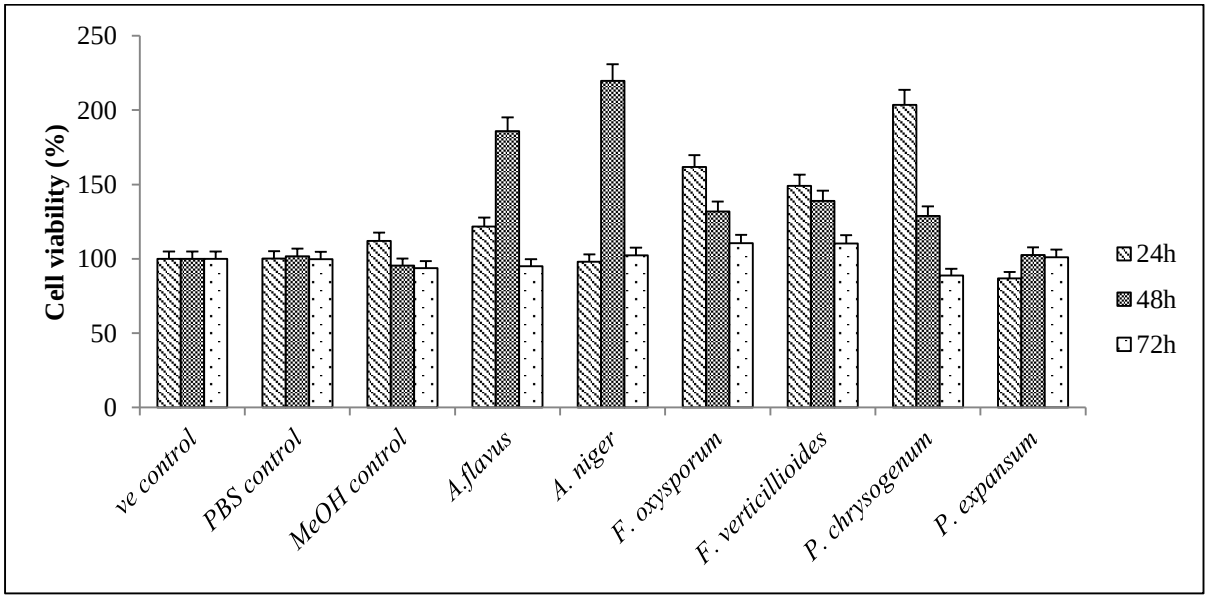


Figure 4.17: Proliferation of renal epithelial cells after exposure to 9-day old MEA cultures of filamentous fungi. PBS- phosphate buffered saline, MeOH- methanol.

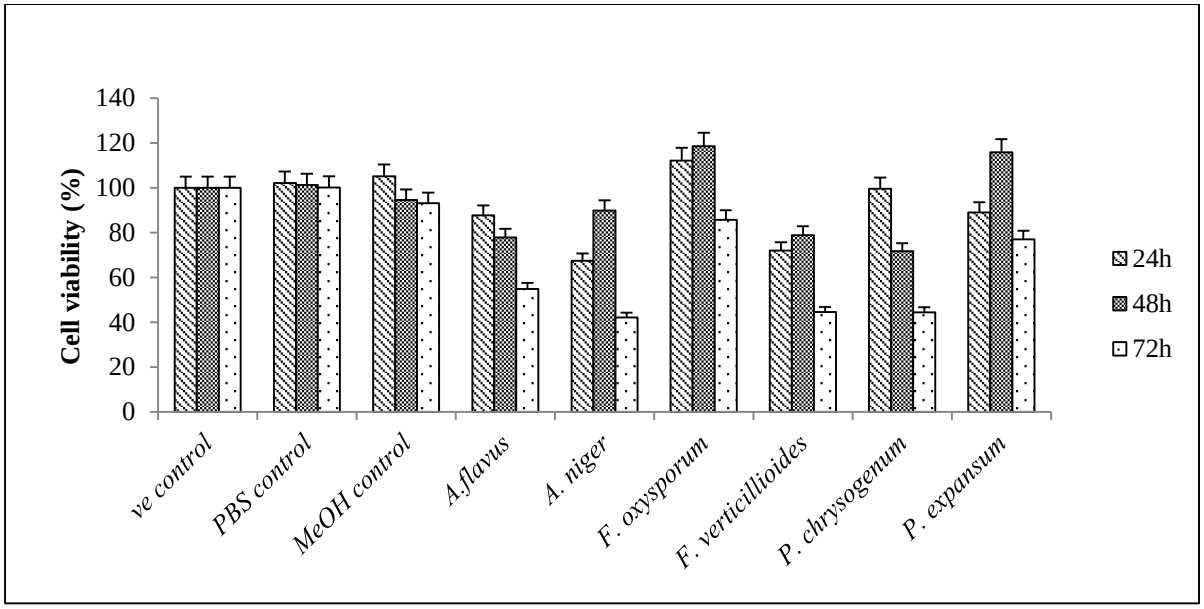


Figure 4.18: Proliferation of renal epithelial cells after exposure to 14-day old MEA cultures of filamentous fungi. PBS- phosphate buffered saline, MeOH- methanol.

For MEA cultures, the response of renal epithelial cells when exposed to filamentous fungi combinations was close to the response recorded for hepatocytes, though it was observed that there was higher proliferation of renal cells at 48h compared to hepatocytes (Fig. 4.19 to 4.24). Some combinations of isolates harvested from MEA at 4 days of culture induced cell proliferation at 48h up to 314.7 above 100% and cell viability thereafter reduced at 72h (Fig. 4.19). The lowest cell viability induced by fungal combinations was recorded as 35.6% for 14-day old *A. flavus* and *P. expansum* combinations, C7 (Fig. 4.24) at 72 h of exposure. The response of renal cells to 9-day old fungal combinations was almost similar to the 4-day old cultures with a slight difference of much reduction of cell viability at 72h. Most of the MEA fungal combinations represented in the following figures induced increased cell proliferation at 48h with the exception of C2, C5 and C13 (Fig. 4.20) which induced reduction in cell viability as exposure increased from 24h to 72h. Highest proliferation of cells by a value of 330 above 100% was recorded for C4 (Fig. 4.20), induced by 9-day old cultures. Combinations

of fungal isolates from 14-day old cultures induced responses similar to 4 and 9 days old culture combinations.

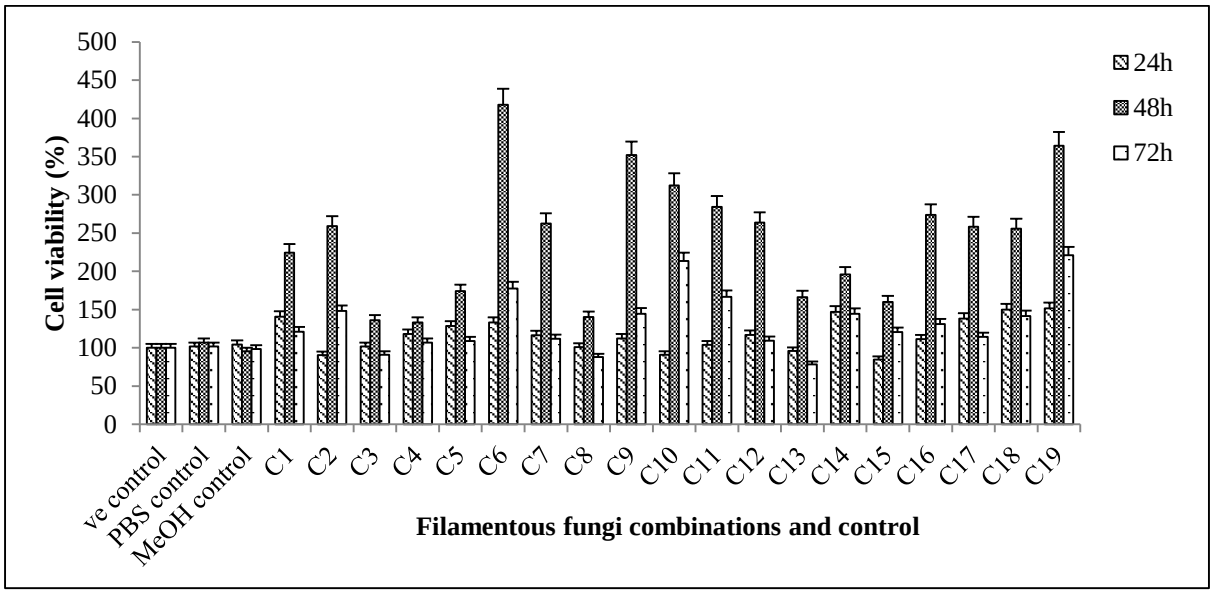


Figure 4.19: Proliferation of renal epithelial cells after exposure to 4-day old MEA cultures of filamentous fungal specie combination

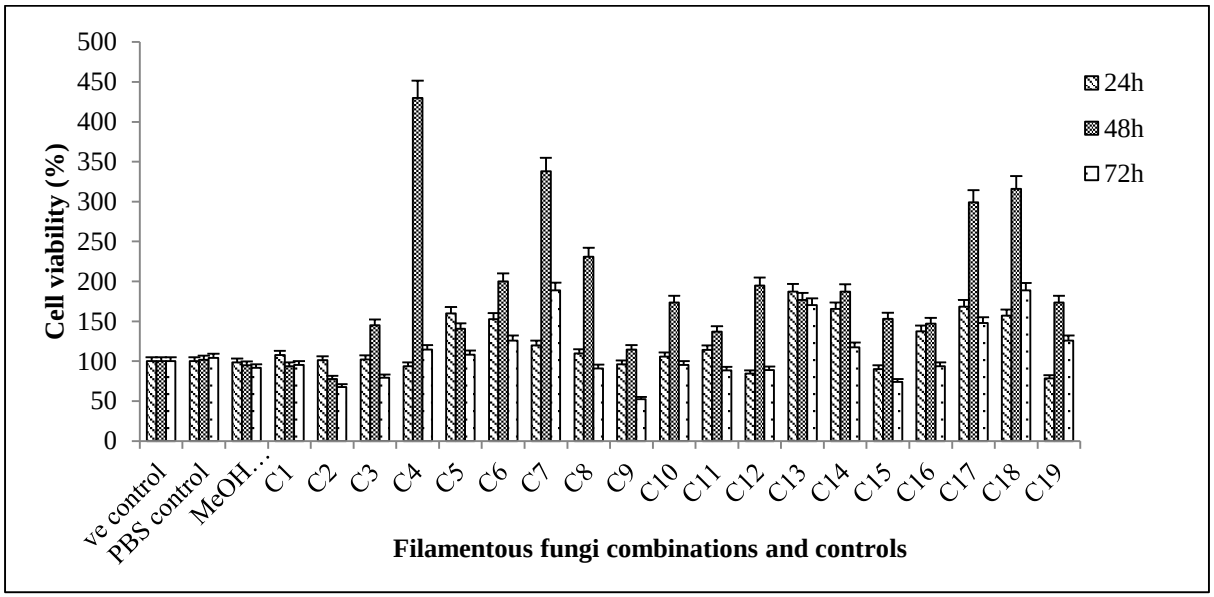


Figure 4.20: Proliferation of renal epithelial cells after exposure to 9-day old MEA cultures of filamentous fungal specie combination

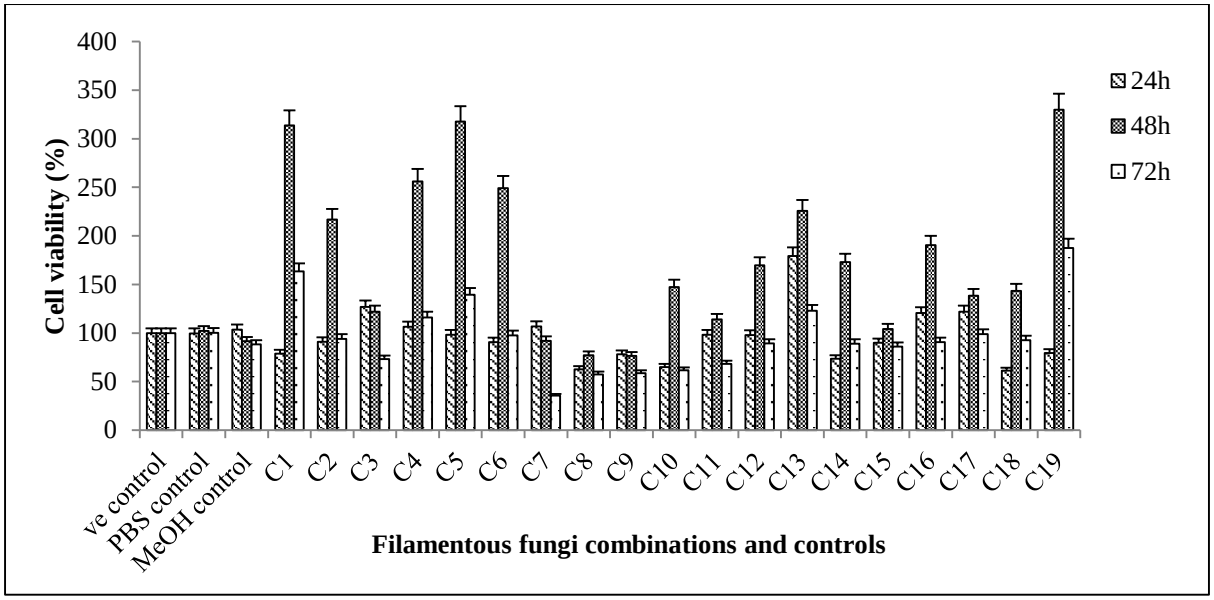


Figure 4.21: Proliferation of renal epithelial cells after exposure to 14-day old MEA cultures of filamentous fungal specie combination

Combinations of filamentous fungi cultured on PDA also induced cell proliferation at 48h and reduced at 72h similar to MEA cultures. Four days old fungal combinations induced cell proliferation up to 260.2 above 100% by C6, while lowest cell viability of 90.3% induced by C13 (Fig. 4.22). Slightly similar to results obtained from MEA cultures, some nine and fourteen days old PDA cultured fungal combinations induced reduction in cell viability (Figures 4.23 and 4.24) as the duration of exposure increased with as low as 43.4% cell viability recorded for C7 14-day old culture combinations at 72h of exposure.

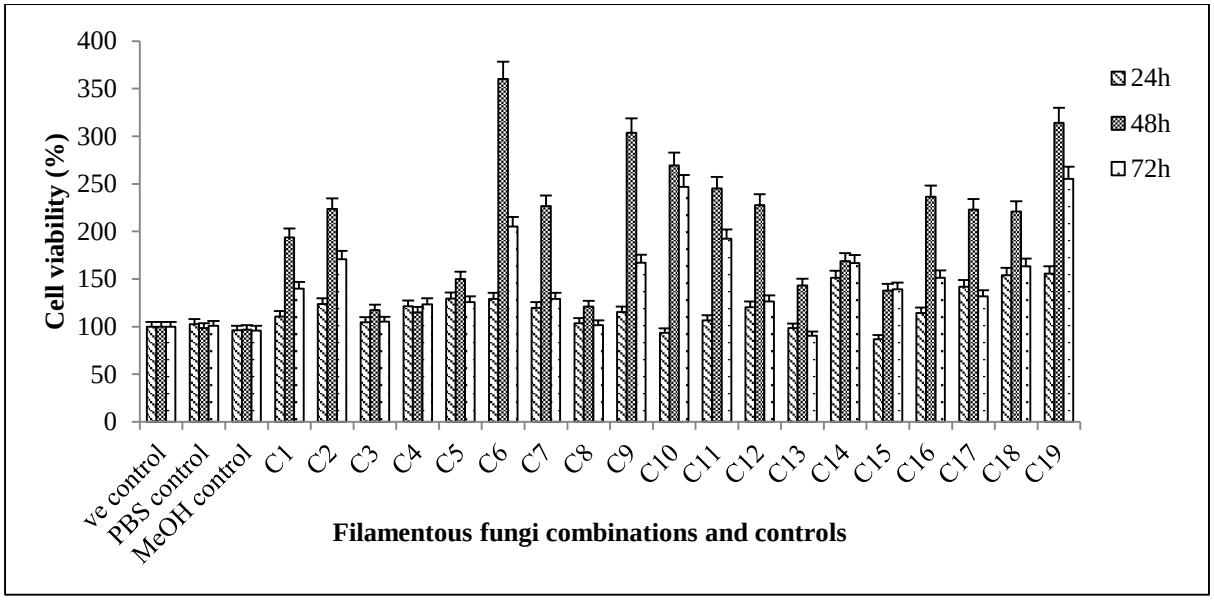


Figure 4.22: Proliferation of renal epithelial cells after exposure to 4-day old PDA cultures of filamentous fungal specie combination

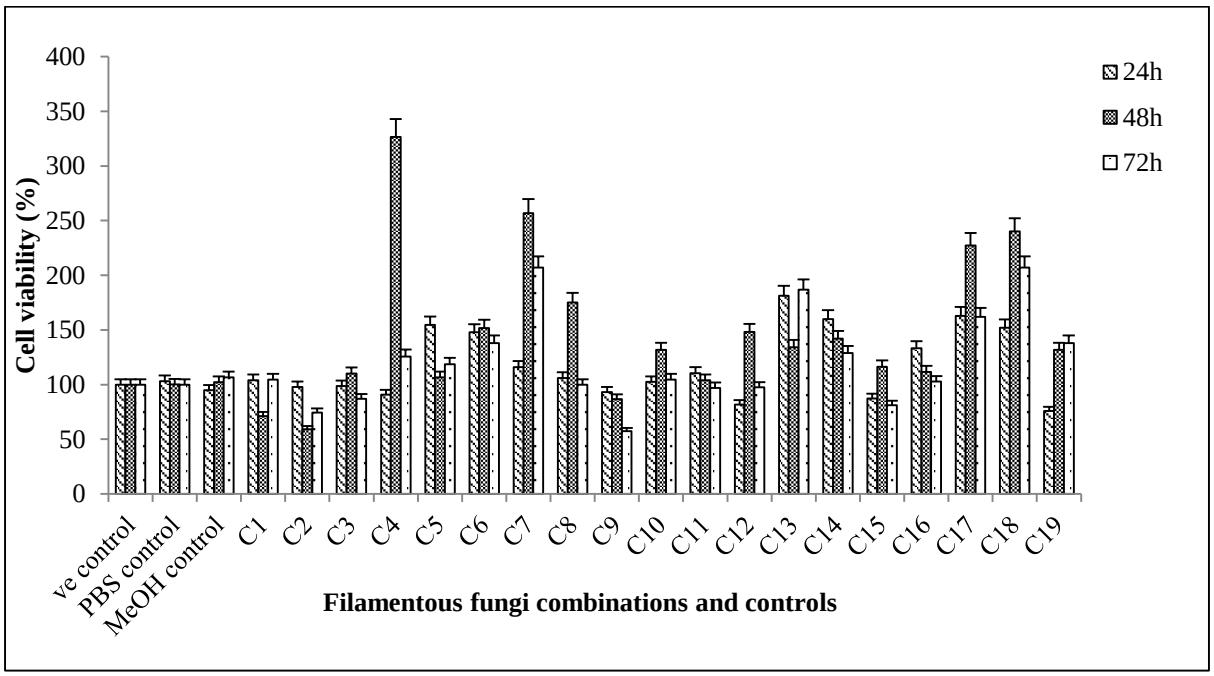


Figure 4.23: Proliferation of renal epithelial cells after exposure to 9-day old PDA cultures of filamentous fungal specie combination

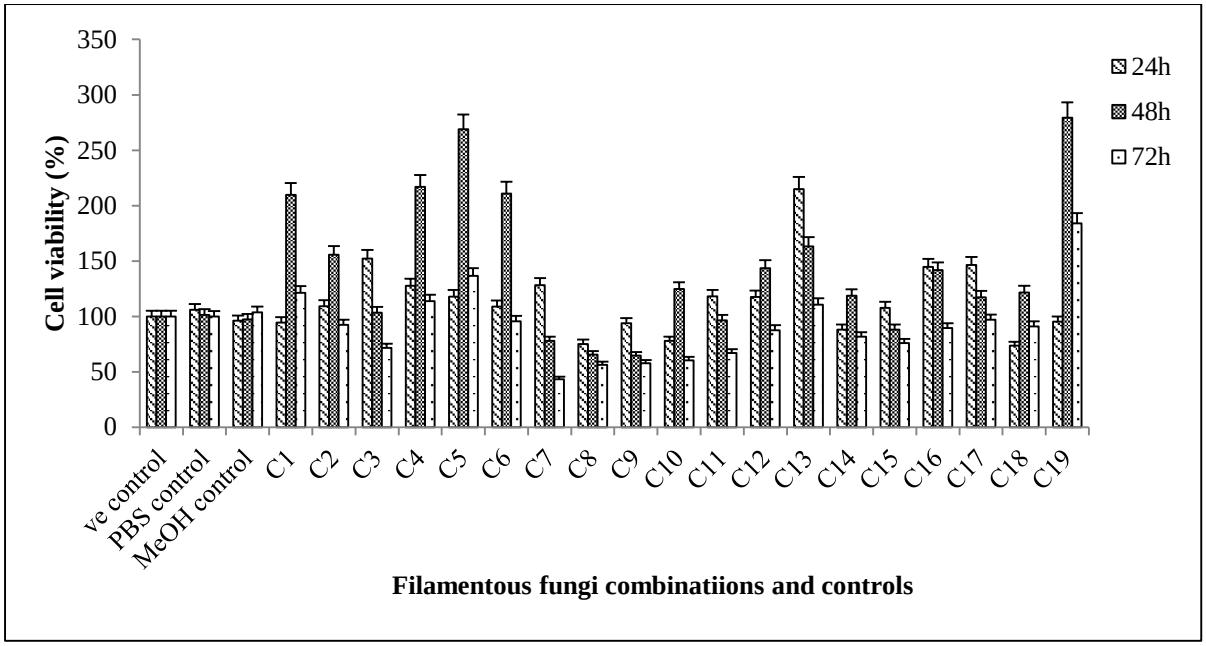


Figure 4.24: Proliferation of renal epithelial cells after exposure to 14-day old PDA cultures of filamentous fungal specie combination

Statistical description of alterations of cell viability of both hepatocytes and renal epithelial cells presented in Tables 4.4 to 4.7 show that there were different minimum and maximum cell viabilities recorded for cells when exposed to filamentous fungi, with 14-day old cultures inducing reduced cell viability less than 50% after 72h of exposure. Data analysed in Table 4.4 shows maximum proliferation observed after cells were exposed to 4-day old fungi for 48h. This pattern shown in the table is consistent with the different ages of fungi cultures. Highest mean proliferation value of 169 is recorded in Table 4.4 induced by 4-day old fungi cultures. The following tables 4.5 to 4.7 also show proliferation of cells following the same pattern as reported in 4.4. Result from this statistical analysis can be summarised by indicating that younger cultures of both individual fungi species and their combinations induced highest proliferation of cells after 48h, whereas, older cultures induced an immense reduction of cell viability.

Table 4.4: Descriptive statistics of hepatocyte cell viability alterations induced by filamentous fungal species and their combinations cultured on PDA

Exposure time in hours and days	N	Range	Minimum (%)	Maximum (%)	Mean (%)
24h exposure to 4 day old filamentous fungi	28	192.53	62.09	254.62	119.30
48h exposure to 4 day old filamentous fungi	28	257.26	91.04	348.30	169.40
72h exposure to 4 day old filamentous fungi	28	76.80	53.90	130.70	85.49
24h exposure to 9 day old filamentous fungi	28	115.54	82.50	198.04	128.61
48h exposure to 9 day old filamentous fungi	28	192.10	68.50	260.60	133.79
72h exposure to 9 day old filamentous fungi	28	89.67	53.03	142.70	104.69
24h exposure to 14 day old filamentous fungi	28	169.60	89.40	259.00	131.03
48h exposure to 14 day old filamentous fungi	28	168.80	80.20	249.00	138.41
72h exposure to 14 day old filamentous fungi	28	90.90	42.03	132.93	92.71
Valid N (list wise)	28				

Table 4.5: Descriptive statistics of hepatocyte cell viability alterations induced by filamentous fungal species and their combinations cultured on MEA

Exposure time in hours and days	N	Range	Minimum (%)	Maximum (%)	Mean (%)
24h eposure to 4 day old filamentous fungi	28	192.20	59.81	252.01	119.68
48h exposure to 4 day old filamentous fungi	28	276.11	79.42	355.53	169.16
72h exposure to 4 day old filamentous fungi	28	64.22	48.12	112.34	81.97
24h exposure to 9 day old filamentous fungi	28	132.92	81.32	214.24	131.60
48h exposure to 9 day old filamentous fungi	28	205.26	79.91	285.17	137.91
72h exposure to 9 day old filamentous fungi	28	194.72	82.66	277.39	134.10
24h exposure to 14 day old filamentous fungi	28	85.36	49.87	135.23	102.44
48h exposure to 14 day old filamentous fungi	28	183.56	70.25	253.81	140.74
72h exposure to 14 day old filamentous fungi	28	86.32	39.85	126.17	90.32
Valid N (list wise)	28				

Table 4.6: Descriptive statistics of renal epithelial cell viability alterations induced by individual filamentous fungal species and their combinations cultured on PDA

Exposure time in hours and days	N	Range	Minimum (%)	Maximum (%)	Mean (%)
24h exposure to 4 day old filamentous fungi	28	76.80	86.80	163.60	119.44
48h exposure to 4 day old filamentous fungi	28	298.60	61.60	360.20	177.23
72h exposure to 4 day old filamentous fungi	28	178.30	77.10	255.40	135.94
24h exposure to 9 day old filamentous fungi	28	267.50	59.10	326.60	136.55
48h exposure to 9 day old filamentous fungi	28	120.90	76.00	196.90	119.60
72h exposure to 9 day old filamentous fungi	28	149.60	57.50	207.10	117.57
24h exposure to 14 day old filamentous fungi	28	218.60	60.70	279.30	126.77
48h exposure to 14 day old filamentous fungi	28	140.60	43.40	184.00	86.78
72h exposure to 14 day old filamentous fungi	28	141.50	73.50	215.00	111.70
Valid N (list wise)	28				

Table 4.7: Descriptive statistics of renal epithelial cell viability alterations induced by individual filamentous fungal species and their combinations cultured on MEA

Exposure time in hours and days	N	Range	Minimum (%)	Maximum (%)	Mean (%)
24h exposure to 4 day old filamentous fungi	28	74.70	84.40	159.10	117.65
48h exposure to 4 day old filamentous fungi	28	346.30	71.40	417.70	204.04
72h exposure to 4 day old filamentous fungi	28	111.70	70.20	181.90	111.28
24h exposure to 9 day old filamentous fungi	28	154.20	66.80	221.00	119.21
48h exposure to 9 day old filamentous fungi	28	352.30	77.80	430.10	176.20
72h exposure to 9 day old filamentous fungi	28	148.90	57.20	206.10	117.19
24h exposure to 14 day old filamentous fungi	28	145.20	75.40	220.60	114.20
48h exposure to 14 day old filamentous fungi	28	232.40	64.50	296.90	139.07
72h exposure to 14 day old filamentous fungi	28	152.00	35.60	187.60	87.78
Valid N (list wise)	28				

4.4 Discussion

In this study, '*in vitro*' cell viability alterations were recorded after hepatocytes and renal epithelial cells were exposed to filamentous fungal species individually and in combinations at different time intervals (24, 48 and 72h). The findings of this study revealed that infections by

individual filamentous fungi species could result in either excessive cell proliferation or reduced cell viability of target cell. This therefore shows that the fungi species analysed in this study initially promoted uncontrolled proliferation of cells and eventually resulted in decreased cell viability as exposure continued, confirming that filamentous fungi can be harmful and even detrimental in some cases (Charles *et al.*, 2011). *Fusarium verticillioides* and *P. expansum* were two species apart from the others which induced continuous cell proliferation on hepatocytes even at 72h of exposure. Observations from this experiment also showed that the response of cells to younger cultures (4 and 9 days) was different from the response to older cultures (14 days). There was further reduction of cell viability when cells were exposed to 14 days old spores compared to when cells were exposed to 4 and 9 days old cultures. It can therefore be assumed that the older the fungal culture, the greater their ability to induce toxicity, thereby resulting in lesser viabilities of cell lines. This assumption is supported with the ability of filamentous fungi spores to produce mycotoxins under certain favourable conditions and in this study, the previous chapter described the production of some mycotoxins by these fungi species at different duration of incubation.

Production of secondary metabolites (mycotoxins) could be responsible for the increase and subsequent reduction in viability of experimental cells when exposed to fungi combinations. Most filamentous fungi produce metabolites at the log and lag phases of their growth which in most cases, falls between the 4th and 14th day of growth (Bellí *et al.*, 2005; Klich, 2002c; Pitt and Hocking, 1997c). Mycotoxins have been reported to induce reduction in cell viabilities in many cell lines from animals and human (Mwanza *et al.*, 2009; Richard, 2007; Stoev *et al.*, 2009), inhibiting cellular activities of target cells. Mycotoxins produced by these filamentous fungi such as the trichothecenes, FB_s, and OTA have the potential to disrupt cell membranes and thereby induce apoptosis (programmed cell death) (Königs *et al.*, 2008). It can thus be suspected that these mycotoxins produced by the fungal species and their combinations must

have contributed to the resultant reduction in cell viability. Independent action of filamentous fungal species was in the order of *A. niger* > *P. chrysogenum* > *P. expansum* > *F. verticillioides* > *F. oxysporum* > *A. flavus* for PDA cultured isolates and *A. niger* > *P. chrysogenum* > *P. expansum* > *A. flavus* > *F. verticillioides* > *F. oxysporum* for MEA isolates.

The combination of *A. niger* and other species elicited a high degree of alteration of cell viability. Many factors could be responsible for the up and down movement in cell viability induced by fungal isolates. One of such factors could be the immune response of the cells which resulted in the proliferation of cells in a bid to combat fungal spores and inhibit their activities. Studies have shown that innate immune response in the liver triggers cell proliferation of hepatocytes (Bieghs and Trautwein, 2013; Malato *et al.*, 2008), as was observed in this work. The kidney has the ability to recover completely from toxic effects (Bonventre, 2003). Dedifferentiation and proliferation of epithelial cells in the kidney is a major response of these cells to injury in order to help the kidney recover (Bonventre, 2003; McCampbell and Wingert, 2012). Most probably, after some longer hours of exposure, fungal spores were able to overcome and suppress the inhibitory activities of the immune system of the cells thus causing a disruption in the cell membrane, which eventually resulted in reduced cell viabilities.

Response of hepatocytes and renal epithelial cells to combined filamentous fungi exposure varied slightly between the two cell types. In the case of hepatocytes, cells proliferated after 24 and 48h of exposure with subsequent reduction of cell viability less than 80% in some cases, even with younger cultures (4 days). Renal epithelial cells on the other hand, proliferated extensively after 24 and 48h of exposure to younger cultures (4 and 9 days) and reduced slightly at 72h of exposure but in most cases, not less than 100%. Higher reduction of cell viability less than 100% was recorded when cells were exposed to 14-day old cultures. This slight variation in response indicate that hepatocytes were more susceptible to both old and young cultures of filamentous fungi compared with renal cells, which were more susceptible to older cultures

than younger ones. A possible explanation for the different reactions of both cell lines could be attributed to genes expressed or inflammatory response of cells. Despite these slight difference in responses of both cell lines, filamentous fungi combinations of four or more species induced proliferations which did not fall below 100 percent after 72h of exposure. Proliferation of cells in the kidney, which is one characteristic of the kidney when responding to injury, can be a good explanation for the continuous increase in proliferation of renal cells induced by some fungal species even at 72h. This response of the human cells to fungal combinations gives an indication that additive synergism of mostly *A. niger* isolates and other isolates induced epileptic cell alterations, resulting in very reduced cell viability (Chou, 2006). The pattern of fungal combination effects was C11 > C9 > C8 > C19 > C2 > C10 > C3 > C16 > C13 > C1 > C15 > C6 > C14 > C4 > C12 > C5 > C7 > C18 > C17 for both PDA and MEA cultured isolates.

It can be inferred from this study, that there were some alterations in cellular activities of both hepatocytes and renal epithelial cells which resulted in the eventual reduction of their cell viability when they were exposed to spores of fungal isolates and their combinations. One of such cellular activities is oxidative stress which is an imbalance between the production of free radicals by cells when exposed to harmful substances and their ability to neutralize such harmful effects (Filomeni *et al.*, 2015). Oxidative stress in cells normally increased in cells when they are exposed to harmful substances (Davies, 2000; Filomeni *et al.*, 2015; Turkez *et al.*, 2012) .The relationship between oxidative stress and reduced cell viability can therefore explain the mode of action of fungi species in the experiment causing an initial increased oxidative stress to facilitate eventual cell viability reduction. Another cellular activity which is suspected to have been affected by exposure of the cells to these fungal species is protein synthesis. Protein synthesis is crucial for proper functioning of metabolic processes of every organ in a system (de la Parra *et al.*, 2015). There is also some form of correlation between

protein synthesis in a cell and its cell viability. Findings by de la Parra *et al.* (2015), Wang *et al.* (2011) and Dennis *et al.* (1984) indicate that alterations in protein synthesis can cause reduction of cell viability and possible total cell death if exposure persists. It is therefore suspected that the filamentous fungal species used could have induced alterations in protein synthesis of target cells and thus contribute to the reduction of cell viability. The possibility of competition for space by replicating fungal spores of filamentous fungi combinations could also have played a role in response of the cells

The continued exposure of cells to these fungi and their combinations could be very detrimental to the hepatocytes and renal epithelial cells in vitro (Malhi *et al.*, 2010). Also, the response of the cells upon exposure to fungi species was time dependent as well as dose dependent. Nutritional composition of culture media also contributes to cytotoxic effects with MEA fungi spores inducing more toxic effects in comparison to PDA fungi spores. The resultant effect of reduced or dead hepatocytes to the liver is that most of the metabolic functions of the liver will be epileptic, reduced or halted which would eventually result in liver disorders or failure. The epithelial cells make up a greater portion of the nephron which is a functional unit of the kidney, thus, kidney diseases can be as a result of a damaged nephron (McCampbell and Wingert, 2012). The exposure of cells could also result in induction of cancers and tumours because some of the fungal species used are producers of mycotoxins that have been classified as carcinogens by the International Agency of Research on Cancer (IARC, 1993a-b; IARC, 1993b-a; IARC, 2002a; IARC, 2012b).

4.5 Conclusion

The effect of filamentous fungi on test cells in combination during this study was higher compared to when cells were exposed to individual strains. Response of cells which was time and dose-dependent suggests that there is the possibility of positive synergistic activity between

reference strains during toxicity induction, an indication that the toxic capacity of one specie was enhanced by the other. The study was also able to show that exposure of human hepatocytes and renal epithelial cells to filamentous fungi could result in alterations in their viability and may result in abnormal functions of the liver and kidney.

CHAPTER FIVE

Expression of Human Th1/Th2 cytokines by human hepatocytes exposed to filamentous fungi and combinations

Abstract

Th1 and Th2 cytokines produced by T helper cells are essential for maintaining innate immunity in the human body, particularly in the presence of infection or injury. Interferon gamma IFN-γ, tumour necrosis factor (TNF), interleukin 10 (IL-10), interleukin 5 (IL-5), interleukin 4 (IL-4) and interleukin 2 (IL-2) are some Th1 and Th2 cytokines that play important roles in the innate immune system. Healthy human hepatocytes were exposed to 4, 9 and 14 days old individual filamentous fungi and their combinations "*in vitro*" for 3 to 24h and cytokine expression determined with a cytometric bead array (CBA) human Th1/Th2 cytokine kit and flow cytometry. It was found that hepatocytes exposed to individual filamentous fungi elicited production of cytokines IFN-γ, TNF, IL-10, IL-4 and IL-2 at a range of 0.017-4.863, 0.023-0.460, 0.103-0.367, 0.107-4.183 and 0.203-2.680 respectively. *Fusarium* species induced the highest level of cytokine production by hepatocytes compared to other filamentous fungal genera used. Their combinations also elicited the production of the five cytokines at a range of 0.010-4.720, 0.020-2.093, 0.017-0.623, 0.020-3.693 and 0.037-3.217pg/ml respectively. Furthermore, combinations of two fungal species induced production of high levels of cytokines whereas, combinations of more than two fungal species induced low levels or no production of cytokines at all. The study revealed that filamentous fungi has the ability to induce "*in vitro*" production of cytokines from hepatocytes when the cells are infected and the production of cytokines is an innate immune response by the hepatocytes to combat effects of micro-organisms.

Keywords: Liver, immune response, Interferon gamma, Interleukin 2 and cells

5.1 Introduction

The human body is constantly exposed to infectious substances that induce negative effects, prompting immune responses to remove such compounds from the body. Cells of the innate immune system produce cytokines which act as hormonal messengers communicating with other cells to initiate immune responses to harmful substances or pathogens (Berger, 2000; Lacy and Stow, 2011). Due to the function of cytokines (inducing cell to cell communication as well as immunological and host responses of cells to infectious agents), regulation of cytokine synthesis and release from cells is crucial for the appropriate functioning of the immune system (Lacy and Stow, 2011). Cytokines include lymphocytes, monokines, chemokines and interleukins (Zhang and An, 2007). They are classified into two groups - pro-inflammatory and anti-inflammatory cytokines. Pro-inflammatory cytokines promote inflammation in the presence of infectious agents in the body, making disease worse (Dinarello, 2000), while anti-inflammatory cytokines suppress the activity of genes for pro-inflammatory cytokines, reducing inflammation in order to promote healing (Opal and DePalo, 2000).

Cytokines (also referred to as soluble signalling proteins), are synthesised at the early stages of infections (Reihill *et al.*, 2011) by nearly every cell (Dinarello, 2000; Zhang and An, 2007) to induce mechanisms against infectious agents including bacteria, fungi and viruses. Th1 and Th2 cytokines are two major groups of cytokines (Ku and Lin, 2013) released by CD4 T lymphocytes T-helper type 1(Th1) and T-helper type 2 (Th2). T helper 1 cytokines which comprise of interferon gamma (IFN-γ), interleukin 2 (IL-2), tumour necrosis factor (TNF-α/β) and interleukin 3 (IL-3), mediate cell immunity thus promoting phagocyte-dependent inflammation and help against intracellular infections caused by micro-organisms (Ku and Lin, 2013; Romagnani, 2000). T helper 2 cytokines, made up of interleukin 4 (IL-4), interleukin 5

(IL-5), interleukin 6 (IL-6), interleukin 9 (IL-9), interleukin 10 (IL-10) and interleukin 13 (IL-13) evoke strong antibody responses as well as enhance immune responses against extracellular pathogens (Mahajan and Mehta, 2011; Romagnani, 2000). T helper 1 and Th2 cytokines are involved in the innate immune system and it is observed that Th1 cytokines (TNF-α) enhance inflammatory responses while some Th2 cytokines such as IL-10 and IL-6 inhibit the activity of inflammatory cytokines (Kidd, 2003; Ku and Lin, 2013; Scheller *et al.*, 2011).

Due to the contradicting activities of some Th1 and Th2 cytokines, a balance of production of these hormonal messengers is essential in immune response by cells in order to combat infections by micro-organisms (Kidd, 2003; Torre *et al.*, 2002). The release or production of cytokines by cells in the presence of pathogens can be described as cell-specific or pathogen specific. Some fungal species have been reported to trigger the release of cytokines with Th1 and Th2 cytokines from macrophages, epithelial cells and monocytes when exposed (Figueiredo *et al.*, 2011; Reihill *et al.*, 2011; Warris *et al.*, 2005). Tumour necrosis factor-alpha (TNF-α), IL-6 and IL-8 are common cytokines that have been reported to be released by cells when exposed to some fungal species. Cytokine release in the liver can be associated with inducing inflammation or reducing inflammation (Gao, 2012; Yang, 2011). Interleukin 6 (IL-6), IL-10 and TNF-α are some common cytokines associated with immune response in the liver. Along with the Kupffer cells and other cells in the liver, hepatocytes are able to produce pro-inflammatory and hepato-protective cytokines when there is injury to the liver (Kong *et al.*, 2012).

Most studies on the release of cytokine in the liver have been associated with alcohol-induced injury (Gao, 2012; Kawaratani *et al.*, 2013) and other liver diseases (Braunersreuther *et al.*, 2012; Kong *et al.*, 2012) not associated with fungal infections. The liver is prone to infections by micro-organisms such as fungi and bacteria (Hassan *et al.*, 2014; Kawecki *et al.*, 2014; Liu *et al.*, 2011; Rolando *et al.*, 1991; Rolando *et al.*, 1996) which could result in fatal health

consequences. Filamentous fungi are ubiquitous and in most cases, species and strains co-occur in the same environment. There is limited knowledge of cytokine expression in the liver induced by exposure to filamentous fungi. In order to fill the knowledge gap in terms of cytokine production and filamentous fungi in the liver, this study was conducted in order to determine the degree of cytokine expression induced "*in vitro*" by selected filamentous fungi species on healthy human hepatocytes. This is a novel approach, with special reference to understanding the synergistic effect of two or more filamentous fungal species on Th1/Th2 cytokines production by hepatocytes.

5.2 Methodology

5.2.1 Ethical approval

Ethical approval was sought from the ethics committee of the North-West University, Mafikeng Campus for this study.

5.2.2 Preparation of selected filamentous fungi species

The following filamentous fungi: *Aspergillus flavus, A. niger, F. oxysporum, F. verticillioides, P. chrysogenum* and *P. expansum* species were used for this work. Spore preparations from MEA cultures used previously to determine effect on cell viability by filamentous fungi combinations on proliferation of hepatocytes were used.

5.2.3 Culture of primary human hepatocytes

Healthy human hepatocytes cultured in 96-well TC-treated plates were exposed to spores of 4, 9 and 14 days old cultures of filamentous fungi and their combinations in sterile phosphate buffered solution (PBS) for a duration of 3, 6, 12 and 24h. After exposure, cell suspensions were collected and transferred to sterile 1.5ml eppendorf vials and stored at -80^{o}C for further use.

5.2.4 Preparation of human TH1/TH2 cytokine standards

Levels of Th1 (IFN-γ, TNF, IL-2) and Th2 (IL-10, IL-5, IL-4) cytokines expressed by healthy human hepatocytes upon exposure to filamentous fungi and their combinations were determined using a cytometric Bead Array (CBA) Human Th1/Th2 cytokine kit purchased from (BD Biosciences, USA) The BD CBA assay provides a method for detecting cytokines by capturing analytes (cytokines) with beads of known sizes and fluorescence, making analyte detection possible by flow cytometry. The technique in the assay is such that complexes of capture beads + analyte + detection reagent are formed and are measured by flow cytometry. Lyophilized cytokine standards were reconstituted following the manufacturer's instruction with 2.0ml of assay diluent and serially diluted to produce 9 standard concentrations in the order of top standard (5,000pg/ml), 1:2 (2,500pg/ml), 1:4 (1,250pg/ml), 1:8 (625pg/ml), 1:16 (312.5pg/ml), 1:32 (156pg/ml), 1:64 (80pg/ml), 1:128 (40pg/ml) and 1:256 (20pg/ml). A negative standard vial (0pg/ml) was prepared containing only assay diluent. Capture beads for the six cytokines were mixed in a 50ml centrifuge tube and kept for further work.

5.2.5 Human TH1/TH2 cytokine assay on primary human hepatocytes

Cell suspensions from exposed human hepatocytes were centrifuged at 7,500 g for 3 min. The supernatant of cell suspensions were collected in sterile eppendorf vials. Fifty millilitres of mixed capture beads was transferred to individual sterile 1.5ml eppendorf vials. Another 50µl of the standard dilutions and supernatants of exposed hepatocytes were added to the vials containing capture beads. This was followed by the addition of 50µl of human Th1/Th2 PE detection reagents to the tubes. Assay vials were incubated at room temperature for 3h and kept in the dark. After 3h of incubation, 1ml of wash buffer was added to each assay tube, centrifuged at 200g for 5 min and supernatant from the tubes carefully aspirated and discarded. After aspirating the supernatant from each vial and 300µl of wash buffer was added to the vials to re-suspend the beads. Samples were further acquired on a BD Accuri C6 flow cytometer

provided by Scientific Group (SG), South Africa. Acquisition started with the lowest concentration of standards to the highest concentration followed by the samples.

5.2.6 Analysis of data

Readings derived from fluorescence detection on the flow cytometer were analysed using the FCAP Array v3 software from BD Biosciences, USA to calculate concentration of cytokines. Microsoft Excel, 2010 was implored to determine mean values of cytokine positive replicates and standard deviation. Descriptive statistics was done using a SPSS software, version 22.

5.3 Result

5.3.1 Effect of individual filamentous fungi on hepatocytes

Healthy human hepatocytes cultured with fungal species elicited the production of some Th1 and Th2 cytokines after 3 to 24h of incubation, while *Aspergillus* specie, *A. niger* did not induce production of any of the targeted Th1 and Th2 cytokines. On the other hand, *Aspergillus flavus* was able to elicit the production of some cytokines (Table 5.1 to 5.3) with 4 days old cultures of the fungi eliciting production of highest concentration of IL-2 (1.767pg/ml) and IL-4 (1.453pg/ml). Four-day old *F. oxysporum* spore solution incubated with hepatocytes resulted in production of all the targeted cytokines at varying concentration with the highest concentration (3.140pg/ml) by IL-4. Cytokine expression induced by *F. verticillioides* was different from the expression induced by *F. oxysporum*, producing more concentration of IL-2 and less of IL-4. Spore solution from 4-day old *F. verticillioides* induced highest production of only IL-2 (1.913pg/ml) and IL-4 (1.177pg/ml). *Penicillium* species induced production of fewer of the targeted cytokines, with 4 days old *P. chrysogenum* isolates inducing highest production of IL-2 (0.987pg/ml) and IL-4 (1.343pg/ml); and *Penicllium expansum* spore solution stimulating the production of IL-2 up to 1.243pg/ml after 12h of incubation.

Table 5.1: Cytokine expression of human hepatocytes exposed to 4-day old filamentous fungal spore suspension (pg/ml)

Filamentous fungi	Duration of exposure (h)	IFN-Y	TNF	IL-10	IL-5	IL-4	IL-2
Aspergillus flavus	3	N.D	N.D	0.130 ± 0.02	N.D	1.453 ± 0.117	0.440 ± 0.026
	6	N.D	N.D	N.D	N.D	1.440 ± 0.030	0.960 ± 0.036
	12	N.D	N.D	N.D	N.D	1.330 ± 0.035	1.767 ± 0.023
	24	N.D	N.D	N.D	N.D	N.D	0.333 ± 0.040
Aspergillus niger	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
Fusarium oxysporum	3	0.427 ± 0.015	0.040 ± 0.030	N.D	N.D	2.053 ± 0.095	0.940 ± 0.030
	6	1.057 ± 0.006	0.157 ± 0.032	0.103 ± 0.012	N.D	2.743 ± 0.023	1.243 ±0.032
	12	2.590 ± 0.050	0.460 ± 0.026	0.367 ± 0.032	0.027 ± 0.031	3.140 ± 0.078	1.870 ± 0.066
	24	1.207 ± 0.006	N.D	N.D	N.D	1.617 ± 0.015	0.477 ± 0.023
Fusarium verticillioides	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	0.357 ± 0.023	0.940 ± 0.030
	12	N.D	N.D	N.D	N.D	1.177 ± 0.006	1.913 ± 0.015
	24	N.D	N.D	N.D	N.D	0.057 ± 0.015	0.203 ± 0.006
Penicillium chrysogenum	3	N.D	N.D	N.D	N.D	0.603 ± 0.012	0.240 ± 0.017
	6	N.D	N.D	N.D	N.D	1.343 ± 0.012	0.987 ± 0.029
	12	N.D	N.D	N.D	N.D	0.107 ± 0.080	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
Penicillium expansum	3	N.D	N.D	N.D	N.D	N.D	0.690 ± 0.036
	6	N.D	N.D	N.D	N.D	N.D	0.690 ± 0.569
	12	N.D	N.D	N.D	N.D	N.D	1.243 ± 0.032
	24	N.D	N.D	N.D	N.D	N.D	N.D

N.D- none detected, values presented as mean value ± standard deviation

Nine days old *A. flavus* spores induced expression of IFN-γ, IL-2 and IL-4 up to 2.393, 1.847 and 1.657pg/ml respectively. Fourteen-day old *A. flavus* induced cytokine expression of IFN-γ, IL-2 and IL-4 up to1.830, 1.840 and 2.753pg/ml respectively. Nine and 14 days old *F. oxysporum* spore solution induced expression of all cytokines with the exception of IL-5. Interferon gamma (IFN-γ) was the most expressed at a concentration of 4.863pg/ml by 9-day old spore solution while IL-4 was the most expressed at a concentration of 4.183pg/ml by 14-day old spore solution. Nine-day old spore solution of *F. verticillioides* elicited the production of all cytokines with the exception of IL-10 and IFN-γ was the most expressed (1.460pg/ml) after 3h, whereas, 14-day old spore solution of the isolate induced the production of all cytokines with the exception of IL-10 and IL-5. Nine-day old isolates did not induce the production of any of the cytokines investigated, although 14-day old isolates of *P. chrysogenum* were able to induce the production of IL-2 up to 1.830pg/ml after 12h incubation. Nine-day old isolate spore solution of *P. expansum* induced the production of IFN-γ up to 1.087pg/ml, whereas, 14-day old isolates induced the production of IFN-γ, Il-2 and IL-4 up to 2.130, 0.863 and 1.930pg/ml respectively.

Table 5.2: Cytokine expression of human hepatocytes exposed to 9-day old filamentous fungal spore suspension (pg/ml)

Filamentous fungi	Duration of exposure (h)	IFN-Y	TNF	IL-10	IL-5	IL-4	IL-2
Aspergillus flavus	3	1.927 ± 0.021	N.D	N.D	N.D	1.493 ± 0.119	N.D
	6	2.393 ± 0.035	N.D	N.D	N.D	1.657 ± 0.015	1.223 ± 0.012
	12	0.377 ± 0.015	N.D	N.D	N.D	N.D	1.847 ± 0.012
	24	1.927 ± 0.021	N.D	N.D	N.D	1.493 ± 0.119	N.D
Aspergillus niger	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
Fusarium oxysporum	3	0.870 ± 0.017	N.D	N.D	N.D	2.743 ± 0.055	1.327 ± 0.075
	6	3.443 ± 0.182	N.D	N.D	N.D	3.023 ± 0.085	1.707 ±0.078
	12	4.863 ± 0.050	N.D	0.290 ± 0.053	N.D	2.167 ± 0.068	2.090 ± 0.095
	24	1.550 ± 0.104	0.047 ± 0.045	N.D	N.D	N.D	0.213 ± 0.148
Fusarium verticillioides	3	1.460 ± 0.380	0.047 ± 0.050	N.D	0.027 ± 0.015	1.193 ± 0.061	0.233 ± 0.086
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
Penicillium chrysogenum	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
Penicillium expansum	3	1.087 ± 0.050	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D

N.D- none detected, values presented as mean value ± standard deviation

Table 5.3: Cytokine expression of human hepatocytes exposed to 14-day old filamentous fungal spore suspension (pg/ml)

Filamentous fungi	Duration of exposure (h)	IFN-Ɣ	TNF	IL-10	IL-5	IL-4	IL-2
Aspergillus flavus	3	0.550 ± 0.053	N.D	N.D	N.D	1.810 ± 0.079	N.D
	6	1.830 ± 0.165	N.D	N.D	N.D	2.753 ± 0.093	0.587 ± 0.080
	12	0.527 ± 0.015	N.D	N.D	N.D	1.780 ± 0.219	1.840 ± 0.085
	24	N.D	N.D	N.D	N.D	0.133 ± 0.130	0.090 ± 0.050
Aspergillus niger	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
Fusarium oxysporum	3	2.307 ± 0.200	N.D	N.D	N.D	0.380 ± 0.154	0.363 ± 0.050
	6	2.967 ± 0.074	N.D	N.D	N.D	2.967 ± 0.117	1.130 ± 0.056
	12	3.153 ± 0.071	0.023 ± 0.025	0.180 ± 0.171	N.D	4.183 ± 0.146	2.680 ± 0.193
	24	N.D	N.D	N.D	N.D	1.047 ± 0.095	N.D
Fusarium verticillioides	3	1.810 ± 0.089	N.D	N.D	N.D	0.830 ± 0.108	0.337 ± 0.310
	6	0.017 ± 0.029	N.D	N.D	N.D	1.430 ± 0.272	1.033 ± 0.107
	12	0.790 ± 0.035	0.247 ± 0.222	N.D	N.D	2.180 ± 0.148	2.167 ± 0.144
	24	N.D	0.063 ± 0.060	N.D	N.D	0.623 ± 0.250	0.367 ± 0.365
Penicillium chrysogenum	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	1.350 ± 0.195
	12	N.D	N.D	N.D	N.D	N.D	1.830 ± 0.236
	24	N.D	N.D	N.D	N.D	N.D	N.D
Penicillium expansum	3	1.237 ± 0.115	N.D	N.D	N.D	N.D	1.177 ± 0.144
	6	1.527 ± 0.431	N.D	N.D	N.D	0.863 ± 0.055	1.930 ± 0.046
	12	2.130 ± 0.111	N.D	N.D	N.D	N.D	0.357 ± 0.038
	24	N.D	N.D	N.D	N.D	N.D	N.D

N.D- none detected, values presented as mean value ± standard deviation

5.3.2 Effect of four days old filamentous fungi combinations on hepatocytes

Filamentous fungi combinations induced the production of Th1 and Th2 cytokines in almost the same manner as individual fungal species (Table 5.4 to 5.6). Production of cytokine varied with age of filamentous fungi cultures and combination. Combinations of 4-day old cultures of *A. niger*, *A. flavus*, *F. oxysporum* and *F. verticillioides* (C1); *A. niger*, *A. flavus*, *P. chrysogenum* and *P. expansum* (C2) as well as *A. niger* and *A. flavus* (C3) did not induce production of any of the targeted cytokines. Expression of IFN-γ (3.073pg/ml) and IL-2 (1.223pg/ml) was observed when hepatocytes were incubated with a combination of *A. flavus* and *F. oxysporum* (C4). Some 4-day old filamentous fungi combinations induced varying cytokine production with combinations of *Fusarium* species and other genera inducing production of almost all target cytokines with the exception of IL-5 and in fewer cases, IL-10 (Table 6.4). Combination of *F. oxysporum* and *F. verticilliodes* (C12) induced the production of IFN-γ, TFN, IL-10, IL-4 and IL-2 up to 2.793, 0.070, 0.143, 3.123 and 1.810pg/ml respectively. Another combination of *F. verticillioides* and *P. chrysogenum* (C15) induced the production of five out of the six cytokines up to 4.670, 2.093, 0.623, 1.823 and 2.103pg/ml respectively for IFN-γ, TFN, IL-10, IL-4 and IL-2. Combinations of *A. niger* and the two *Penicillium* species, *C. chrysogenum* and *P. expansum* (C10 and C11) did not elicit any form of cytokine expression by the hepatocytes. The same was observed for the combination of the reference fungal species (C19), with no expression of target cytokines.

Table 5.4: Cytokine expression of human hepatocytes exposed to 4-day old fungal cultures (pg/ml)

Combined filamentous fungi species	Duration of exposure (h)	IFN-Y	TNF	IL-10	IL-5	IL-4	IL-2
C1	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
C2	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
C3	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
C4	3	1.643 ± 0.085	N.D	N.D	N.D	N.D	1.023 ± 0.168
	6	2.007 ± 0.164	N.D	N.D	N.D	N.D	1.223 ± 0.045
	12	3.073 ± 0.135	N.D	N.D	N.D	N.D	0.443 ± 0.168
	24	0.287 ± 0.268	N.D	N.D	N.D	N.D	N.D
C5	3	N.D	N.D	N.D	N.D	N.D	0.300 ± 0.267
	6	N.D	N.D	N.D	N.D	N.D	1.920 ± 0.075
	12	N.D	N.D	N.D	N.D	N.D	3.217 ± 0.201
	24	N.D	N.D	N.D	N.D	N.D	0.217 ± 0.210
C6	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
C7	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
C8	3	1.630 ± 0.044	N.D	N.D	N.D	N.D	0.077 ± 0.071
	6	1.893 ± 0.374	N.D	N.D	N.D	0.040 ± 0.040	0.523 ± 0.234
	12	1.163 ± 0.101	N.D	N.D	N.D	0.133 ± 0.146	1.350 ± 0.331
	24	N.D	N.D	N.D	N.D	N.D	0.183 ± 0.180
C9	3	N.D	N.D	N.D	N.D	N.D	N.D

	6	0.940 ± 0.105	N.D	N.D	N.D	N.D	1.097 ± 0.067
	12	1.357 ± 0.266	0.293 ± 0.311	N.D	N.D	N.D	2.117 ± 0.157
	24	0.067 ± 0.065	N.D	N.D	N.D	N.D	1.230 ± 0.156
C10	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
C11	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
C12	3	2.793 ± 0.040	N.D	N.D	N.D	3.123 ± 0.072	1.810 ± 0.104
	6	1.303 ± 0.133	0.070 ± 0.062	N.D	N.D	0.787 ± 0.107	1.280 ± 1.109
	12	2.343 ± 0.057	0.020 ± 0.035	0.143 ± 0.100	N.D	2.877 ± 0.123	1.443 ± 0.083
	24	0.167 ± 0.289	N.D	0.017 ± 0.029	N.D	1.497 ± 0.229	0.083 ± 0.055
C13	3	0.293 ± 0.159	0.540 ±0.056	N.D	N.D	N.D	N.D
	6	1.917 ± 0.059	1.687 ± 0.045	N.D	N.D	N.D	0.467 ± 0.435
	12	2.960 ± 0.210	0.190 ± 0.190	N.D	N.D	N.D	1.787 ± 0.146
	24	1.853 ± 0.096	N.D	N.D	N.D	N.D	N.D
C14	3	0.497 ± 0.215	N.D	N.D	N.D	1.020 ± 0.157	1.630 ± 0.046
	6	1.050 ± 0.056	N.D	N.D	N.D	1.480 ± 0.195	2.940 ± 0.185
	12	N.D	N.D	N.D	N.D	1.913 ± 0.075	2.620 ± 0.046
	24	N.D	N.D	N.D	N.D	0.753 ± 0.059	1.507 ± 0.032
C15	3	1.893 ± 0.080	0.970 ± 0.137	N.D	N.D	0.027 ± 0.046	0.793 ± 0.032
	6	3.393 ± 0.159	1.710 ± 0.256	0.047 ± 0.045	N.D	1.823 ± 0.076	1.410 ± 0.144
	12	4.670 ± 0.183	2.093 ± 0.070	0.623 ± 0.055	N.D	1.103 ± 0.100	2.103 ± 0.055
	24	2.790 ± 0.161	0.703 ± 0.083	N.D	N.D	N.D	1.237 ± 0.090
C16	3	0.010 ± 0.017	N.D	N.D	N.D	N.D	0.040 ± 0.040
	6	0.777 ± 0.060	N.D	N.D	N.D	0.067 ± 0.07	0.787 ± 0.075
	12	0.143 ± 0.140	N.D	N.D	N.D	0.173 ± 0.081	0.900 ± 0.779
	24	N.D	N.D	N.D	N.D	N.D	0.910 ± 0.046
C17	3	N.D	N.D	N.D	N.D	N.D	0.773 ± 0.047
	6	N.D	N.D	N.D	N.D	0.043 ± 0.040	1.693 ± 0.042
	12	N.D	N.D	N.D	N.D	0.367 ± 0.040	0.973 ± 0.061
	24	N.D	N.D	N.D	N.D	N.D	N.D
C18	3	0.013 ± 0.023	0.037 ± 0.035	N.D	N.D	0.807 ± 0.170	1.457 ± 0.380
	6	0.423 ± 0.101	1.200 ± 0.151	N.D	N.D	1.447 ± 0.197	1.980 ± 0.056

	12	1.327 ± 0.035	1.437 ±0.115	N.D	N.D	2.503 ±0.202	2.787 ± 0.059
	24	N.D	0.827 ± 0.057	N.D	N.D	0.987 ± 0.131	1.127 ± 0.055
C19	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D

N.D- not detected, values presented as mean value ± standard deviation

5.3.3 Effect of nine-day old filamentous fungi combinations on hepatocytes

Nine-day old filamentous fungi combinations which included *A. niger* (C1, C2 and C3) incubated with hepatocytes were not able to stimulate the production of target cytokines. Other filamentous fungi combinations of *A. flavus* with either *Fusarium* or *Penicillium* species induced the expression of some target cytokines. The combination of *A. flavus* and *F. oxysporum* (C4) induced the expression of IFN-γ and IL-2 up to 2.940 and 2.727pg/ml respectively. Combinations of *Aspergillus flavus* and *F. verticillioides* (C5) elicited the expression of up to 2.780pg/ml of IL-2. Combinations of *A. flavus* and *Penicillium* species (Table 5.5) induced the expression of IFN-γ and IL-2 cytokines from hepatocytes when incubated with cells for 3 and 6h. Although *A. niger* species did not elicit cytokine expression by the cells, combinations of *A. niger* and *F. oxysporum* (C8) and *F. verticillioides* (C9) induced expression of varying concentrations of four cytokines (IFN-γ, TNF, IL-4 and IL-2 and three cytokines (IFN-γ, TNF and IL-2) respectively. *Aspergillus niger* and *F. oxysporum* combination (C8) produced lower TNF concentration (0.037pg/ml) when compared to the concentration (0.503pg/ml) produced by the combination of *A. niger* and *F. verticillioides* (C9). Combinations of *A. niger* and *Penicillium* species (C10 and C11) did not elicit the production of any of the target cytokines. It was also observed that combinations of nine-day old *Fusarium* species induced expression of cytokines in the same manner as four-day old fungi combinations. There was expression of IFN-γ, TNF, IL-10, IL-4 and IL-2 at highest

concentration of 3.123 pg/ml, 0.307pg/ml, 0.143pg/ml, 3.693pg/ml and 2.343pg/ml respectively by these combinations containing *Fusarium*. Expression of cytokine was also observed for *Fusarium* and *Penicillium* species combinations (C13, C14, C15 and C16) which followed the same pattern as the four-day old combinations.

Table 5.5: Cytokine expression of human hepatocytes exposed to 9-day old fungal cultures (pg/ml)

Combined filamentous fungi species	Duration of exposure (h)	IFN-Y	TNF	IL-10	IL-5	IL-4	IL-2
C1	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
C2	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
C3	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
C4	3	1.880 ± 0.069	N.D	N.D	N.D	N.D	2.107 ± 0.035
	6	2.583 ± 0.127	N.D	N.D	N.D	N.D	2.727 ± 0.155
	12	2.940 ± 0.075	N.D	N.D	N.D	N.D	0.880 ± 0.070
	24	0.880 ± 0.030	N.D	N.D	N.D	N.D	N.D
C5	3	N.D	N.D	N.D	N.D	N.D	1.673 ± 0.051
	6	N.D	N.D	N.D	N.D	N.D	2.780 ± 0.061
	12	N.D	N.D	N.D	N.D	N.D	1.120 ± 0.066
	24	N.D	N.D	N.D	N.D	N.D	0.763 ± 0.076
C6	3	0.223 ± 0.045	N.D	N.D	N.D	N.D	0.037 ± 0.040
	6	1.407 ± 0.078	N.D	N.D	N.D	N.D	0.790 ±0.053
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
C7	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	1.697 ± 0.042	N.D	N.D	N.D	N.D	N.D

	12	3.597 ± 0.169	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
C8	3	0.123 ± 0.045	N.D	N.D	N.D	0.760 ± 0.036	1.113 ± 0.065
	6	0.780 ± 0.089	N.D	N.D	N.D	1.227 ± 0.045	1.817 ± 0.040
	12	1.790 ± 0.070	0.037 ± 0.035	N.D	N.D	1.593 ± 0.091	2.040 ± 0.193
	24	N.D	N.D	N.D	N.D	N.D	N.D
C9	3	0.040 ± 0.036	0.010 ± 0.017	N.D	N.D	N.D	0.273 ± 0.065
	6	1.120 ± 0.066	0.133 ± 0.006	N.D	N.D	N.D	0.937 ± 0.031
	12	1.473 ± 0.146	0.503 ± 0.470	N.D	N.D	N.D	1.697 ± 0.083
	24	N.D	N.D	N.D	N.D	N.D	0.737 ±0.090
C10	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
C11	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
C12	3	3.123 ± 0.096	N.D	N.D	N.D	3.150 ± 0.193	2.270 ± 0.079
	6	1.570 ± 0.142	0.307 ± 0.163	N.D	N.D	1.083 ± 0.025	2.343 ± 0.045
	12	2.257 ± 0.110	0.057 ± 0.098	0.143 ± 0.127	N.D	3.693 ± 0.112	1.783 ± 0.146
	24	N.D	N.D	N.D	N.D	N.D	N.D
C13	3	1.033 ± 0.057	0.520 ± 0.452	N.D	0.027 ± 0.038	N.D	0.880 ± 0.098
	6	2.387 ± 0.090	1.650 ± 0.066	N.D	1.157 ± 0.099	N.D	1.823 ± 0.060
	12	3.290 ± 0.150	0.523 ± 0.465	N.D	0.013 ± 0.023	N.D	2.197 ± 0.139
	24	N.D	N.D	N.D	N.D	N.D	N.D
C14	3	0.870 ± 0.056	N.D	N.D	N.D	0.450 ± 0.394	1.163 ± 0.206
	6	0.953 ± 0.130	N.D	N.D	N.D	1.123 ± 0.051	2.817 ± 0.108
	12	N.D	N.D	N.D	N.D	1.347 ± 0.065	1.383 ± 0.220
	24	N.D	N.D	N.D	N.D	N.D	1.167 ± 0.071
C15	3	1.310 ± 0.156	0.127 ± 0.125	N.D	N.D	1.017 ± 0.055	0.837 ± 0.085
	6	2.537 ± 0.352	0.883 ± 0.025	N.D	N.D	1.777 ± 0.208	1.753 ± 0.059
	12	4.720 ± 0.090	1.173 ± 0.071	N.D	N.D	0.297 ±0.042	2.043 ± 0.087
	24	N.D	N.D	N.D	N.D	N.D	N.D
C16	3	0.070 ± 0.075	N.D	N.D	N.D	N.D	N.D
	6	0.400 ± 0.101	N.D	N.D	N.D	0.127 ± 0.142	0.617 ± 0.070
	12	N.D	N.D	N.D	N.D	N.D	1.870 ± 0.053

	24	N.D	N.D	N.D	N.D	N.D	0.807 ± 0.075
C17	3	N.D	N.D	N.D	N.D	N.D	0.400 ± 0.347
	6	N.D	N.D	N.D	N.D	0.727 ± 0.093	1.377 ± 0.178
	12	N.D	N.D	N.D	N.D	N.D	0.500 ± 0.123
	24	N.D	N.D	N.D	N.D	N.D	N.D
C18	3	N.D	N.D	N.D	N.D	N.D	0.787 ± 0.059
	6	0.410 ± 0.092	0.047 ± 0.045	N.D	N.D	1.173 ± 0.060	1.387 ± 0.146
	12	0.767 ± 0.060	1.000 ± 0.866	N.D	N.D	2.667 ± 0.040	0.793 ± 0.040
	24	N.D	0.160 ± 0.140	N.D	N.D	0.030 ± 0.036	1.900 ± 0.056
C19	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D

N.D- not detected, values presented as mean value ± standard deviation

5.3.4 Effect of fourteen days old filamentous fungi combinations on hepatocytes

Combinations of filamentous fungi spores harvested after 14-day of incubation elicited some of the target Th1/Th2 cytokines investigated. Some fungal combinations including *A. niger* (C1, C2, C3, C10 and C11) did not induce the production of cytokines. *Aspergillus flavus* and *Fusarium* species combinations (C4 and C5) induced the expression of cytokines (Table 5.6). Combinations of *A. flavus* and *P. chrysogenum* (C6) elicited cytokines IFN-γ and IL-2 up to 0.750 and 0.983pg/ml respectively, while *A. flavus* and *P. expansum* (C7) combinations did not induce the production of any cytokines. Combinations of *F. oxysporum*/*A. niger* (C8) and *F. verticillioides*/*A. niger* (C9) induced production of four cytokines (IFN-γ, TNF, IL-4 and IL-2) and two cytokines (IFN-γ, IL-2) respectively. The combination of *F. verticillioides* and *F. oxysporum* (C12) induced the production of cytokines IFN-γ, TNF, IL-10, IL-4 and IL-2 up to 1.847, 1.283, 0.107, 3.307 and 1.880pg/ml respectively. Production of cytokines was also induced by combinations of *F. oxysporum* and *P. chrysogenum* (C13) as well as *F. oxysporum* and *P. expansum* (C14). Interferon gamma (IFN-γ), TNF, IL-4 and IL-2 were expressed up to 2.547, 1.100, 0.100pg/ml and 1.760pg/ml respectively by C13 filamentous fungi combination, while C14 induced the production of IFN-γ, IL-4 and IL-2 up to 0.033, 1.383 and 2.097pg/ml respectively. Combinations of *F. verticillioides* and *Penicillium* species, *P. chrysogenum* (C15) and *P. expansum* (C16) also induced the production of cytokines as well as C17 (*P. chrysogenum* and *P. expansum*) and C18 (*F. oxysporum*, *F. verticillioides*, *P. chrysogenum* and *P. expansum*). Combinations of all the isolates (C19) used in this study did not elicit the production of any cytokine as observed in 4 and 9-day old spore exposure.

Table 5.6: Cytokine expression of human hepatocytes exposed to 14 days old fungal cultures in pg/ml

Combined filamentous fungi species	Duration of exposure (h)	IFN-Y	TNF	IL-10	IL-5	IL-4	IL-2
C1	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
C2	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
C3	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
C4	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	0.870 ± 0.040	N.D	N.D	N.D	N.D	0.037 ± 0.035
	12	2.750 ± 0.120	N.D	N.D	N.D	N.D	1.080 ± 0.066
	24	0.207 ± 0.268	N.D	N.D	N.D	N.D	0.583 ± 0.093
C5	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	0.613 ± 0.095
	12	N.D	N.D	N.D	N.D	N.D	1.647 ± 0.172
	24	N.D	N.D	N.D	N.D	N.D	0.113 ± 0.106
C6	3	0.063 ± 0.071	N.D	N.D	N.D	N.D	0.160 ± 0.072
	6	0.750 ± 0.096	N.D	N.D	N.D	N.D	0.983 ± 0.070
	12	N.D	N.D	N.D	N.D	N.D	0.037 ± 0.040
	24	N.D	N.D	N.D	N.D	N.D	N.D
C7	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
C8	3	0.080 ± 0.092	0.053 ± 0.076	N.D	N.D	N.D	1.607 ± 0.025
	6	1.267 ± 0.093	0.223 ± 0.038	N.D	N.D	0.153 ± 0.025	1.993 ± 0.081
	12	1.240 ± 0.175	0.570 ± 0.095	N.D	N.D	N.D	0.870 ± 0.056
	24	0.217 ± 0.193	N.D	N.D	N.D	N.D	N.D

C9	3	N.D	N.D	N.D	N.D	N.D	0.043 ± 0.045
	6	0.127 ± 0.045	N.D	N.D	N.D	N.D	0.700 ± 0.082
	12	0.823 ± 0.060	N.D	N.D	N.D	N.D	1.497 ± 0.060
	24	0.133 ± 0.135	N.D	N.D	N.D	N.D	0.587 ± 0.060
C10	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
C11	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D
C12	3	2.620 ± 0.046	N.D	0.017 ± 0.029	N.D	3.307 ± 0.367	1.880 ± 0.070
	6	1.093 ± 0.067	0.820 ± 0.123	0.107 ± 0.095	N.D	2.427 ± 0.234	1.820 ± 0.110
	12	1.847 ± 0.106	1.283 ± 0.138	0.020 ± 0.035	N.D	2.263 ± 0.150	0.223 ± 0.085
	24	0.523 ± 0.096	N.D	N.D	N.D	0.953 ± 0.106	N.D
C13	3	0.077 ± 0.071	N.D	N.D	N.D	0.020 ± 0.035	N.D
	6	1.303 ± 0.110	0.880 ± 0.056	N.D	N.D	0.100 ± 0.089	0.660 ± 0.070
	12	2.547 ± 0.107	1.100 ± 0.151	N.D	N.D	N.D	1.760 ± 0.075
	24	1.040 ± 0.111	0.077 ± 0.071	N.D	N.D	N.D	0.040 ± 0.036
C14	3	0.033 ± 0.031	N.D	N.D	N.D	0.450 ± 0.394	0.700 ± 0.066
	6	N.D	N.D	N.D	N.D	0.640 ± 0.053	1.290 ± 0.036
	12	N.D	N.D	N.D	N.D	1.383 ± 0.031	2.097 ± 0.040
	24	N.D	N.D	N.D	N.D	N.D	1.323 ± 0.060
C15	3	0.730 ± 0.046	0.140 ± 0.036	N.D	N.D	0.250 ± 0.030	0.153 ± 0.038
	6	1.950 ± 0.036	0.887 ± 0.031	N.D	N.D	0.263 ± 0.045	1.060 ± 0.036
	12	3.700 ± 0.036	1.810 ± 0.050	N.D	N.D	1.323 ± 0.040	1.637 ± 0.055
	24	0.953 ± 0.163	0.107 ± 0.025	N.D	N.D	N.D	0.703 ± 0.038
C16	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	0.197 ± 0.047	N.D	N.D	N.D	N.D	0.147 ± 0.035
	12	0.697 ± 0.081	N.D	N.D	N.D	N.D	0.837 ± 0.045
	24	N.D	N.D	N.D	N.D	N.D	N.D
C17	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	0.083 ± 0.042
	12	N.D	N.D	N.D	N.D	N.D	0.523 ± 0.038
	24	N.D	N.D	N.D	N.D	N.D	N.D
C18	3	0.047 ± 0.042	N.D	N.D	N.D	0.153 ± 0.031	0.720 ± 0.050

	6	0.833 ± 0.060	0.030 ± 0.052	N.D	N.D	1.620 ± 0.040	1.430 ± 0.056
	12	1.237 ± 0.127	0.100 ± 0.044	N.D	N.D	1.710 ± 0.080	2.093 ± 0.078
	24	N.D	N.D	N.D	N.D	0.623 ± 0.040	0.673 ± 0.015
C19	3	N.D	N.D	N.D	N.D	N.D	N.D
	6	N.D	N.D	N.D	N.D	N.D	N.D
	12	N.D	N.D	N.D	N.D	N.D	N.D
	24	N.D	N.D	N.D	N.D	N.D	N.D

N.D- not detected, values presented as mean value ± standard N.D- not detected, values presented as mean value ± standard deviation

Statistical significance calculated for cytokines expressed by hepatocytes (Table 5.7) shows that values obtained ranged between 0.014 – 0.941 with standard error value 0.797 – 1.589. The statistical outcome can be attributed to low concentration of cytokines expressed and in some cases, no cytokine expressed.

Table 5.7: Statistical calculation of concentration of cytokines expressed by hepatocytes after exposure to combination of filamentous fungi

Cytokines expressed by hepatocytes	Unstandardized Coefficients		Standardized Coefficients			95.0% Confidence Interval for B	
	B	Std. Error	Beta	t	Sig.	Lower Bound	Upper Bound
(Constant)	3.222	1.589		2.028	.057	-.103	6.547
IFN	-2.303	.856	-.798	-2.691	.014	-4.094	-.512
TNF	.515	1.063	.156	.485	.633	-1.709	2.739
IL-10	-.091	1.203	-.021	-.076	.941	-2.609	2.427
IL-4	.636	.797	.223	.798	.434	-1.032	2.305
IL-2	.687	.876	.233	.784	.443	-1.147	2.521

5.4 Discussion

Mycotoxin producing filamentous fungi which induced abnormal proliferation of hepatocytes and resultant reduction in cell viability after 72h of exposure in the previous chapters were used to investigate their individual and synergistic ability to induce cytokine production "*in vitro*" by the hepatocytes. Common cytokines associated with the innate immune system include

tumour necrosis factor (TNF-α), interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 4 (IL-4), interleukin 10 (IL-10), interleukin 12 (IL-12), interferon alpha (IFN-α) and interferon gamma (IFN-γ). These cytokines perform different activities in immune response and have the ability to work in synergy to induce or reduce inflammation (Dinarello, 2000; Lacy and Stow, 2011; Opal and DePalo, 2000). Also, some cytokines such as IL-6 inhibit the production of TNF and IL-1 (Opal and DePalo, 2000; Scheller *et al.,* 2011). Although cytokines are reported to be produced mainly by monocytes, lymphocytes and some epithelial cells (Reihill *et al.*, 2011; Warris *et al.*, 2005; Zhang and An, 2007) in response to microbial infections, cytokine production has been reported to be possible in almost every cell in mammals (Dinarello, 2000; Zhang and An, 2007). In this study, the production of cytokine by human hepatocytes was induced through individual filamentous fungi and their combinations. This finding, therefore, correlates with reports by Dong *et al.* (1998a); Panesar *et al.* (1999) and Joshi-Barve *et al.* (2007) that hepatocytes can produce cytokines when stimulated by certain compounds or substances. Out of the six cytokines screened for, five were expressed by hepatocytes at certain duration of exposure.

5.4.1 IFN-γ production by hepatocytes

Production of interferon gamma by hepatocytes exposed to individual filamentous fungi species increased with the age of fungal cultures harvested. It was observed that 4-day old fungi did not induce the production of IFN-γ as much as 9-day and 14-day old cultures. This is because only 4-day old *Fusarium oxysporum* isolates were able to induce production of the cytokine. It is therefore assumed that 4-day old cultures of most filamentous fungi species do not have the capacity to trigger release of IFN-γ from hepatocytes. Interferon gamma encourages fungus-specific CD4 T cell differentiation and promotes antifungal immunity (Espinosa and Rivera, 2012). This could explain the increase in production of IFN-γ when hepatocytes were exposed to older cultures of filamentous fungi, as an antifungal activity. The

production of cytokine by hepatocytes in this study is also supported by the fact that in the liver, IFN-γ is expressed to induce immune modulation thereby regulating hepatocyte apoptosis and cell cycle progression (Horras *et al.*, 2011). The function of this cytokine in the liver is therefore important in suppressing or controlling tumour progression when the liver is exposed to compounds or organisms that could trigger such a reaction as well as regulating the size of the liver and hepatic immune responses (Horras *et al.*, 2011). It can thus be suggested that the release of IFN-γ by hepatocytes exposed to filamentous fungi in this work was an anti-fungal response by the hepatocytes to regulate abnormal reactions that could occur as a result of exposure to fungal species. The non-production of IFN-γ by hepatocytes exposed to *A. niger* and *P. chrysogenum* species which was also observed is supported by the fact that there is no documented production of IFN-γ stimulated by these species. There are several studies on *A. fumigatus* and *C. albicans* inducing IFN-γ expression by cells (Brown and Netea, 2007; Levitz and North, 1996; Liebhart *et al.*, 2002; Wiseman and Mody, 2007) which confirms that filamentous fungi can induce the production of cytokine.

The effect of combinations of filamentous fungi on human hepatocytes showed a similar pattern to that of individual filamentous fungal species (production of IFN-γ by hepatocytes increased with age of fungal spores). Although individual *A. niger* species did not induce the production of IFN-γ, its combination with *F. oxysporum* and *F. verticillioides* elicited cytokine expression. *Fusarium oxysporum* and *F. verticillioides* were able to induce the production of IFN-γ from hepatocytes individually and it is thus believed that their combination elicited the production of cytokines. The combination of *A. flavus* and *F. oxysporum* (C4) induced the production of IFN-γ while the combination of *A. flavus* and *F. verticillioides* (C5) did not induce the production of IFN-γ. The study also revealed that there was synergistic activity between combinations of filamentous fungal species to elicit the expression of IFN-γ by the hepatocytes. A combination of *F. verticillioides* and *P. chrysogenum* (C15) induced the highest

expression of IFN-γ (4.72pg/ml) although not as high as the lowest concentration of standard cytokines (20pg/ml) used in the experiment. This complements earlier results of the work which showed that *F. verticilloides* produced fumonisin and DON (Fig. 4.5 and 4.6). Both mycotoxins (FBs and DON) are reported to be cytotoxic and immune suppressive (Gutleb *et al.*, 2002; Pestka *et al.*, 2004; Rotter *et al.*, 1996; Theumer *et al.*, 2002). The production of these mycotoxins by the fungal species while exposed to cells might explain the expression of IFN-γ. Deoxynivalenol stimulates expression of cytokines when present in low doses and immune suppression when the dose is increased (Pestka *et al.*, 2004). The outcome of this study thus correlates with the finding as IFN-γ was expressed even though DON and FB concentration was not very high.

Expression of IFN-γ by the hepatocytes induced by the effect of both filamentous fungi and combinations probably started after 3h of incubation and gradually increased up to 12h. There was a decline in the concentration of expressed IFN-γ by 24 hours and in some cases, there was no expression at 24h. The increase in cytokine expression between 3 and 12h of incubation is because IFN-γ has been reported to be rapidly produced after infection by fungi (Brown and Netea, 2007) and its production continues after 18h of exposure with dead cells being able to continue releasing cytokine (Levitz and North, 1996). Though concentration of IFN-γ produced by the hepatocytes was lower than the least standard concentration (20pg/ml), it is remarkable to know that some of these filamentous fungi were able to elicit expression of the cytokine irrespective of the minimal concentration.

5.4.2 Production of TNF by hepatocytes

Expression of TNF was observed in hepatocytes exposed to filamentous fungal species and their combination. However, the degree of TNF production by cells in response to the spore suspension of filamentous fungi was less compared to production of IFN-γ in this work. Micro-

organisms (bacteria and viruses) are reported to induce the "*in vitro*" production of TNF by hepatocytes and also "in vivo" (González-Amaro *et al.*, 1994; Santos *et al.*, 2011; Takano *et al.*, 2012) with as much as 146pg/ml expressed by hepatocytes exposed to extracts of the bacterium *Salmonella typhimurium* (Santos *et al.*, 2011). Although there are no reports of the production of TNF by hepatocytes exposed to filamentous fungi, other cells (leukocytes, monocytes, macrophages and mononuclear cells) have been reported to express TNF when exposed to filamentous fungi such as *A. fumigatus* (Figueiredo *et al.*, 2011; Liebhart *et al.*, 2002; Warris *et al.*, 2005). The production of TNF by hepatocytes exposed to some of the filamentous fungi and their combinations complement these reports. Tumour necrosis factor (TNF), which is a cytokine that contributes to the immune system by promoting migration of neutrophils, is very important in resisting invasive filamentous fungi infections (Borish *et al.*, 1989; Mehrad *et al.*, 1999; Warris *et al.*, 2005).

The study revealed that some *Fusarium* and *Penicillium* species have the capacity to induce production of TNF. This cytokine, which affects the expression of systemic cytokines when expressed by hepatocytes (Takano *et al.*, 2012), was produced by hepatocytes in this work which were exposed to filamentous fungi and their combinations. Individual filamentous fungi species were not able to induce the production of TNF from the hepatocytes except *F. oxysporum* and *F. verticilliodes* at very low concentrations. Expression of TNF from hepatocytes increased when 9-day old individual filamentous fungi species were exposed to the hepatocytes. This could be because of age of the harvested fungal spores or other cytokines expressed because some cytokines act as inhibitors for the production of other cytokines in the immune system (Lacy and Stow, 2011; Scheller *et al.*, 2011). Filamentous fungi combinations of *Fusarium*/*Aspergillus* species and *Fusarium*/*Penicillium* species (C8, C9, C13, C15 and C18) induced the production of TNF with higher concentrations observed from filamentous fungi combinations compared to individual fungi species. It can thus be assumed that a

synergistic action of the different fungal species increased the production of TNF which reduced as age of fungal spores increased. Production of TNF by hepatocytes after incubation with filamentous fungi was rapid after 3h incubation and in some cases, 6h (Table 5.4 and 5.6). This conforms to reports of rapid TNF production by hepatocytes with subsequent reduction after some hours up to 48 hours (Glezerman *et al.*, 1998; Warris *et al.*, 2005; Wollenberg *et al.*, 1993). Generally, cytokines are produced rapidly by cells in response to injury or infection (Lacy and Stow, 2011).

5.4.3 Production of IL-10 by hepatocytes

The function of interleukin 10 in the immune system is to inhibit acute inflammation and limit proliferative response of hepatocytes when there is injury or infection (Louis *et al.*, 1998). Production of IL-10 is common with many cells involved in the immune system (Wagener *et al.*, 2014; Zhang and Wang, 2006) and hepatocytes have also been reported to produce this cytokine (Ishizaka *et al.*, 1996; Nishitani *et al.*, 2007; Zhang and Wang, 2006). Out of the six filamentous fungi species used, only *F. oxysporum* was able to induce expression of the cytokine and the level of IL-10 produced reduced as age of fungal cultures increased. Interleukin-10 promotes susceptibility to infections by micro-organisms (Zhang and Wang, 2006), thereby suggesting that lack of production of this cytokine by hepatocytes in the experiment increases the ability of the cells (hepatocytes) to withstand infections by the fungi. The combination of filamentous fungi *F. oxysporum* and *F. verticillioides* (C12) was able to induce the production of IL-10 which reduced as the age of the fungal spores increased. Like other cytokines, the production of IL-10 was rapid, in most cases, after 3 or 6h of incubation confirming the report that production of IL-10 in response to infection is an early regulatory mechanism that controls abnormal proliferative response after liver injury or infection (Louis *et al.*, 1998).

5.4.4 Production of IL-4 by hepatocytes

The hepatocytes expressed IL-4, one of the most influential cytokines in the immune system (Brown, 2008). Production of IL-4, a cytokine involved in multiple immune responses in the system, was observed from hepatocytes incubated with both filamentous fungal species and their combinations, although there is little or no report of hepatocyte producing this cytokine when exposed to filamentous fungi. Production of interleukin 4 by hepatocytes exposed to *A. flavus*, *F. oxysporum* and *F. verticillioides* spores increased gradually as the age of the culture increased with 14 days old spores eliciting the highest concentrations of IL-4 (4.183pg/ml). Older cultures of filamentous fungi had more viable spores thus, the tendency to induce more expression of the cytokine. *Penicillium expansum* on the other hand, induced production of IL-4 which reduced with increase in age of fungal spores. The production of IL-4 had earlier been reported to be cytokine dependent or independent (Hu-Li *et al.*, 1997).

Combinations of *Fusarium* species and species of other genera (C8, C12, C14, C15, C16, C17 & C18) induced production of IL-4 and highest concentration of 3.693pg/ml (Table 5.5) observed from hepatocytes incubated with *F. verticillioides* and *F. oxysporum* combination (C12). This, therefore, suggests that there was synergistic action between the two fungi species which induced production of IL-4 as the fungal species were able to elicit expression of the cytokine singularly. The production of mycotoxins (FB1, DON and NIV) by the two fungal species (*F. verticillioides* and *F. oxysporum*) could be another explanation for expression of IL-4. Fumonisin produced by *Fusarium* species is reported to induce increased level of cytokine by cells (Theumer *et al.*, 2002). Expression of IL-4 which was induced by filamentous fungi combinations increased up to 9-day old fungi combinations but reduced with 14-day old combination. It can be assumed that combinations of older spores antagonised themselves and brought about reduced expression of the cytokine. Also, combinations of two filamentous fungi induced higher concentrations of IL-4 compared to more than two combinations indicating that

the more the filamentous fungi species combinations, the lesser the possibility of production of IL-4. As observed with other cytokines, expression of IL-4 began at the 3rd hour of incubation and continued until the 24th hour of incubation with most combinations. This is because IL-4 is reported to be produced by cells immediately after infection by micro-organisms (Lee *et al.*, 2013). There are previous reports of production of IL-4 by other cells aside from hepatocytes (Brandt *et al.*, 2000; Kamogawa *et al.;* Lee *et al.*, 2013) but none has been reported about the production of IL-4 by hepatocytes. This study therefore shows that hepatocytes can produce IL-4 when exposed to filamentous fungi.

5.4.5 Production of IL-2 by hepatocytes

IL-2, another major cytokine produced by T cells in response to infection or injury is important for controlling autoimmunity in the body system by influencing production of $CD4^+$ and $CD25^+$ T regulatory cells (Boyman and Sprent, 2012; Malek, 2003; Zelante *et al.*, 2012). Aside from IFN-γ, IL-2 is another cytokine expressed by hepatocytes incubated with most fungi spore suspension in this work. All individual filamentous fungi spores with the exception of *A. niger*, induced the expression of IL-2 by hepatocytes and *Fusarium* species induced expression of most of the IL-2. Expression of IL-2 by hepatocytes when incubated with 4 and 9 day old fungal cultures but a reduced concentration at 14-day old cultures, an indication that spores from older cultures of individual filamentous fungi induced less expression of IL-2. Although there are several reports on the production of IL-2 by other cells apart from hepatocytes during infections Gaffen and Liu (2004) and Rojo *et al.* (1986) reported fungal-influenced production of IL-2 by mice lymphocytes.

Combinations of filamentous fungi species induced similar responses with some combinations of *Fusarium* and *Penicillium* species not eliciting production of IL-2. Also, it was observed that combinations of more than two fungal species did not elicit any production of IL-2

suggesting that the more the combination of fungal species, the lesser the possibility of production of IL-2 by hepatocytes. A comparison of production of IL-2 induced by 4, 9 and 14 days old filamentous fungal spore combinations revealed that there was not much difference in production of the cytokine, although with some combinations, expression decreased as spores got older. Production of IL-2 in most instances, was optimal at 12h of incubation and decreased at 24h of incubation. Like most cytokines, IL-2 is usually expressed after 2 to 3h of incubation (Granucci *et al.*, 2003; Kaplan, 2010; Zelante *et al.*, 2012) with a stimulant. This study also confirms that production of IL-2 began at the stage of exposure of hepatocytes to the filamentous fungi.

5.5 Conclusion

Th1 and Th2 cytokines were produced by hepatocytes exposed to filamentous fungi and their combinations. Although the level of production of these cytokines by the hepatocytes was low when compared to standards used and reports of cytokine expression induced by other micro-organisms, it was proven that hepatocytes can produce cytokines when exposed to filamentous fungi or their combinations. A novel finding of this work is the ability of *F. verticilioides* and *F. oxysporum* to induce production of cytokines in hepatocytes. This is an indication that hepatocytes contribute to the innate immune system when exposed to fungi contributing to combating infections. Finally, it could be concluded that there is a balance in production of Th1 and Th2 cytokines by hepatocytes upon exposure to filamentous fungi with more production of IFN-γ, Il-4 and IL-2.

CHAPTER SIX

General discussion and conclusion

Micro-organisms cause infections and diseases in the human body resulting in alteration of different cellular functions or activities in the system. The ability of micro-organisms to induce cell proliferation or reduction in cell viability has been attributed to both bacteria and filamentous fungi (Rowell *et al.*, 1997) as well as the ability to elicit the expression of inflammatory cytokines (Brown and Netea, 2007; Wiseman and Mody, 2007). Uncontrolled cell proliferation as a response of cells to microbial infections can be detrimental to the body if not controlled resulting in tumours and cancerous growths. On the other hand, reduction in cell viability of cells exposed to microbial infection can eventually result in complete cell death and complete shutdown of the organ to which the affected cells belong (Stoev *et al.*, 2009; Stoev *et al.*, 2002). Also, the expression of cytokines in the human body in the presence of microbial infections can either promote progression or decrease of infections, depending on the type of cytokines expressed by producing cells (Torre *et al.*, 2002). This study showed that some fungi are able to induce abnormal cellular response in hepatocytes and renal epithelial cells with regard to cell multiplication. Also, they are able to induce the expression of certain cytokines by target cells when exposed to them.

6.1 Production of metabolite by fungi and reduction of cell viability reduction

The study revealed that fungi are able to induce reduction of cell viability of healthy cells if exposure is prolonged. Although, *P. chrysogenum* and *P. expansum* did not produce metabolites (mycotoxins) screened for, it will be inappropriate to conclude that these fungi were free of other metabolites not screened for. It can be assumed that production of metabolites contributed to induction of initial cell proliferation and subsequent reduction in cell viability of hepatocytes and renal epithelial cells. It was further observed that younger

cultures (4 and 9 day old) were able to induce increased cell proliferation and reduction of cell viability responses from both renal epithelial cells and hepatocytes compared to 14-day old cultures even though spore concentration of 14-day old fungal cultures were more than 4 and 9 days old fungal cultures.

Mycotoxins are known to cause health disorders in different parts of the human body, including the digestive, immune, reproductive, urinary and central nervous system (Doi and Uetsuka, 2011). Mycotoxins possess membrane-active properties that enable them to penetrate membranes thereby resulting to changes in physiological functions of organs in the system (Doi and Uetsuka, 2011). Bouaziz *et al.* (2008) maintained that mycotoxins affect cells in terms of modulating proliferation, differentiation, cell reduction and cell death in both human and animal systems.

Although *Penicillium* species did not produce the mycotoxins screened for, they elicited the same response from experimental cells as the other fungal species. It will be safe to conclude that the *Penicillium* species produced other metabolites that were not screened for and that they probably contributed to the responses of the cells. The ability of filamentous fungi to induce reduction of cell viability is supported by Bensassi *et al.* (2012) who maintained that *Alternaria* species induced reduction of cell viability and subsequent cell death. There was synergistic activity between the fungal species in combination with combinations of *A. niger* and species of the genera *Fusarium* and *Penicillium* inducing higher responses with regard to initial cell proliferation and subsequent reduction of cell viability. This response may be due to the synergistic ability of OTA and fumonisins to exert additive deleterious effects (Klarić *et al.*, 2013; Pfohl-Leszkowicz, 2009; Stoev and Denev, 2013). The combination of isolates producing AFs, OTA, FUMs, NIV and DON (chromatograms represented in the appendix) induced reduction in cell viability at 24 hours of incubation and further reduction in cell

viability after 72 hours, indicating that the synergistic action of mycotoxins contributed to the effects.

6.2 Production of cytokines by hepatocytes and metabolites by filamentous fungi

The expression of cytokines in response to injury or infection is commonly associated with certain cells in the body system including monocytes (Johannessen *et al.*, 2009), mononuclear cells (Warris *et al.*, 2005), leucocytes (Elsässer-Beile *et al.*, 1996) and kupffer cells (Kawaratani *et al.*, 2013). In some cases, hepatocytes have been reported to produce cytokines either when there is injury to the liver or in the presence of an infection (Gutierrez-Ruiz *et al.*, 2001; Joshi-Barve *et al.*, 2007; Kawaratani *et al.*, 2013). This study confirms previous findings as it showed the ability of filamentous fungi species and their combinations to induce expression of both Th1 and Th2 human cytokines.

As regards the production of metabolites by filamentous fungi and expression of cytokines by hepatocytes when exposed to filamentous fungal species, the study revealed that even though *A. niger* produced OTA at low concentration, it did not exert production of any of the cytokines. Furthermore, it was observed in this study that filamentous fungi producing FBs, DON, NIV and AFs as well as combinations of these filamentous fungi induced expression of different cytokines at different concentrations, eliciting most of the target cytokines immediately after incubation. This is an indication that mycotoxins produced by the fungi, contributed to eliciting cytokine expression by the cells. The findings of this study could be supported by reports of human hepatocytes expressing cytokines in the presence of microbial infections (Rowell *et al.*, 1997) or toxic substances (Dong *et al.*, 1998a; Dong *et al.*, 1998b) and expression of antigen-specific cytokines by hepatocytes in response to stimuli (Rowell *et al.*, 1997).

6.3 Cell proliferation or reduction and production of cytokines by hepatocytes

Individual fungal species which induced high degrees of responses regarding cell proliferation were able to induce the production of cytokines at different concentrations with the exception of *A. niger* which did not induce expression of any of the target cytokines even though it induced a high degree of cell proliferation of hepatocytes. *Penicillium* species which also induced proliferation of cells at 24h, although not as high as *A. niger*, induced expression of some of the cytokines including IL-2 and IL-4. *Fusarium* species which induced the lowest rate of cell proliferation compared to other fungal species were able to induce production of most of the cytokines.

The response of hepatocytes with regard to cell viability and the expression of cytokines when exposed to filamentous fungi species followed the pattern of individual filamentous fungi where there was subsequent increase in cell proliferation and subsequent reduction of cell viability as incubation time increased. Combinations of *A. niger* and *Penicillium* species as well as *A. flavus* species which induced higher rates of cell proliferation of hepatocytes at 24h of exposure induced reduced cytokine expression and sometimes, no production of cytokines. *Fusarium* species, in combination with all the other filamentous fungi species, induced the lowest rate of cell proliferation at 24h of exposure to the cells. This therefore suggests of a possible enhancement of *Fusarium* species to induce such reactions in the cells because of the synergistic action of most filamentous fungi when they co-occur. Although there is no documentary evidence that compares cell viability of cells to production of cytokine by cells, statistical correlation analysis showed a generally moderate to weak positive relationship between cell proliferation of hepatocytes at 24h exposure to filamentous fungi and the different cytokines. This therefore indicates that as hepatocyte cell viability increased in response to the exposure of fungal species, production of cytokines by the same cells also increased.

In conclusion, toxicity induction by filamentous fungi on human cells can be expressed in different forms. The study was able to show that exposure of human hepatocytes and renal epithelial cells to mycotoxin-producing filamentous fungi and their combinations can result in epileptic replication of cells as well as expression of cytokines to a certain degree. This study was able to evaluate the relationship between toxicity induction by filamentous fungi on healthy human cells and the ability of these cells to produce cytokine as an immune response to them. The ability of these cells to produce cytokines in response to infection by fungal species shows that they play immune-regulatory roles in the organs and the whole body. Similarly, production of Th1 and Th2 cytokines within the first 3 to 12h of exposure by the experimental cells indicated a balance in immune response such that anti-inflammatory and pro-inflammatory cytokines were evenly expressed to prevent progression of the infection, although cytokine expression stopped mostly at 12h of exposure. Subsequent reduction of cell viability of both hepatocytes and renal epithelial cells induced by filamentous fungi at 72h of exposure is an indication that continuous exposure to these filamentous fungi, either individually or in combinations, could result in complete cell death and subsequent shut down of the organs. Finally, production of deoxynivalenol (DON) by *F. oxysporum* and expression of cytokines by hepatocytes upon exposure to the fungi species are novel findings that emerged from this study. There is therefore the need for more research into molecular reactions such as DNA stability, gene expression, and other cellular activities involved in the reaction of these cells to filamentous fungi combinations.

References

Abarca, M. L., Bragulat, M. R., Castellá, G. & Cabañes, F. J. 1994. Ochratoxin A production by strains of *Aspergillus niger* var. *niger*. *Applied and Environmental Microbiology,* 60**,** 2650-2652.

Abbas, A. K. & Lichtman, A. H. 2010. *Basic immunology: functions and disorders of the immune system,* Saunders/Elsevier.

Abbas, H. K., Ocamb, C. M., Xie, W., Mirocha, C. J. & Shier, W. T. 1995. First report of fumonisin B1, B2 and B3 production by *Fusarium oxysporum var. redolens*. *Plant Disease,* 79.

Abbas, H. K., Weaver, M. A., Horn, B. W., Carbone, I., Monacell, J. T. & Shier, W. T. 2011. Selection of *Aspergillus flavus* isolates for biological control of aflatoxins in corn. *Toxin Reviews,* 30**,** 59-70.

Abbas, H. K., Yoshizawa, T. & Shier, W. T. 2013. Cytotoxicity and phytotoxicity of trichothecene mycotoxins produced by *Fusarium* spp. *Toxicon,* 74**,** 68-75.

Abdulkadar, A. H. W., Al-Ali, A. A., Al-Kildi, A. M. & Al-Jedah, J. H. 2004. Mycotoxins in food products available in Qatar. *Food Control,* 15**,** 543-548.

Abeywickrama, K. & Bean, G. A. 1992. Cytotoxicity of *Fusarium* species mycotoxins and culture filtrates of *Fusarium* species isolated from the medicinal plant *Tribulus terrestris* to mammalian cells. *Mycopathologia,* 120**,** 189-193.

Ahmadi, B., Hashemi, S. J., Zaini, F., Shidfar, M. R., Moazeni, M., Mousavi, B., Noorbakhsh, F., Gheramishoar, M., Hossein pour, L. & Rezaie, S. 2012. A case of onychomycosis caused by *Aspergillus candidus*. *Medical Mycology Case Reports,* 1**,** 45-48.

Ahmed, H., Strub, C., Hilaire, F. & Schorr-Galindo, S. 2015. First report: *Penicillium adametzioides,* a potential biocontrol agent for ochratoxin-producing fungus in grapes, resulting from natural product pre-harvest treatment. *Food Control,* 51**,** 23-30.

Aktaş, A. H., Yılmazer, M. & Demirci, Ş. 2004. Determination of patulin in apple juice produced in Isparta, Turkey by HPLC with diode array detection. *Journal of Food and Drug Analysis,* 12**,** 228-231.

Amadio, J., Casey, E. & Murphy, C. 2013. Filamentous fungal biofilm for production of human drug metabolites. *Applied Microbiology and Biotechnology,* 97**,** 5955-5963.

Antony, M., Shukla, Y. & Janardhanan, K. K. 2003. Potential risk of acute hepatotoxicity of kodo poisoning due to exposure to cyclopiazonic acid. *Journal of Ethnopharmacology,* 87**,** 211-214.

Araújo, K. C. F., de M.B. Costa, E. M., Pazini, F., Valadares, M. C. & de Oliveira, V. 2013. Bioconversion of quercetin and rutin and the cytotoxicity activities of the transformed products. *Food and Chemical Toxicology,* 51**,** 93-96.

Archer, D. B. 2000. Filamentous fungi as microbial cell factories for food use. *Current Opinion in Biotechnology,* 11**,** 478-483.

Arivudainambi, U., Kanugula, K., Kotamraju, S., Karunakaran, C. & Rajendran, A. 2014. Cytotoxic and antibacterial activities of secondary metabolites from endophytic fungus *Pestalotiopsis virgatula* VN2. *Current Research in Environmental & Applied Mycology* 4**,** 107-115.

Arvanitidou, M., Kanellou, K., Constantinidis, T. & Katsouyannopoulos, V. 2000. Higher prevalence of *Alternaria* spp. in marine and river waters than in potable samples. *Microbiological Research,* 155**,** 49-51.

Asan, A. 2011. Checklist of *Fusarium* species reported from Turkey. *Mycotaxon,* 116.

Astoreca, A., Magnoli, C., Ramirez, M. L., Combina, M. & Dalcero, A. 2007. Water activity and temperature effects on growth of Aspergillus niger, A. awamori and A. carbonarius isolated from different substrates in Argentina. *International Journal of Food Microbiology,* 119**,** 314-318.

Barkai-Golan, R. 2008. Chapter 6 - *Aspergillus* Mycotoxins. *In:* Rivka, B.-G. & Nachman, P. (eds.) *Mycotoxins in Fruits and Vegetables.* San Diego: Academic Press.

Barthomeuf, C., Regerat, F. & Pourrat, H. 1991. Production of inulinase by a new mold *Penicillium rugulosum*. *Journal of Fermentation and Bioengineering,* 72**,** 491-494.

Baud, L. 2003. Renal Epithelial Cells: Differentiation and Plasticity. *Journal of the American Society of Nephrology,* 14**,** S1-S2.

Bellí, N., Ramos, A. J., Coronas, I., Sanchis, V. & Marín, S. 2005. *Aspergillus carbonarius* growth and ochratoxin A production on a synthetic grape medium in relation to environmental factors. *Journal of Applied Microbiology,* 98**,** 839-844.

Benndorf, D., Muller, A., Bock, K., Manuwald, O., Herbarth, O. & von Bergen, M. 2008. Identification of spore allergens from the indoor mould *Aspergillus versicolor*. *Allergy,* 63**,** 454-460.

Bennett, J. W. 1998. Mycotechnology: the role of fungi in biotechnology1. *Journal of Biotechnology,* 66**,** 101-107.

Bennett, J. W. & Klich, M. 2003. Mycotoxins. *Clinical Microbiology Reviews,* 16**,** 497-516.

Bennett, J. W. & Klich, M. 2009. Mycotoxins. *In:* Schaechter, M. (ed.) *Encyclopedia of Microbiology (Third Edition).* Oxford: Academic Press.

Bensassi, F., Gallerne, C., Sharaf El Dein, O., Hajlaoui, M. R., Bacha, H. & Lemaire, C. 2012. Cell death induced by the *Alternaria* mycotoxin Alternariol. *Toxicology In Vitro,* 26**,** 915-923.

Berger, A. 2000. Th1 and Th2 responses: what are they? *BMJ,* 321**,** 424.

Berkeley, M. J. 1857. Introduction to cryptogamic botany. *London: Bailliere.* **,** 604.

Bieghs, V. & Trautwein, C. 2013. The innate immune response during liver inflammation and metabolic disease. *Trends in Immunology,* 34**,** 446-452.

Bonventre, J. V. 2003. Dedifferentiation and Proliferation of Surviving Epithelial Cells in Acute Renal Failure. *Journal of the American Society of Nephrology,* 14**,** S55-S61.

Bony, S., Olivier-Loiseau, L., Carcelen, M. & Devaux, A. 2007. Genotoxic potential associated with low levels of the *Fusarium* mycotoxins nivalenol and fusarenon X in a human intestinal cell line. *Toxicology In Vitro,* 21**,** 457-465.

Borish, L., Rosenbaum, R., Albury, L. & Clark, S. 1989. Activation of neutrophils by recombinant interleukin 6. *Cellular Immunology,* 121**,** 280-289.

Bouaziz, C., Sharaf el dein, O., El Golli, E., Abid-Essefi, S., Brenner, C., Lemaire, C. & Bacha, H. 2008. Different apoptotic pathways induced by zearalenone, T-2 toxin and ochratoxin A in human hepatoma cells. *Toxicology,* 254**,** 19-28.

Boyman, O. & Sprent, J. 2012. The role of interleukin-2 during homeostasis and activation of the immune system. *Nature Reviews: Immunology,* 12**,** 180-190.

Brandt, E., Woerly, G., Younes, A. B., Loiseau, S. & Capron, M. 2000. IL-4 production by human polymorphonuclear neutrophils. *Journal of Leukocyte Biology,* 68**,** 125-130.

Braunersreuther, V., Viviani, G. L., Mach, F. & Montecucco, F. 2012. Role of cytokines and chemokines in non-alchoholic fatty liver disease *World Journal of Gastroenterology,* 18**,** 727-735.

Brown, D. W., Butchko, R. A. E., Baker, S. E. & Proctor, R. H. 2012. Phylogenomic and functional domain analysis of polyketide synthases in *Fusarium*. *Fungal Biology,* 116**,** 318-331.

Brown, G. D. & Netea, M. G. 2007. *Immunology of fungal infections,* Springer Science and Business Media.

Brown, M. A. 2008. IL-4 Production by T Cells: You Need a Little to Get a Lot. *The Journal of Immunology,* 181**,** 2941-2942.

Burdock, G. A. & Flamm, W. G. 2000. Review Article: Safety assessment of the mycotoxin cyclopiazonic acid. *International Journal of Toxicology,* 19**,** 195-218.

Butinar, L., Zalar, P., Frisvad, J. C. & Gunde-Cimerman, N. 2005. The genus *Eurotium* – members of indigenous fungal community in hypersaline waters of salterns. *FEMS Microbiology Ecology,* 51**,** 155-166.

Calvo, A. M., Wilson, R. A., Bok, J. W. & Keller, N. P. 2002. Relationship between Secondary Metabolism and Fungal Development. *Microbiology and Molecular Biology Reviews,* 66**,** 447-459.

Cetin, Y. & Bullerman, L. B. 2005. Cytotoxicity of *Fusarium* mycotoxins to mammalian cell cultures as determined by the MTT bioassay. *Food and Chemical Toxicology,* 43**,** 755–764.

Chakravarthi, B., Sujay, R., Kuriakose, G., Karande, A. & Jayabaskaran, C. 2013. nhibition of cancer cell proliferation and apoptosis-inducing activity of fungal taxol and its precursor baccatin III purified from endophytic *Fusarium solani*. *Cancer Cell International,* 13**,** 1-11.

Charles, M. V. P., Joseph, N. M., Easow, J. M. & Ravishankar, M. 2011. Invasive pulmonary aspergillosis caused by *Aspergillus versicolor* in a patient on mechanical ventilation. *Australiasian Medical Journal* 4**,** 632-634.

Chaudhuri, R., Ansari, F. A., Raghunandanan, M. V. & Ramachandran, S. 2011. FungalRV: adhesin prediction and immunoinformatics portal for human fungal pathogens. *BMC Genomics,* 12**,** 1-14.

Chávez, R., Roa, A., Navarrete, K., Trebotich, J., Espinosa, Y. & Vaca, I. 2010. Evaluation of properties of several cheese-ripening fungi for potential biotechnological applications. *Mycoscience,* 51**,** 84-87.

Chithra, V., Rao, T., Sathiavathy, K., Suseela, K. & Binoy, K. 2008. Onychomycosis due to *Fusarium oxysporum*. *The Internet Journal of Infectious diseases,* 7.

Chou, T.-C. 2006. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacological Reviews,* 58**,** 621-681.

Chu, F. S. & Li, G. Y. 1994. Simultaneous occurrence of fumonisin B1 and other mycotoxins in moldy corn collected from the People's Republic of China in regions with high incidence of esophageal cancer. *Applied Environmental Microbiology,* 60**,** 847-852.

Cocchi, S., Codeluppi, M., Venturelli, C., Bedini, A., Grottola, A., Gennari, W., Cavrini, F., Di Benedetto, F., De Ruvo, N., Rumpianesi, F., Gerunda, G. E. & Guaraldi, G. 2011. *Fusarium verticillioides* fungemia in a liver transplantation patient: successful

treatment with voriconazole. *Diagnostic Microbiology and Infectious Disease,* 71**,** 438-441.

Consigny, S., Dhedin, N., Datry, A., Choquet, S., Leblond, V. r. & Chosidow, O. 2003. Successsful Voriconazole Treatment of Disseminated *Fusarium* Infection in an Immunocompromised Patient. *Clinical Infectious Diseases,* 37**,** 311-313.

Corrier, D. E. 1991. Mycotoxicosis: mechanisms of immunosuppression. *Verterinary immunology and immunopathology,* 30**,** 73-87.

Craddock, V. M. 1992. Aetiology of oesophageal cancer: some operative factors. *European Journal of Cancer Prevention,* 1**,** 89-103.

Creppy, E. E., Chiarappa, P., Baudrimont, I., Borracci, P., Moukha, S. & Carratù, M. R. 2004. Synergistic effects of fumonisin B1 and ochratoxin A: are in vitro cytotoxicity data predictive of in vivo acute toxicity? *Toxicology,* 201**,** 115-123.

Cruz, R., Santos, C., Silva de Lima, J., Aparecida Moreira, K. & Maria de Souza-Motta, C. 2013. Diversity of *Penicillium* in soil of Caatinga and Atlantic Forest areas of Pernambuco, Brazil: an ecological approach. *Nova Hedwigia,* 97**,** 543-556.

D'Annibale, A., Rosetto, F., Leonardi, V., Federici, F. & Petruccioli, M. 2006. Role of Autochthonous Filamentous Fungi in Bioremediation of a Soil Historically Contaminated with Aromatic Hydrocarbons. *Applied and Environmental Microbiology,* 72**,** 28-36.

D' Mello, J. P. F. & Macdonald, A. M. C. 1997. Mycotoxins. *Animal Feed Science and Technology,* 69**,** 155-166.

Davies, K. J. A. 2000. Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. *IUBMB Life,* 50**,** 279-289.

Dayalan, S. A. J., Darwin, P. & Prakash, S. 2011. Comparative study on production, purification of penicillin by *Penicillium chrysogenum* isolated from soil and citrus samples. *Asian Pacific Journal of Tropical Biomedicine,* 1**,** 15-19.

de la Parra, C., Borrero-Garcia, L. D., Cruz-Collazo, A., Schneider, R. J. & Dharmawardhane, S. 2015. Equol, an Isoflavone Metabolite, Regulates Cancer Cell Viability and Protein Synthesis Initiation via c-Myc and eIF4G. *Journal of Biological Chemistry,* 290**,** 6047-6057.

Deacon, J. 2006. *Fungal biology,* Oxford, UK, Blackwell Publishing.

Dennis, H. J. S., van Walraven, H. S., Frederik, A. C. W., Wilbert, A. M. L., van Rijn, J., van den Berg, J. & van Wijk, R. 1984. Thermotolerance in Cultured Hepatoma Cells: Cell

Viability, Cell Morphology, Protein Synthesis, and Heat-Shock Proteins. *Radiation Research,* 98**,** 82-95.

Deshpande, S. D. & Koppikar, G. V. 1999. A study of mycotic keratitis in Mumbai. *Indian Journal of Pathololology and Microbiology,* 42**,** 81-87.

DeWitte-Orr, S. J. & Bols, N. C. 2005. Gliotoxin-induced cytotoxicity in three salmonid cell lines: Cell death by apoptosis and necrosis. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* 141**,** 157-167.

Dinarello, C. A. 2000. Proinflammatory cytokines*. *Chest,* 118**,** 503-508.

Doi, K. & Uetsuka, K. 2011. Mechanisms of Mycotoxin-Induced Neurotoxicity through Oxidative Stress-Associated Pathways. *International Journal of Molecular Sciences,* 12**,** 5213-5237.

Dong, W., Simeonova, P. P., Gallucci, R., Matheson, J., Fannin, R., Montuschi, P., Flood, L. & Luster, M. I. 1998a. Cytokine Expression in Hepatocytes: Role of Oxidant Stress. *Journal of Interferon & Cytokine Research,* 18**,** 629-638.

Dong, W., Simeonova, P. P., Gallucci, R., Matheson, J., Flood, L., Wang, S., Hubbs, A. & Luster, M. I. 1998b. Toxic Metals Stimulate Inflammatory Cytokines in Hepatocytes through Oxidative Stress Mechanisms. *Toxicology and Applied Pharmacology,* 151**,** 359-366.

Dutton, M. F. 1996. Fumonisins, mycotoxins of increasing importance: Their nature and their effects. *Pharmacology and Therapeutics,* 70**,** 137-161.

Dutton, M. F. 2009. The African *Fusarium*/maize disease. *Mycotoxin Research,* 25**,** 29-39.

Edel-Hermann, V., Gautheron, N., Mounier, A. & Steinberg, C. 2015. *Fusarium* diversity in soil using a specific molecular approach and a cultural approach. *Journal of Microbiological Methods,* 111**,** 64-71.

Egbuta, M. A., Chilaka, C. A., Phoku, J. Z., Mwanza, M. & Dutton, M. F. 2013. Co-contamination of Nigerian cocoa and cocoa-based powder beverages destined for human consumption by mycotoxins. *Ethno Medicine,* 7**,** 187-194.

Egbuta, M. A., Mwanza, M. & Dutton, M. F. 2015a. Evaluation of five major mycotoxins co-contaminating two cereal grains from Nigeria. *International Journal of Biochemistry Research and Review,* 6**,** 160-169.

Egbuta, M. A., Mwanza, M., Njobeh, P. B., Phoku, J. Z. & A, C. C. 2015b. Isolation of Filamentous Fungi Species Contaminating Some Nigerian Food Commodities. *Journal of Food Research,* 4**,** 38-50.

Egner, P. A., Wang, J. B., Zhu, Y. R., Zhang, B. C., Wu, Y., Zhang, Q. N., Qian, G. S., Kuang, S. Y., Gange, S. J., Jacobson, L. P., Helzlsouer, K. J., Bailey, G. S., Groopman, J. D. & Kensler, T. W. 2001. Chlorophyllin intervention reduces aflatoxin-DNA adducts in individuals at high risk for liver cancer. *Proceedings of the National Academy of Science USA,* 98**,** 14601-14606.

Elsässer-Beile, U., Willenbacher, W., Bartsch, H. H., Gallati, H., Mönting, J. S. & von Kleist, S. 1996. Cytokine production in leukocyte cultures during therapy with echinacea extract. *Journal of Clinical Laboratory Analysis,* 10**,** 441-445.

Engelhart, S., Loock, A., Skutlarek, D., Sagunski, H., Lommel, A., Fa¨rber, H. & Exner, M. 2002. Occurrence of toxigenic *Aspergillus versicolor* isolates and sterigmatocystin in carpet dust from damp indoor environments. *Applied and Environmental Microbiology,* 68**,** 3886-3890.

Esnakula, A. K., Summers, I. & Naab, T. J. 2013. Fatal disseminated *Fusarium* infection in a Human Immunodeficiency Virus positive patient. *Case Reports in infectious diseases,* 2013**,** 1-5.

Espinosa, V. & Rivera, A. 2012. Cytokines and the regulation of fungus-specific CD4 T cell differentiation. *Cytokine,* 58**,** 100-106.

Fakruddin, M., Chowdhury, A., Hossain, M. N. & Ahmed, M. M. 2015. Characterization of aflatoxin producing *Aspergillus flavus* from food and feed samples. *SpringerPlus,* 4**,** 159.

Fausto, N. 2000. Liver regeneration. *Journal of Hepatology,* 32, Supplement 1**,** 19-31.

Fausto, N. & Campbell, J. S. 2003. The role of hepatocytes and oval cells in liver regeneration and repopulation. *Mechanisms of Development,* 120**,** 117-130.

Fehr, M., Baechler, S., Kropat, C., Mielke, C., Boege, F., Pahlke, G. & Marko, D. 2010. Repair of DNA damage induced by the mycotoxin alternariol involves tyrosyl-DNA phosphodiesterase 1. *Mycotoxin Research,* 26**,** 247-256.

Figueiredo, R. T., Carneiro, L. A. M. & Bozza, M. T. 2011. Fungal Surface and Innate Immune Recognition of Filamentous Fungi. *Frontiers in Microbiology,* 2**,** 248.

Filomeni, G., De Zio, D. & Cecconi, F. 2015. Oxidative stress and autophagy: the clash between damage and metabolic needs. *Cell Death and Differentiation,* 22**,** 377-388.

Fisher, G., Müller, T., Schwalbe, R., Ostrowski, R. & Dott, W. 2000. Exposure to airborne fungi, MVOC and mycotoxins in biowaste-handling facilities. *International Journal of Hygiene and Environmental Health,* 203**,** 37-104.

Fog Nielsen, K. 2003. Mycotoxin production by indoor molds. *Fungal Genetics and Biology,* 39**,** 103-117.

Fontaine, T., Delangle, A., Simenel, C., Coddeville, B., van Vliet, S. J., van Kooyk, Y., Bozza, S., Moretti, S., Schwarz, F., Trichot, C., Aebi, M., Muriel Delepierre, M., Elbim, C., Romani, L. & Latge, J.-P. 2011. Galactosaminogalactan, a new immunosuppressive polysaccharide of *Aspergillus fumigatus*. *PLoS Pathogens,* 7.

Frisvad, J. C., Smedsgaard, J., Larsen, T. O. & Samson, R. A. 2004. Mycotoxins, drugs and other extrolites produced by species in *Penicillium* subgenus *Penicillium*. *Studies in Mycology,* 49**,** 201-241.

Funa, N., Awakawa, T. & Horinouchi, S. 2007. Pentaketide resorcylic acid synthesis by type III polyketide synthase from *Neurospora crassa The Journal of Biological Chemistry,* 282**,** 14476-14481.

Funnell-Harris, D. L. & Pedersen, J. F. 2011. Presence of *Fusarium* spp. in Air and Soil Associated with Sorghum Fields. *Plant Disease,* 95**,** 648-656.

Gaffen, S. L. & Liu, K. D. 2004. Overview of interleukin-2 function, production and clinical applications. *Cytokine,* 28**,** 109-123.

Galimberti, R., Torre, A. C., Baztán, M. C. & Rodriguez-Chiappetta, F. 2012. Emerging systemic fungal infections. *Clinics in Dermatology,* 30**,** 633-650.

Gammelsrud, A., Solhaug, A., Dendelé, B., Sandberg, W. J., Ivanova, L., Kocbach Bølling, A., Lagadic-Gossmann, D., Refsnes, M., Becher, R., Eriksen, G. & Holme, J. A. 2012 Enniatin B-induced cell death and inflammatory responses in RAW 267.4 murine macrophages. *Toxicology and Applied Pharmacology,* 261**,** 74-87.

Gangadevi, V. & Muthumary, J. 2007. Preliminary studies on cytotoxic effect of fungal taxol on cancer cell lines *African Journal of Biotechnology,* 6**,** 1382-1386.

Gao, B. 2012. Hepatoprotective and anti-inflammatory cytokines in alcoholic liver disease. *Journal of Gastroenterology and Hepatology,* 27**,** 89-93.

Gebala, B. & Sandle, T. 2013. Comparison of Different Fungal Agar for the Environmental Monitoring of Pharmaceutical-Grade Cleanrooms. *PDA Journal of Pharmaceutical Science and Technology,* 67**,** 621-633.

Geiger, M., Guitton, Y., M. Vansteelandt, M., Kerzaon, I., Blanchet, E., Robiou du Pont, T., Frisvad, J. C., Hess, P., Pouchus, Y. F. & Grovel, O. 2013. Cytotoxicity and mycotoxin production of shellfish-derived *Penicillium* spp., a risk for shellfish consumers. *Letters in Applied Microbiology,* 57**,** 385-392

Georgiadou, S. P. & Kontoyiannis, D. P. 2012. Concurrent lung infections in patients with hematological malignancies and invasive pulmonary aspergillosis: How firm is the *Aspergillus* diagnosis? *Journal of Infection,* 65**,** 262-268.

Georgiadou, S. P., Velegraki, A., Arabatzis, M., Neonakis, I., Chatzipanagiotou, S., Dalekos, G. N. & Petinaki, E. 2014. Cluster of *Fusarium verticillioides* bloodstream infections among immunocompetent patients in an internal medicine department after reconstruction works in Larissa, Central Greece. *Journal of Hospital Infection,* In press.

Glezerman, M., Maymon, B. P., Prinsloo, I., Benharroch, D., Yanai-Inbar, I. & Huleihel, M. 1998. Tumor necrosis factor-alpha and interleukin-6 are differently expressed by fresh human cancerous ovarian tissue and primary cell lines. *European Cytokine Network,* 9**,** 171-179.

Gniadek, A., Macura, A. B. & Gorkiewicz, M. 2011. Cytotoxicity of *Aspergillus* fungi isolated from hospital environment. *Polish Journal of Microbiology,* 60**,** 59-63.

Goldbeck, R., Ramos, M. M., Pereira, G. A. G. & Maugeri-Filho, F. 2013. Cellulase production from a new strain *Acremonium strictum* isolated from the Brazilian Biome using different substrates. *Bioresource Technology,* 128**,** 797-803.

Gonçalves, A. B., Paterson, R. R. M. & Lima, N. 2006. Survey and significance of filamentous fungi from tap water. *International Journal of Hygiene and Environmental Health,* 209**,** 257-264.

Gonçalves, S. S., Cano, J. F., Stchigel, A. M., Melo, A. S., Godoy-Martinez, P. C., Correa, B. & Guarro, J. 2012. Molecular phylogeny and phenotypic variability of clinical and environmental strains of *Aspergillus flavus*. *Fungal Biology,* 116**,** 1146-1155.

González-Amaro, R., García-Monzón, C., García-Buey, L., Moreno-Otero, R., Alonso, J. L., Yagüe, E., Pivel, J. P., López-Cabrera, M., Fernández-Ruiz, E. & Sánchez-Madrid, F. 1994. Induction of tumor necrosis factor alpha production by human hepatocytes in chronic viral hepatitis. *The Journal of Experimental Medicine,* 179**,** 841-848.

Gordon, T. R. & Martyn, R. D. 1997. The evolutionary biology of *Fusarium oxysporum*. *Annual Review Phytopathology,* 35**,** 111-128.

Gordon, T. R., Swett, C. L. & Wingfield, M. J. 2015. Management of *Fusarium* diseases affecting conifers. *Crop Protection,* 73**,** 28-39.

Gordon, T. R. a. M., R.D. . . 1997. The evolutionary biology of *Fusarium oxysporum*. *Annual Review Phytopathology,* 35**,** 111-128.

Goswami, S., Rani, A., Priyadarshini, R., Bhunia, B. & Mandal, T. 2012a. A review on production of echinocandins by *Aspergillus* sp. *Journal of Biochemical Technology*, 4**,** 568-575.

Goswami, S., Vidyarthi, A. S., Bhunia, B. & Mandal, T. 2012b. A review on lovastatin and its production. *Journal of Biochemical technology*, 4**,** 581-587.

Granucci, F., Feau, S., Angeli, V., Trottein, F. & Ricciardi-Castagnoli, P. 2003. Early IL-2 production by mouse dendritic cells is the result of microbial-induced priming. *Journal of Immunology,* 170**,** 5075-5081.

Grollman, P. A. & Jelakovic, B. 2007. Role of environmental toxins in endemic (Balkan) nephropathy. *Journal of the American Society of Nephrology,* 18.

Gugnani, H. C., Talwar, R. S., Njoku-Obi, A. N. U. & Kodilinye, H. C. 1976. Mycotic keratitis in Nigeria: A study of 21 cases. *British Journal of Opthalmology,* 60**,** 607-613.

Guimarães, L. H. S., Peixoto-Nogueira, S. C., Michelin, M., Rizzatti, A. C. S., Sandrim, V. C., Zanoelo, F. F., Aquino, A. C. M. M., Junior, A. B. & Polizeli, M. d. L. T. M. 2006. Screening of filamentous fungi for production of enzymes of biotechnological interest. *Brazilian Journal of Microbiology,* 37**,** 474-480.

Gutierrez-Ruiz, M. C., Gomez Quiroz , L. E., Hernandez, E., Bucio, L., Souza, V., Llorente, L. & Kershenobich, D. 2001. Cytokine response and oxidative stress produced by ethanol, acetaldehyde and endotoxin treatment in HepG2 cells. *Israel Medical Association Journal,* 3**,** 131-136.

Gutleb, A. C., Morrison, E. & Murk, A. J. 2002. Cytotoxicity assays for mycotoxins produced by *Fusarium* strains: a review. *Environmental Toxicology and Pharmacology,* 11**,** 309-320.

Hageskal, G., Lima, N. & Skaar, I. 2009. The study of fungi in drinking water. *Mycological Research,* 113**,** 165-172.

Hameed, S., Sultana, V., Ara, J., Ehteshamul-Haque, S. & Athar, M. 2009. Toxicity of *Fusarium solani* strains on brine shrimp (*Artemia salina*). *Zoological Research,* 30**,** 468-472.

Hassan, E. A., El-Rehim, A. S. A., Hassany, S. M., Ahmed, A. O., Elsherbiny, N. M. & Mohammed, M. H. 2014. Fungal infection in patients with end-stage liver disease: low frequency or low index of suspicion. *International Journal of Infectious Diseases,* 23**,** 69-74.

Hayette, M. P., Christiaens, G., Mutsers, J., Barbier, C., Huynen, P., Melin, P. & de Mol, P. 2010. Filamentous fungi recovered from the water distribution system of a Belgian university hospital. *Medical Mycology,* 48**,** 969-974.

He, H., Yang, H. Y., Bigelis, R., Solum, E. H., Greenstein, M. & Carter, G. T. 2002. Pyrrocidines A and B, new antibiotics produced by a filamentous fungus. *Tetrahedron Letters,* 43**,** 1633-1636.

Hedayati, M. T., Pasqualotto, A. C., Warn, P. A., Bowyer, P. & D. W. Denning, D. W. 2007a. *Aspergillus flavus*: human pathogen, allergen and mycotoxin producer. *Microbiology,* 153**,** 1677-1692.

Hedayati, M. T., Pasqualotto, A. C., Warn, P. A., Bowyer, P. & Denning, D. W. 2007b. *Aspergillus flavus*: Human pathogen, allergen and mycotoxin producer. *Microbiology,* 153**,** 1677-1692.

Hedayati, M. T., Pasqualotto, A. C., Warn, P. A., Bowyer, P. & Denning, D. W. 2007c. Aspergillus flavus: human pathogen, allergen and mycotoxin producer. *Microbiology,* 153**,** 1677-1692.

Heussner, A. H., Dietrich, D. R. & O'Brien, E. 2006. In vitro investigation of individual and combined cytotoxic effects of ochratoxin A and other selected mycotoxins on renal cells. *Toxicology In Vitro,* 20**,** 332-341.

Horn, B. W. & Dorner, J. W. 1998. Soil Populations of *Aspergillus* Species from Section *Flavi* along a Transect through Peanut-Growing Regions of the United States. *Mycologia,* 90**,** 767-776.

Horras, C. J., Lamb, C. L. & Mitchell, K. A. 2011. Regulation of Hepatocyte Fate by Interferon-γ. *Cytokine & Growth Factor Reviews,* 22**,** 35-43.

Hou, H., Zhou, R., Li, A., Li, C., Li, Q., Liu, J. & Jiang, B. 2014. Citreoviridin inhibits cell proliferation and enhances apoptosis of human umbilical vein endothelial cells. *Environmental Toxicology and Pharmacology,* 37**,** 828-836.

Howard, D. H. 2002. Pathogenic Fungi in Humans and Animals Ist Ed. *Marcel and Dekker Inc. New York,* 422-424.

Howard, D. H. 2003. Pathogenic Fungi in Humans and Animals. 2nd ed. *Marcel Dekker Inc. New York,* 237-430.

Hu-Li, J., Huang, H., Ryan, J. & Paul, W. E. 1997. In differentiated CD4+ T Cells, interleukin 4 production is cytokine-autonomous, whereas interferon γ production is cytokine-dependent. *Proceedings of the National Academy of Sciences,* 94**,** 3189-3194.

Huang, S., Wang, J., Xing, L., Shen, H., Yan, X., Wang, J. & Zhang, X. 2014. Impairment of cell cycle progression by sterigmatocystin in human pulmonary cells in vitro. *Food and Chemical Toxicology,* 66**,** 89-95.

Hubka, V., Kolařík, M., Kubátová, A. & Peterson, S. W. 2013. Taxonomic revision of *Eurotium* and transfer of species to *Aspergillus*. *Mycologia,* 105**,** 912-937.

Hughes, K. A., Bridge, P. & Clark, M. S. 2007. Tolerance of Antarctic soil fungi to hydrocarbons. *Science of the Total Environment,* 372**,** 539-548.

Hussein, H. S. & Brasel, J. M. 2001. Toxicity, metabolism, and impact of mycotoxins on humans and animals. *Toxicology,* 167**,** 101-134.

Hymery, N., Léon, K., Carpentier, F. G., Jung, J. L. & Parent-Massin, D. 2009. T-2 toxin inhibits the differentiation of human monocytes into dendritic cells and macrophages. *Toxicology In Vitro,* 23**,** 509-519.

IARC 1993a-a. Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. *International Agency for Research on Cancer, Lyon, France,* Monograph 56.

IARC. 1993a-b. *RE: Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins.*

IARC. 1993b-a. *RE: Ochratoxin A. Monographs on the evaluation of carcinogenic risks to humans.*

IARC 1993b-b. Ochratoxin A. Monographs on the evaluation of carcinogenic risks to humans, Vol. 56, some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. *Int. Agency Res. Can. Lyon, France***,** 489-521.

IARC. 2002a. *RE: Traditional herbal medicines, some mycotoxins, naphthalene and styrene. Monographs on the evaluation of carcinogenic risks to humans.*

IARC 2002b. Traditional herbal medicines, some mycotoxins, naphthalene and styrene. Monographs on the evaluation of carcinogenic risks to humans. *IARC,* **,** 82-171.

IARC 2012a. Aflatoxins. *IARC monographs on chemical agents and related occupations,* 100F**,** 225-248.

IARC. 2012b. *RE: Aflatoxins.*

Imwidthaya, P., Thipsuvan, K., Chaiprasert, A., Danchaivijitra, S., Sutthent, R. & Jearanaisilavong, J. 2001. *Penicillium marneffei*: Types and drug susceptibility. *Mycopathologia,* 149**,** 109-115.

Inglis, P. W. & Tigano, M. S. 2006. Identification and taxonomy of some entomopathogenic *Paecilomyces* spp. (*Ascomycota*) isolates using rDNA-ITS Sequences. *Genetics and Molecular Biology,* 29**,** 132-136.

Irzykowska, L., Bocianowski, J., Waśkiewicz, A., Weber, Z., Karolewski, Z., Goliński, P., Kostecki, M. & Irzykowski, W. 2012. Genetic variation of *Fusarium oxysporum* isolates forming fumonisin B(1) and moniliformin. *Journal of Applied Genetics,* 53**,** 237-247.

Ishizaka, S., Saito, S., Yoshikawa, M., Kimoto, M. & Nishiyama, T. 1996. IL-10 PRODUCTION IN MOUSE HEPATOCYTES AUGMENTED BY TGF-β. *Cytokine,* 8**,** 837-843.

Jahromi, M. F., Liang, J. B., Ho, Y. W., Mohamad, R., Goh, Y. M. & Shokryazdan, P. 2012. Lovastatin Production by*Aspergillus terreus* Using Agro-Biomass as Substrate in Solid State Fermentation. *Journal of Biomedicine and Biotechnology,* 2012**,** 11-22.

Jain, P. K., Gupta, V. K., Misra, A. K., Gaur, R., Bajpai, V. & Issar, S. 2011. Current status of *Fusarium* infection in human and animal. *Asain Journal of Animal and Verterinary Advances,* 6**,** 201-227.

Jerusik, R. J. 2010. Fungi and paper manufacture. *Fungal Biology Reviews,* 24**,** 68-72.

Johannessen, L., LØVik, M., Lydersen, S. & Nilsen, A. M. 2009. Combined cell wall polysaccharide, mycotoxin and bacterial lipopolysaccharide exposure and inflammatory cytokine responses. *APMIS,* 117**,** 507-517.

Joshi-Barve, S., Barve, S. S., Amancherla, K., Gobejishvili, L., Hill, D., Cave, M., Hote, P. & McClain, C. J. 2007. Palmitic acid induces production of proinflammatory cytokine interleukin-8 from hepatocytes. *Hepatology,* 46**,** 823-830.

Juan-García, A., Manyes, L., Ruiz, M.-J. & Font, G. 2013. Involvement of enniatins-induced cytotoxicity in human HepG2 cells. *Toxicology Letters,* 218**,** 166-173.

Jussila, J., Komulainen, H., Kosma, V.-M., Pelkonen, J. & Hirvonen, M.-R. 2002. Inflammatory potential of the spores of *Penicillium spinulosum* isolated from indoor air of a moisture-damaged building in mouse lungs. *Environmental Toxicology and Pharmacology,* 12**,** 137-145.

Kamei, K., Watanabe, A., Nishimura , K. & Miyaji, M. 2002. Cytotoxicity of *Aspergillus fumigatus* culture filtrate against macrophages. *Japanese Journal of Medical Mycology,* 43**,** 37-41.

Kamogawa, Y., Minasi, L.-a. E., Carding, S. R., Bottomly, K. & Flavell, R. A. The relationship of IL-4- and IFNγ-producing T cells studied by lineage ablation of IL-4-producing cells. *Cell,* 75**,** 985-995.

Kaplan, D. H. 2010. "In vivo" function of Langerhans cells and dermal dendritic cells. *Trends in Immunology,* 31**,** 446-451.

Karwowska, W., Pierzynowska, J., Janicki, A., Waszkiewicz-Robak, B. e. & Przybylska, A. 2004. Qualitative and quantitative analysis of filamentous fungi in air, food and ochratoxin a in human milk. *Polish Journal of Food and Nutrition Sciences,* 13/54**,** 41-44.

Katoch, M., Singh, G., Sharma, S., Gupta, N., Sangwan, P. L. & Saxena, A. K. 2014. Cytotoxic and antimicrobial activities of endophytic fungi isolated from *Bacopa monnieri* (*L.*) *Pennell* (*Scrophulariaceae*). *BMC Complementary and Alternative Medicine,* 14**,** 1-8.

Katona, S. J. 2002. Sensitivity to Quorn mycoprotein (*Fusarium venenatum*) in a mould allergic patient. *Journal of Clinical Pathology,* 55**,** 876-877.

Kaur, R., Kaur, J. & Singh, R. S. 2010. Nonpathogenic *Fusarium* as a biological control agent. *Plant Pathology Journal,* 9**,** 79-91.

Kawaratani, H., Tsujimoto, T., Douhara, A., Takaya, H., Moriya, K., Namisaki, T., Noguchi, R., Yoshiji, H., Fujimoto, M. & Fukui, H. 2013. The Effect of Inflammatory Cytokines in Alcoholic Liver Disease. *Mediators of Inflammation,* 2013**,** 10.

Kawecki, D., Pacholczyk, M., Lagiewska, B., Sawicka-Grzelak, A., Durlik, M., Mlynarczyk, G. & Chmura, A. 2014. Bacterial and Fungal Infections in the Early Post-transplantation Period After Liver Transplantation: Etiologic Agents and Their Susceptibility. *Transplantation Proceedings,* 46**,** 2777-2781.

Khan, H. A. A. & Karuppayil, M. S. 2012. Fungal pollution of indoor environments and its management. *Saudi Journal of Biological Sciences,* 19**,** 405-426.

Khokhar, I., Haider, M. S., Mushtaq, S. & Mukhtar, I. 2012. Isolation and screening of highly cellulolytic filamentous fungi. *Journal of Applied Sciences and Environmental Management,* 16**,** 223-226.

Kidd, P. 2003. Th1/Th2 balance: The hypothesis, its limitations, and implications for health and disease. *Alternative Medicine Review,* 8**,** 223-246.

Kirk, P. M., Cannon, P. F., Minter, D. W. & Stalpers, J. A. 2008. *Dictionary of the Fungi,* Australia, CSIRO publishing.

Klarić, M. Š., Rašić, D. & Peraica, M. 2013. Deleterious Effects of Mycotoxin Combinations Involving Ochratoxin A. *Toxins,* 5**,** 1965-1987.

Klich, M. A. 2002a. Biogeography of *Aspergillus* species in soil and litter. *Mycologia,* 94**,** 21-27.

Klich, M. A. 2002b. *Identification of common Aspergillus spp,* The Netherlands, Ponson and Looijen, Wageningen.

Klich, M. A. 2002c. Introduction; economic and medical importance of *Aspergillus*. . *Identification of common Aspergillus species.* The Netherlands: Centraalbureau voor Schimmelculture, Utrecht.

Klich, M. A. 2002d. Introduction; economic and medical importance of *Aspergillus*. In: Identification of common *Aspergillus* species. *Centraalbureau voor Schimmelculture, Utrecht, The Netherlands (Publishers)***,** 1-16.

Klich, M. A. 2007. Aspergillus flavus: the major producer of aflatoxin. *Molecular Plant Pathology,* 8**,** 713-722.

Knutsen, A. P., Bush, R. K., Demain, J. G., Denning, D. W., Dixit, A., Fairs, A., Greenberger, P. A., Kariuki, B., Kita, H., Kurup, V. P., Moss, R. B., Niven, R. M., Pashley, C. H., Slavin, R. G., Vijay, H. M. & Wardlaw, A. J. 2012. Fungi and allergic lower respiratory tract diseases. *Journal of Allergy and Clinical Immunology,* 129**,** 280-291.

Kong, X., Horiguchi, N., Mori, M. & Gao, B. 2012. Cytokines and STATs in Liver Fibrosis. *Frontiers in Physiology,* 3**,** 69.

Königs, M., Schwerdt, G., Gekle, M. & Humpf, H.-U. 2008. Effects of the mycotoxin deoxynivalenol on human primary hepatocytes. *Molecular Nutrition & Food Research,* 52**,** 830-839.

Kramer, R. & Abraham, W.-R. 2012. Volatile sesquiterpenes from fungi: what are they good for? *Phytochemistry Reviews,* 11**,** 15-37.

Kriek, N. P. J., Marasas, W. F. O. & Thiel, P. G. 1981. Hepato- and cardiotoxicity of *Fusarium verticillioides* (F.moniliforme) isolates from Southern African maize. *Food and Cosmetics Toxicology,* 19**,** 447-456.

Krysinska-Traczyk, E. & Dutkiewicz, J. 2000. *Aspergillus candidus*: A respiratory hazard associated with grain dust. *Annals of Agricultural and Environmental Medicine,* 7 101-109.

Ku, C.-M. & Lin, J.-Y. 2013. Anti-inflammatory effects of 27 selected terpenoid compounds tested through modulating Th1/Th2 cytokine secretion profiles using murine primary splenocytes. *Food Chemistry,* 141**,** 1104-1113.

Kubosaki, A., Aihara, M., Park, B. J., Sugiura, Y., Shibutani, M., Hirose, M., Suzuki, Y., Takatori, K. & Sugita-Konishi, Y. 2008. Immunotoxicity of nivalenol after subchronic dietary exposure to rats. *Food and Chemical Toxicology,* 46**,** 253-258.

Kuiper-Goodman, T. 1994. Prevention of human mycotoxicosis through risk assessment and risk management. *In:* Miller, J. D. & Trenholm, H. L. (eds.) *Mycotoxins in grain.* St. Paul, MN: Eagan Press.

Kuiper-Goodman, T. 1995. Mycotoxins: risk assessment and legislation. *Toxicology Letters,* 82/83**,** 853-859.

Kumar, V., Basu, M. S. & Rajendran, T. P. 2008. Mycotoxin research and mycoflora in some commercially important agricultural commodities. *Crop Protection,* 27**,** 891-905.

Kupfahl, C., Michalka, A., Lass-Flörl, C., Fischer, G., Haase, G., Ruppert, T., Geginat, G. & Hof, H. 2008. Gliotoxin production by clinical and environmental *Aspergillus fumigatus* strains. *International Journal of Medical Microbiology,* 298**,** 319-327.

Kurek, E., Teresa Korniłłowicz−Kowalska, T., Słomka, A. & Melke, J. 2007. Characteristics of soil filamentous fungi communities isolated from various micro−relief forms in the high Arctic tundra (Bellsund region, Spitsbergen). *Polish Polar Research,* 28**,** 57-73.

Kurniati, E., Arfarita, N., Imai, T., Higuchi, T., Kanno, A., Yamamoto, K. & Sekine, M. 2014. Potential bioremediation of mercury-contaminated substrate using filamentous fungi isolated from forest soil. *Journal of Environmental Sciences,* 26**,** 1223-1231.

Lacy, P. & Stow, J. L. 2011. Cytokine release from innate immune cells: association with diverse membrane trafficking pathways. *Blood,* 118**,** 9-18.

Lai, X., Zhang, H., Liu, R. & Liu, C. 2015. Potential for aflatoxin B(1) and B(2) production by *Aspergillus flavus* strains isolated from rice samples. *Saudi Journal of Biological Sciences,* 22**,** 176-180.

Laich, F., Fierro, F. & Martı´n, J. F. 2002. Production of *Penicillin* by fungi growing on food products: Identification of a complete *Penicillin* gene cluster in *Penicillium griseofulvum* and a truncated cluster in *Penicillium verrucosum*. *Applied and Environmental Microbiology,* 68**,** 1211-1219.

Langseth, W., Bernhoft, A., Rundberget, T., Kosiak, B. & Gareis, M. 1999. Mycotoxin production and cytotoxicity of *Fusarium* strains isolated from Norwegian cereals. *Mycopathologia,* 144**,** 103-113.

LatgÉ, J.-P. 2003. *Aspergillus fumigatus,* a saprotrophic pathogenic fungus. *Mycologist,* 17**,** 56-61.

Lee, U. S., Jang, H. S., Tanaka, T., Toyasaki, N., Sugiura, Y., Oh, Y. J., Cho, C. M. & Ueno, Y. 1986. Mycological survey of Korean cereals and production of mycotoxins by *Fusarium* isolates. *Applied and Environmental Microbiology,* 52**,** 1258-1260.

Lee, Y. J., Holzapfel, K. L., Zhu, J., Jameson, S. C. & Hogquist, K. A. 2013. Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nature Immunology,* 14**,** 1146-1154.

Lei, M., Zhang, N. & Qi, D. 2013. In vitro investigation of individual and combined cytotoxic effects of aflatoxin B1 and other selected mycotoxins on the cell line porcine kidney 15. *Experimental and Toxicologic Pathology,* 65**,** 1149-1157.

Leslie, J. F. & Summerell, B. A. 2006. The *Fusarium* laboratory manual. *Blackwell publishing, Iowa, U.S.A,* Ist ed.**,** 1-2.

Levitz, S. M. & North, E. A. 1996. gamma Interferon gene expression and release in human lymphocytes directly activated by *Cryptococcus neoformans* and *Candida albicans*. *Infection and Immunity,* 64**,** 1595-1599.

Li, B.-K., Wang, X. & Ding, Q. 2009. *RE: A case report of severe Aspergillus flavus penile infection.*

Li, M., Harkema, J. R., Islam, Z., Cuff, C. F. & Pestka, J. J. 2006. T-2 toxin impairs murine immune response to respiratory reovirus and exacerbates viral bronchiolitis. *Toxicology and Applied Pharmacology,* 217**,** 76-85.

Liebhart, J., Cembrzyńska-Nowak, M., Bieńkowska, M., Liebhart, E., Dobek, R., Zaczyńska, E., Panaszek, B., Obojski, A. & Małolepszy, J. 2002. Relevance of the selected cytokine release (TNF-alpha, IL-6, IFN-gamma, and IFN-alpha) to the exacerbation of bronchial asthma from airway mycotic infections. Predominant role of TFN-alpha? *Journal of Investigational Allergology and Clinical Immunology,* 12**,** 182-191.

Lillards-Roberts, S. 2011. Mycotoxin list. *Boletim Cientifico,* 46**,** 1-7.

Liu, D. 2011a. Molecular detection of human fungal pathogens. *Taylor and Francis group, CRC press***,** 329-343.

Liu, X., Ling, Z., Li, L. & Ruan, B. 2011. Invasive fungal infections in liver transplantation. *International Journal of Infectious Diseases,* 15**,** e298-e304.

Louis, H., Van Laethem, J.-L., Wu, W., Quertinmont, E., Degraef, C., Van den Berg, K., Demols, A., Goldman, M., Le Moine, O., Geerts, A. & Devière, J. 1998. Interleukin-10 controls neutrophilic infiltration, hepatocyte proliferation, and liver fibrosis induced by carbon tetrachloride in mice. *Hepatology,* 28**,** 1607-1615.

Luangsa-ard, J., Houbraken, J., van Doorn, T., Hong, S.-B., Borman, A. M., Hywel-Jones, N. L. & Samson, R. A. 2011. *Purpureocillium, a new genus for the medically important Paecilomyces lilacinus*.

Lucas, E. M. F., Abreu, L. M., Marriel, I. E., Pfenning, L. H. & Takahashi, J. A. 2008. Phthalates production from *Curvularia senegalensis* (*Speg*.) *Subram*, a fungal species associated to crops of commercial value. *Microbiological Research,* 163**,** 495-502.

Mahajan, S. G. & Mehta, A. A. 2011. Suppression of ovalbumin-induced Th2-driven airway inflammation by β-sitosterol in a guinea pig model of asthma. *European Journal of Pharmacology,* 650**,** 458-464.

Majumder, L., Khalil, I., Munshi, M. K., Alam, K., Rashid, H.-O., Begum, R. & Alam, N. 2010. Citric acid production by *Aspergillus niger* using molasses and pumpkin as substrates. *European Journal of Biological Sciences,* 2**,** 1-8.

Makun, H. A., Dutton, M. F., Njobeh, P. B., Mwanza, M. & Kabiru, A. Y. 2011. Natural multi-mycotoxin occurrence in ricefrom Niger State, Nigeria. *Mycotoxin Research,* 27**,** 97-104.

Malato, Y., Sander, L. E., Liedtke, C., Al-Masaoudi, M., Tacke, F., Trautwein, C. & Beraza, N. 2008. Hepatocyte-specific inhibitor-of-kappaB-kinase deletion triggers the innate immune response and promotes earlier cell proliferation during liver regeneration. *Hepatology,* 46**,** 2036-2050.

Malek, T. R. 2003. The main function of IL-2 is to promote the development of T regulatory cells. *Journal of Leukocyte Biology,* 74**,** 961-965.

Malhi, H., Guicciardi, M. E. & Gores, G. J. 2010. Hepatocyte Death: A Clear and Present Danger. *Physiological Reviews,* 90**,** 1165-1194.

Mally, A. 2012. Ochratoxin A and Mitotic Disruption: Mode of Action Analysis of Renal Tumor Formation by Ochratoxin A. *Toxicological Sciences,* 127**,** 315-330.

Mancera-López, M. E., Esparza-García, F., Chávez-Gómez, B., Rodríguez-Vázquez, R., Saucedo-Castañeda, G. & Barrera-Cortés, J. 2008. Bioremediation of an aged hydrocarbon-contaminated soil by a combined system of biostimulation–bioaugmentation with filamentous fungi. *International Biodeterioration & Biodegradation,* 61**,** 151-160.

Manzoni, M. & Rollini, M. 2002. Biosynthesis and biotechnological production of statins by filamentous fungi and application of these cholesterol-lowering drugs. *Applied Microbiology and Biotechnology,* 58**,** 555-564.

Marasas, W. F. O., Kellerman, T. S., Gelderblom, W. C., Coetzer, J. A., Thiel, P. G. & van der Lugt, J. J. 1988. Leukoencephalomalacia in a horse induced by fumonisin B1 isolated from *Fusarium moniliforme*. *Journal of Veterinary Research,* 55**,** 197-203.

Marin, S., Ramos, A. J., Cano-Sancho, G. & Sanchis, V. 2013. Mycotoxins: Occurrence, toxicology, and exposure assessment. *Food and Chemical Toxicology,* 60**,** 218-237.

Marino, A., Fiorentino, C., Spataro, F. & Nostro, A. 2014. Effect of Temperature on Production of Ochratoxin A by *Aspergillus niger* in Orange Juice. *Journal of Toxins,* 2014**,** 5.

Matny, O. N. 2013. Screening of Mycotoxin Produced by *Fusarium verticillioides* and *F. proliferatum* in Culture Media. *Asian Journal of Agriculture and Rural Development,* 3**,** 1001-1006.

Matsuzawa, T., Tanaka, R., Horie, Y., Gonoi, T. & Yaguchi, T. 2010. Development of Rapid and Specific Molecular Discrimination Methods for Pathogenic <I>Emericella</I> Species. *Nippon Ishinkin Gakkai Zasshi,* 51**,** 109-116.

Max, B., Salgado, J. M., Rodríguez, N., Cortés, S., Converti, A. & Domínguez, J. M. 2010. Biotechnological production of citric acid. *Brazilian Journal of Microbiology,* 41**,** 862-875.

Mbata, T. I. 2008. Isolation of fungi in hyper saline Dead Sea water. *Sudanese Journal of Public Health,* 3**,** 170-172.

McCampbell, Kristen K. & Wingert, Rebecca A. 2012. Renal stem cells: fact or science fiction? *Biochemical Journal,* 444**,** 153-168.

McLean, K. J., Hans, M., Meijrink, B., van Scheppingen, W. B., Vollebregt, A., Tee, K. L., van der Laan, J.-M., Leys, D., Munro, A. W. & van den Berg, M. A. 2015. Single-step fermentative production of the cholesterol lowering drug pravastatin via reprogramming of *Penicillium chrysogenum*. *Proceedings of the National Academy of Sciences,* 112**,** 2847- 2852.

McLean, M. & Dutton, M. F. 1995. Cellular interactions and metabolism of aflatoxin: An update. *Pharmacology & Therapeutics,* 65**,** 163-192.

Mehrad, B., Strieter, R. M. & Standiford, T. J. 1999. Role of TNF-α in Pulmonary Host Defense in Murine Invasive Aspergillosis. *The Journal of Immunology,* 162**,** 1633-1640.

Michelson, P. 2010. *Cheese: Exploring Taste and Tradition*.

Miller, J. D. & Trenholm, H. l. 1994. Mycotoxins in grain: Compounds other than aflatoxin. *Eagan press, USA***,** 3-541.

Mochizuki, K., Shiraki, I., Murase, H., Ohkusu, K. & Nishimura, K. 2012. Identification and sensitivity of two rare fungal species isolated from two patients with *Fusarium keratomycosis*. *Journal of Infection and Chemotherapy,* 18**,** 939-944.

Mohamed, S. A., Hamed, S. R. & Al-Wasify, R. S. 2014. Relative diversity of filamentous fungi and yeasts in groundwater and their correlation to fecal pollution indicators and physicochemical parameters. *International Journal of Current Microbiology and Applied Sciences,* 3**,** 905-919.

Mohammad, N., Alam, M. Z., Kabbashi, N. A. & Ahsan, A. 2012. Effective composting of oil palm industrial waste by filamentous fungi: A review. *Resources, Conservation and Recycling,* 58**,** 69-78.

Mok, T., Koehler, A. P., Yu, M. Y., Ellis, D. H., Johnson, P. J. & Wickham, N. W. R. 1997. Fatal *Penicillium citrinum* pneumonia with pericarditis in a patient with acute leukemia. *Journal of Clinical Microbiology,* 35**,** 2654–2656.

Morath, S. U., Hung, R. & Bennett, J. W. 2012. Fungal volatile organic compounds: A review with emphasis on their biotechnological potential. *Fungal Biology Reviews,* 26**,** 73-83.

More, T. T., Yan, S., Tyagi, R. D. & Surampalli, R. Y. 2010. Potential use of filamentous fungi for wastewater sludge treatment. *Bioresource Technology,* 101**,** 7691-7700.

Moretti, A. N. 2009. Taxonomy of *Fusarium* genus, a continuous fight between lumpers and splitters. . *Proceedings of the National Academy of Sciences,* 117**,** 7-13.

Morrissey, R. E., Norred, W. P., Cole, R. J. & Dorner, J. 1985. Toxicity of the mycotoxin, cyclopiazonic acid, to Sprague-Dawley rats. *Toxicology and Applied Pharmacology,* 77**,** 94-107.

Morrissey, R. E. & Vesonder, R. F. 1986. Teratogenic potential of the mycotoxin, citreoviridin, in rats. *Food and Chemical Toxicology,* 24**,** 1315-1320.

Mounjouenpou, P., Gueule, D., Fontana-Tachon, A., Guyot, B., Tondje, P. R. & Guiraud, J.-P. 2008. Filamentous fungi producing ochratoxin a during cocoa processing in Cameroon. *International Journal of Food Microbiology,* 121**,** 234-241.

Mueller, A., Schlink, U., Wichmann, G., Bauer, M., Graebsch, C., Schüürmann, G. & Herbarth, O. 2013. Individual and combined effects of mycotoxins from typical indoor moulds. *Toxicology In Vitro,* 27**,** 1970-1978.

Mwanza, M., Kametler, L., Bonai, A., Rajli, V., Kovacs, M. & Dutton, M. F. 2009. The cytotoxic effect of fumonisin B1 and ochratoxin A on human and pig lymphocytes using the Methyl Thiazol Tetrazolium (MTT) assay. *Mycotoxin Research,* 25**,** 233-238.

Negedu, A., Atawodi, S. E., Ameh, J. B., Umoh, V. J. & Tanko, H. Y. 2011. Economic and health perspectives of mycotoxins. *Continental J. Biomedical Sciences* , , 5 5 - 26.

Nelson, J. H. 1970. Production of Blue Cheese Flavor via Submerged ermentation by *Penicillium roqueforti*. *Journal of Agriculture and Food Chemistry,* 18**,** 567-569.

Nieminen, S. M., Mäki-Paakkanen, J., Hirvonen, M.-R., Roponen, M. & von Wright, A. 2002. Genotoxicity of gliotoxin, a secondary metabolite of *Aspergillus fumigatus,* in a battery of short-term test systems. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis,* 520**,** 161-170.

Niide, O., Suzuki, Y., Yoshimaru, T., Inoue, T., Takayama, T. & Ra, C. 2006. Fungal metabolite gliotoxin blocks mast cell activation by a calcium- and superoxide-dependent mechanism: Implications for immunosuppressive activities. *Clinical Immunology,* 118**,** 108-116.

Nikaeen, M. & Mirhendi, H. 2008. Inactivation of *Aspergillus flavus* Spores in Water by Ultraviolet Irradiation. *World Applied Sciences Journal,* 4**,** 594-595.

Nishitani, Y., Okazaki, S., Imabayashi, K., Katada, R., Umetani, K., Yajima, H. & Matsumoto, H. 2007. Saturated and monounsaturated fatty acids increase interleukin-10 production in rat hepatocytes. *Japanese Journal of Alcohol Studies and Drug Dependence,* 42**,** 32-35.

Nowicki, M., Nowakowska, M., Niezgoda, A. & Kozik, E. U. 2012. *Alternaria* black spot of crucifers: Symptoms, importance of disease, and perspectives of resistance breeding 76**,** 5-19.

Nucci, M. & Anaissie, E. 2002. Cutaneous infection by *Fusarium species* in healthy and immunocompromised hosts: implications for diagnosis and management. *Clinical Infectious Diseases,* 35**,** 909-920.

Nucci, M. & Anaissie, E. 2007a. *Fusarium* infections in immuno-compromised patients. *Clinical Microbiology Reviews,* 20**,** 695-704.

Nucci, M. & Anaissie, E. 2007b. Fusarium Infections in Immunocompromised Patients. *Clinical Microbiology Reviews,* 20**,** 695-704.

O'Donnell, K., Sutton, D. A., Rinaldi, M. G., Magnon, K. C., Cox, P. A., Revankar, S. G., Sanche, S., Geiser, D. M., Juba, J. H., van Burik, J.-A. H., Padhye, A., Anaissie, E. J., Francesconi, A., Walsh, T. J. & Robinson, J. S. 2004. Genetic diversity of human pathogenic members of the *Fusarium oxysporum* complex inferred from multilocus DNA sequence data and amplified fragment length polymorphism analyses: Evidence

for the recent dispersion of a geographically widespread clonal lineage and nosocomial origin. *Journal of Clinical Microbiology,* 42**,** 5109–5120.

Ogawa, A., Morita, Y., Tanaka, T., Sakiyama, T. & Nakanishi, K. 1995 Production of kojic acid from *Aspergillus oryzae* var.*oryzae* by membrane-surface liquid culture. *Biotechnology Techniques,* 9**,** 153-156.

Ogórek, R., Lejman, A., Pusz, W., Miłuch, A. & Miodyńska, P. 2012. Characteristics and taxonomy of *Cladosporium* fungi. *Mikologia Lekarska,* 19**,** 80-85.

Ogorek, R., Pusz, W., Matkowski, K. & Pla˛Skowska, E. b. 2014. Assessment of abundance and species composition of filamentous fungi in the underground Rzeczka complex in Sowie Mountains (Lower Silesia, Poland). *Geomicrobiology Journal,* 31**,** 900-906.

Oh, S.-H., Hatch, H. M. & Petersen, B. E. 2002. Hepatic oval 'stem' cell in liver regeneration. *Seminars in Cell & Developmental Biology,* 13**,** 405-409.

Oh, S.-Y., Boermans, H. J., Swamy, H. V. L. N., Sharma, B. S. & Karrow, N. A. 2012. Immunotoxicity of *Penicillium* mycotoxins on viability and proliferation of Bovine macrophage cell line (BOMACs). *The open Mycology Journal,* 6**,** 11-16.

Okpako, E. C., Osuagwu, A. N., Duke, A. E. & Ntui, V. O. 2009. Prevalence and significance of fungi in sachet and borehole drinking water in Calabar, Nigeria. *African Journal of Microbiological Research,* 3**,** 56-61.

Oliveira, L. G., Cavalcanti, M. A. Q., Fernandes, M. J. S. & Lima, D. M. M. 2013. Diversity of filamentous fungi isolated from the soil in the semiarid area, Pernambuco, Brazil. *Journal of Arid Environments,* 95**,** 49-54.

Opal, S. M. & DePalo, V. A. 2000. Anti-inflammatory cytokines*. *Chest,* 117**,** 1162-1172.

Oshikata, C., Tsurikisawa, N., Saito, A., Watanabe, M., Kamata, Y., Tanaka, M., Tsuburai, T., Mitomi, H., Takatori, K., Yasueda, H. & Akiyama, K. 2013. Fatal pneumonia caused by *Penicillium digitatum*. *BMC Pulmonary Medicine.*

Ostry, V., Malir, F. & Ruprich, J. 2013. Producers and Important Dietary Sources of Ochratoxin A and Citrinin. *Toxins,* 5**,** 1574-1586.

Pahl, H. L., KrauB, B., Schulze-Osthoff, K., Decker, T., Traenckner, E. B.-M., Vogt, M., Myers, C., Parks, T., Warring, P., Miihlbacher, A., Czernilofiky, A.-P. & Baeuerle, P. A. 1996. The Immunosuppressive Fungal Metabolite Gliotoxin specifically Inhibits Transcription Factor NF-KB *Journal of Experimental Medicine,* 183**,** 1829-1840.

Palmero, D., Iglesias, C., de Cara, M., Lomas, T., Santos, M. & Tello, J. C. 2009. Species of *Fusarium* Isolated from River and Sea Water of Southeastern Spain and Pathogenicity on Four Plant Species. *Plant Disease,* 93**,** 377-385.

Panagopoulou, P., Filioti, J., Farmaki, E., Maloukou, A. & Roilides, E. 2007. Filamentous fungi in a tertiary care hospital: Environmental surveillance and susceptibility to antifungal drugs. *Infection Control and Hospital Epidemiology,* 28**,** 60-67.

Panagopoulou, P., Filioti, J., Petrikkos, G., Giakouppi, P., Anatoliotaki, M., Farmaki, E., Kanta, A., Apostolakou, H., Avlami, A., Samonis, G. & Roilides, E. 2002. Environmental surveillance of filamentous fungi in three tertiary care hospitals in Greece. *Journal of Hospital Infection,* 52**,** 185-191.

Panesar, N., Tolman, K. & Mazuski, J. E. 1999. Endotoxin Stimulates Hepatocyte Interleukin-6 Production. *Journal of Surgical Research,* 85**,** 251-258.

Pastor, F. J. & Guarro, J. 2006. Clinical manifestations, treatment and outcome of *Paecilomyces lilacinus* infections. *Clinical Microbiology and Infection,* 12**,** 948-960.

Pastor, F. J. & Guarro, J. 2008. *Alternaria* infections: laboratory diagnosis and relevant clinical features. *Clinical Microbiology and Infection,* 14**,** 734-746.

Paterson, R. R. M., Hageskal, G., Skaar, I. & Lima, N. 2009. Occurrence, problems, analysis and removal of filamentous fungi in drinking water. *In:* De Costa, P. A. B., P (ed.) *Fungicides: Chemistry, Environmental Impacts and Health Effects.* Nova Science Publishers, Inc.

Paterson, R. R. M. & Lima, N. 2010. How will climate change affect mycotoxins in food? *Food Research International,* 43**,** 1902-1914.

Pattron, D. D. 2006. *Aspergillus*, Health Implication & Recommendations for Public Health Food Safety. *Journal of Food Safety,* Vol.8**,** 19-23.

Peltonen, K., Jestoi, M. & Eriksen, G. S. 2010. Health effects of moniliformin a poorly understood *Fusarium* mycotoxin. *World Mycotoxin Journal,* 3 403-414.

Perrone, G., Susca, A., Cozzi, G., Ehrlich, K., Varga, J., Frisvad, J. C., Meijer, M., Noonim, P., Mahakarnchanakul, W. & Samson, R. A. 2007. Biodiversity of *Aspergillus* species in some important agricultural products. *Studies in Mycology,* 59**,** 53-66.

Person, A. K., Chudgar, S. M., Norton, B. L., Tong, B. C. & Stout, J. E. 2010. *Aspergillus niger*: an unusual cause of invasive pulmonary aspergillosis. *Journal of Medical Microbiology,* 59 834-838.

Pestka, J. J., Zhou, H.-R., Moon, Y. & Chung, Y. J. 2004. Cellular and molecular mechanisms for immune modulation by deoxynivalenol and other trichothecenes: unraveling a paradox. *Toxicology Letters,* 153**,** 61-73.

Pfohl-Leszkowicz, A. 2009. Ochratoxin A and Aristolochic Acid Involvement in Nephropathies and Associated Urothelial Tract Tumours. *Archives of Industrial Hygiene and Toxicology.*

Pfohl-Leszkowicz, A., Gabryelski, W. & Manderville, R. A. 2009. Formation of 2′-deoxyguanosine-carbon 8-bound ochratoxin A adduct in rat kidney DNA. *Molecular Nutrition & Food Research,* 53**,** 154-155.

Pfohl-Leszkowicz, A. & Manderville, R. A. 2012. An Update on Direct Genotoxicity as a Molecular Mechanism of Ochratoxin A Carcinogenicity. *Chemical Research in Toxicology,* 25**,** 252-262.

Pfohl-Leszkowicz, A., Petkova-Bocharova, T., Chernozemsky, I. N. & Castegnaro, M. 2002. Balkan endemic nephropathy and associated urinary tract tumours: a review on aetiological causes and the potential role of mycotoxins. *Food Additives & Contaminants,* 19**,** 282-302.

Phoku, J. Z., Dutton, M. F., Njobeh, P. B., Mwanza, M., Egbuta, M. A. & Chilaka, C. A. 2012. *Fusarium* infection of maize and maize-based products and exposure of a rural population to fumonisin B1 in Limpopo Province, South Africa. *Food Additives and Contaminants: Part A,* 29**,** 1743-1751.

Picot, A., Barreau, C., Pinson-Gadais, L., Caron, D., Lannou, C. & Richard-Forget, F. 2010. Factors of the *Fusarium verticillioides*-maize environment modulating fumonisin production. *Critical Reviews in Microbiology,* Early online**,** 1-11.

Pitt, J. I. & Hocking, A. D. 1997a. Fungi and mycotoxins in foods. *In:* Orchard, A. E. (ed.) *Fungi of Australia, vol.1B, Introduction Fungi in the environment.* Canberra, Australia: Austr. Boil. Res. Study.

Pitt, J. I. & Hocking, A. D. 1997b. Primary keys and miscellaneous fungi *Fungi and food spoilage.* 2nd ed. London, Weinheim, New York, Tokyo, Melbourne, Madras: Blackie Academic and Professional.

Pitt, J. I. & Hocking, A. D. 1997c. Primary keys and miscellaneous fungi. In: Fungi and Food Spoilage. *Blackie Academic and Professional. London, Weinheim, New York, Tokyo, Melbourne, Madras,* 2nd ed**,** 59-171.

Pitt, J. I. & Hocking, A. D. 2012. *Fungi and Food Spoilage,* USA, Springer Science.

Pitt, J. I., Samson, R. A. & Frisvad, J. C. 2000. List of accepted species and their synonyms in the family Trichocomaceae. *In:* Samson, R. A. & Pitt, J. I. (eds.) *Integration of modern taxonomic methods for Penicillium and Aspergillus classification.* Amsterdam: Harwood.

Polizzi, V., Adams, A., De Saeger, S., Van Peteghem, C., Moretti, A. & De Kimpe, N. 2012. Influence of various growth parameters on fungal growth and volatile metabolite production by indoor molds. *Science of the Total Environment,* 414**,** 277-286.

Potin, O., Rafin, C. & Veignie, E. 2004. Bioremediation of an aged polycyclic aromatic hydrocarbons (PAHs)-contaminated soil by filamentous fungi isolated from the soil. *International Biodeterioration & Biodegradation,* 54**,** 45-52.

Pratt, R. G. 2006. Comparative survival of conidia of eight species of *Bipolaris*, *Curvularia,* and *Exserohilum* in soil and influences of swine waste amendments on survival. *Applied Soil Ecology,* 31**,** 159-168.

Prelusky, D. B., Rotter B.A. & R.G., R. 1994. Toxicology of mycotoxins. In Mycotoxins in grain (Edited by Miller, J.D., and Trenholm H.L.). *Eagan press, St. Paul, MN***,** 359-404.

Proctor, R. H., Busman, M., Seo, J.-A., Lee, Y. W. & Plattner, R. D. 2008. A fumonisin biosynthetic gene cluster in *Fusarium oxysporum* strain O-1890 and the genetic basis for B versus C fumonisin production. *Fungal Genetics and Biology,* 45**,** 1016-1026.

Prosperini, A., Font, G. & Ruiz, M. J. 2014. Interaction effects of *Fusarium* enniatins (A, A1, B and B1) combinations on in vitro cytotoxicity of Caco-2 cells. *Toxicology In Vitro,* 28**,** 88-94.

Pryce-Miller, E., Aanensen, D., Vanittanakom, N. & Fisher, M. C. 2008. Environmental detection of *Penicillium marneffei* and growth in soil microcosms in competition with *Talaromyces stipitatus*. *Fungal Ecology,* 1**,** 49-56.

Rasmussen, R. R., Rasmussen, P. H., Larsen, T. O., Bladt, T. T. & Binderup, M. L. 2011. *In vitro* cytotoxicity of fungi spoiling maize silage. *Food and Chemical Toxicology,* 49**,** 31-44.

Ravichandra, N. G. 2013. *Fundamentals of plant pathology,* PHI Learning.

Reihill, J. A., Moore, J. E., Elborn, J. S. & Ennis, M. 2011. Effect of *Aspergillus fumigatus* and *Candida albicans* on pro-inflammatory response in cystic fibrosis epithelium. *Journal of Cystic Fibrosis,* 10**,** 401-406.

Rheeder, J. P., Marasas, W. F. O. & Vismer, H. F. 2002. Production of fumonisin analogs by *Fusarium* species. *Applied and Environmental Microbiology,* 68**,** 2101-2105.

Ribeiro, S. C. C., Santana, A. N. C., Arriagada, G. H., Martins, J. E. C. & Takagaki, T. Y. 2005. A novel cause of invasive pulmonary infection in an immunocompetent patient: *Aspergillus candidus*. *Journal of Infection,* 51**,** e195-e197.

Richard, J. L. 2007. Some major mycotoxins and their mycotoxicoses—An overview. *International Journal of Food Microbiology,* 119**,** 3-10.

Ritter, A. C., Hoeltz, M. & Noll, I. B. 2011. Toxigenic potential of *Aspergillus flavus* tested in different culture conditions. *Ciência e Tecnologia de Alimentos,* 31**,** 623-628.

Ritz, K. & Young, I. M. 2004. Interactions between soil structure and fungi. *Mycologist,* 18**,** 52-59.

Rivas, S. & Thomas, C. M. 2005. Molecular interactions between tomato and the leaf mold pathogen: *Cladosporium fulvum*. *Annual Review of Phytopathology,* 43**,** 395-436.

Rodríguez-Rodríguez, C. E., Barón, E., Gago-Ferrero, P., Jelić, A., Llorca, M., Farré, M., Díaz-Cruz, M. S., Eljarrat, E., Petrović, M., Caminal, G., Barceló, D. & Vicent, T. 2012. Removal of pharmaceuticals, polybrominated flame retardants and UV-filters from sludge by the fungus *Trametes versicolor* in bioslurry reactor. *Journal of Hazardous Materials,* 233–234**,** 235-243.

Rojo, J. M., Rejas, M. T., Ojeda, G., Portolés, P. & Barasoain, I. 1986. Enhancement of lymphocyte proliferation, interleukin-2 production and NK activity by inmunoferon (AM-3), a fungal immunomodulator: Variations in normal and immunosuppressed mice. *International Journal of Immunopharmacology,* 8**,** 593-597.

Rolando, N., Harvey, F., Brahm, J., Philpott-Howard, J., Alexander, G., Casewell, M., Fagan, E. & Williams, R. 1991. Fungal infection: a common, unrecognised complication of acute liver failure. *Journal of Hepatology,* 12**,** 1-9.

Rolando, N., Philpott-Howard, J. & Williams, R. 1996. Bacterial and Fungal Infection in Acute Liver Failure. *Seminars in Liver Disease,* 16**,** 389-402.

Romagnani, S. 2000. T-cell subsets (Th1 versus Th2). *Annals of Allergy, Asthma & Immunology,* 85**,** 9-18.

Romero, C. M., Urrutia, I. M., Reinoso, E. H. & Kiernan, M. M. 2010. Benzo[a]pyrene degradation by soil filamentous fungi. *Journal of Yeast and Fungal Research,* 1**,** 25-29.

Roncero, M. I. G., n Hera, C., Ruiz-Rubio, M., Maceira, F. I. G. a., Madrid, M. P., Caracuel, Z., Calero, F., Delgado-Jarana, J. s., Rolda´n-Rodrı´guez, R., Martı´nez-Rocha, A. L., Velasco, C., Roa, J., Martı´n-Urdiroz, M., Co´rdoba, D. & Di Pietro, A. 2003. *Fusarium* as a model for studying virulence in soilborne plant pathogens. *Physiological and Molecular Plant Pathology,* 62**,** 87-98.

Rotter, B. A., Prelusky, D. B. & Pestka, J. J. 1996. Toxicology of deoxinivalenol (vomitoxin). *Journal of Toxicology and Environment Health,* 48**,** 1-34.

Rowell, D. L., Eckmann, L., Dwinell, M. B., Carpenter, S. P., Raucy, J. L., Yang, S. K. & Kagnoff, M. F. 1997. Human hepatocytes express an array of proinflammatory

cytokines after agonist stimulation or bacterial invasion. *American Journal of Physiology - Gastrointestinal and Liver Physiology,* 273**,** G322-G332.

Ruiz, M. J., Franzova, P., Juan-García, A. & Font, G. 2011. Toxicological interactions between the mycotoxins beauvericin, deoxynivalenol and T-2 toxin in CHO-K1 cells in vitro. *Toxicon,* 58**,** 315-326.

Runge, D., Michalopoulos, G. K., Strom, S. C. & Runge, D. M. 2000 Recent advances in human hepatocyte culture systems. *Biochemical and Biophysical Research Communications,* 274**,** 1-3.

Sahasrabudhe, N. A., Lackhe, A. H. & Ranjekar, P. K. 1987. Characterization of the purified multifunctional cellulase component of *P. funiculosum*. *FEMS Microbiology Letters,* 40**,** 315-319.

Sahasrabudhe, N. A. & Sankpal, N. V. 2001. Production of organic acids and metabolites of fungi for food industry. *In:* George, G. K. & Dilip, K. A. (eds.) *Applied Mycology and Biotechnology*. Elsevier.

Samapundo, S., Devliehgere, F., De Meulenaer, B. & Debevere, J. 2005. Effect of Water Activity and Temperature on Growth and the Relationship between Fumonisin Production and the Radial Growth of *Fusarium verticillioides* and *Fusarium proliferatum* on Corn. *Journal of Food Protection,* 68**,** 1054-1059.

Sampietro, D. A., Marín, P., Iglesias, J., Presello, D. A., Vattuone, M. A., Catalan, C. A. N. & Gonzalez Jaen, M. T. 2010. A molecular based strategy for rapid diagnosis of toxigenic *Fusarium* species associated to cereal grains from Argentina. *Fungal Biology,* 114**,** 74-81.

Sanchis, V., Marín, S., Magan, N. & Ramos, A. 2006. Ecophysiology of fumonisin producers in *Fusarium* section *Liseola*. *In:* Hocking, A. D., Pitt, J. I., Samson, R. & Thrane, U. (eds.) *Advances in Food Mycology.* Springer US.

Santos, S. A. D., Andrade Júnior, D. R. D. & Andrade, D. R. D. 2011. Tnf-a production and apoptosis in hepatocytes after *listeria* monocytogenes and *salmonella typhimurium* invasion. *Revista do Instituto de Medicina Tropical de São Paulo,* 53**,** 107-112.

Sauer, M., Porro, D., Mattanovich, D. & Branduardi, P. 2008. Microbial production of organic acids: expanding the markets. *Trends in Biotechnology,* 26**,** 100-108.

Sautour, M., Edel-Hermann, V., Steinberg, C., Sixt, N., Laurent, J., Dalle, F., Aho, S., Hartemann, P., L'Ollivier, C., Goyer, M. & Bonnin, A. 2012. *Fusarium* species recovered from the water distribution system of a French university hospital. *International Journal of Hygiene and Environmental Health,* 215**,** 286-292.

Sawane, A. & Saoji, A. 2004. A report on *Penicillium* in the intramural and extramural air of residential areas of Nagpur city (India). *Aerobiologia,* 20**,** 229-236.

Scheel, C., Hurst, S., Barreiros, G., Akiti, T., Nucci, M. & Balajee, S. A. 2013. Molecular analyses of *Fusarium* isolates recovered from a cluster of invasive mold infections in a Brazilian hospital. *BMC Infectious Diseases,* 13**,** 49.

Scheller, J., Chalaris, A., Schmidt-Arras, D. & Rose-John, S. 2011. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research,* 1813**,** 878-888.

Schindler, A. F., Palmer, J. G. & Eisenberg, W. V. 1967. Aflatoxin Production by *Aspergillus flavus* as Related to Various Temperatures. *Applied Microbiology,* 15**,** 1006-1009.

Schmidt-Heydt, M., Magan, N. & Geisen, R. 2008. Stress induction of mycotoxin biosynthesis genes by abiotic factors. *FEMS Microbiology Letters,* 284**,** 142-149.

Schuster, E., Dunn-Coleman, N., Frisvad, J. C. & van Dijck, P. W. M. 2002. On the safety of *Aspergillus niger* - a review. *Applied Microbiology and Biotechnology,* 59**,** 426-435.

Seo, J.-A., Kim, J.-C. & Lee, Y.-W. 1996. Isolation and Characterization of Two New Type C Fumonisins Produced by *Fusarium oxysporum*. *Journal of Natural Products,* 59**,** 1003-1005.

Shah, G. S., Shier, W. T., ·, J., Tahir, N., Hameed, A., Ahmad, S. & Ali, N. 2014. *Penicillium verruculosum* SG: a source of polyketide and bioactive compounds with varying cytotoxic activities against normal and cancer lines. *Archives of Microbiology,* 196**,** 267-278.

Shahan, T. A., Sorenson, W. G., Paulaskis, J. D., Morey, R. & Lewis, D. M. 1998. Concentration- and time-dependent upregulation and release of the cytokines MIP-2 KC, TNF, and MIP-1a in rat alveolar macrophages by fungal spores implicated in airway inflammation. *American Journal of Respiratory Cell and Molecular Biology,* 18**,** 435-440.

Sharmila, K., Thillaimaharani, K. A., Logesh, A. R., Sathishkumar, A. & Kalaiselvam, M. 2012. Production of Cyclosporin- A By Saprophytic Filamentous Fungus *Fusarium oxysporum*. *International Journal of Pharmacy and Pharmaceutical Sciences,* 4**,** 149-153.

Shephard, G. S. 2008. Impact of mycotoxins on human health in developing countries. *Food Additives and Contaminants: Part A,* 25**,** 146-151.

Shepherd, G. S., Thiel, P. G., Sydenham, E. W., Vleggaar, R. & Alberts, J. F. 1994. Determination of the mycotoxin fumonisin B1 and identification of its partially

hydrolysed metabolites in the faeces of nonhuman primates. *Food Chemical Toxicology,* 32**,** 23-29.

Smith, H. A. 2014. *Production of antimicrobials and antioxidants from filamentous fungi.* PhD PhD, National University of Ireland.

Solé, A. & Salavert, M. 2008. Fungal infections after lung transplantation. *Transplantation Reviews,* 22**,** 89-104.

Sonigo, P., De Toni, A. & Reilly, K. 2011. A review of fungi in drinking water and the implications for human health. France: BIO Intelligence Service.

Soriano, J. M. & Dragacci, S. 2004. Occurrence of fumonisins in foods. *Food Research International,* 37**,** 985- 1000.

Sorrenti, V., Di Giacomo, C., Acquaviva, R., Barbagallo, I., Bognanno, M. & Galvano, F. 2013. Toxicity of Ochratoxin A and Its Modulation by Antioxidants: A Review. *Toxins,* 5**,** 1742-1766.

Steiner, B., Aquino, V. R., Paz, A. A., Silla, L. M. d. R., Zavascki, A. & Goldani, L. Z. 2013. *Paecilomyces variotii* as an Emergent Pathogenic Agent of Pneumonia. *Case Reports in Infectious Diseases,* 2013**,** 3.

Sterflinger, K., Tesei, D. & Zakharova, K. 2012. Fungi in hot and cold deserts with particular reference to microcolonial fungi. *Fungal Ecology,* 5**,** 453-462.

Steyn, P. S. 1995. Mycotoxins, general view, chemistry and structure. *Toxicology Letters,* 82/83**,** 843-851.

Stoev, S., Denev, S., Dutton, M. F. & Nkosi, B. 2009. Cytotoxic effect of some mycotoxins and their combinations on human peripheral blood mononuclear cells as measured by the MTT assay. *The Open Toxicology Journal* 2**,** 1-8.

Stoev, S. D. 1998. The role of ochratoxin A as a possible cause of Balkan Endemic Nephropathy and its risk evaluation *Journal of Veterinary and Human Toxicology* 40**,** 352-360.

Stoev, S. D., Daskalov, H., Radic, B., Domijan, A. & Peraica, M. 2002. Spontaneous mycotoxic nephropathy in Bulgarian chickens with unclarified mycotoxin aetiology. *Veterinary Research,* 33**,** 83-94.

Stoev, S. D. & Denev, S. A. 2013. Porcine/Chicken or Human Nephropathy as the Result of Joint Mycotoxins Interaction. *Toxins,* 5**,** 1503-1530.

Sudarmono, P., Utji, R., Kardon, L. & Kumala, S. 2006. Cytotoxic assay of endophytic fungus 1.2.11 secondary metabolites from *Brucea javanica (L) Merr* towards cancer cell in vitro *Cytotoxic assay of secondary metabolite,* 15**,** 137-144.

Suryanarayanan, T. S., Senthilarasu, G. & Muruganandam, V. 2000. Endophytic fungi from *Cuscuta rejlexa* and its host plants *Fungal Diversity* 4**,** 117-123.

Svahn, S. K., Goransson, U., El-Seedi, H., Bohlin, L., Larsson, J. D. G., Olsen, B. & Chryssanthou, E. 2012. Antimicrobial activity of filamentous fungi isolated from highly antibiotic contaminated river sediment. *Infection Ecology and Epidemiology*, 2**,** 11591-11597.

Sweeney, M. J. & Dobson, A. D. 1998a. Mycotoxin production by Aspergillus, Fusarium and Penicillium species. *Inernational Journal of Food Microbiology.*, 43**,** 141-158.

Sweeney, M. J. & Dobson, A. D. 1998b. Mycotoxin production by *Aspergillus*, *Fusarium* and *Penicillium* species. *International Journal of Food Microbiology.* , 43**,** 141-158.

Sydenham, E. W., Thiel, P. G., Marasas, W. F. O., Shephard, G. S., van Schalkwyk, D. J. & Koch, K. R. 1990. Natural occurrence of some *Fusarium* mycotoxins in corn from low and high oesophageal cancer prevalence areas of the Transkei, southern Africa. *Journal of Agriculture and Food Chemistry*, 38**,** 1900-1903.

Takano, M., Sugano, N., Mochizuki, S., Koshi, R. N., Narukawa, T. S., Sawamoto, Y. & Ito, K. 2012. Hepatocytes produce tumor necrosis factor-α and interleukin-6 in response to Porphyromonas gingivalis. *Journal of Periodontal Research*, 47**,** 89-94.

Taniwaki, M. H., Hocking, A. D., Pitt, J. I. & Fleet, G. H. 2009. Growth and mycotoxin production by food spoilage fungi under high carbon dioxide and low oxygen atmospheres. *International Journal of Food Microbiology*, 132**,** 100-108.

Tarkkanen, A., Raivio, V., Anttila, V.-J., Tommila, P., Ralli, R., Merenmies, L. & Immonen, I. 2004. Fungal endophthalmitis caused by *Paecilomyces variotii* following cataract surgery: a presumed operating room air-conditioning system contamination. *Acta Ophthalmologica Scandinavica*, 82**,** 232-235.

Terao, K., Aikawa, T. & Kera, K. 1978. A synergistic effect of nitrosodimethylamine on sterigmatocystin carcinogenesis in rats. *Food and Cosmetics Toxicology*, 16**,** 591-596.

Tezcan, G., Ozhak-Baysan, B., Alastruey-Izquierdo, A., Ogunc, D., Ongut, G., Yıldıran, S. T., Hazar, V., Cuenca-Estrella, M. & Rodriguez-Tudela, J. L. 2009. Disseminated Fusariosis Caused by *Fusarium verticillioides* in an Acute Lymphoblastic Leukemia Patient after Allogeneic Hematopoietic Stem Cell Transplantation. *Journal of Clinical Microbiology*, 47**,** 278-281.

Theumer, M. G., López, A. G., Masih, D. T., Chulze, S. N. & Rubinstein, H. R. 2002. Immunobiological Effects of Fumonisin B1 in Experimental Subchronic Mycotoxicoses in Rats. *Clinical and Diagnostic Laboratory Immunology*, 9**,** 149-155.

Torre, D., Speranza, F., Giola, M., Matteelli, A., Tambini, R. & Biondi, G. 2002. Role of Th1 and Th2 cytokines in immune response to uncomplicated *Plasmodium falciparum* malaria. *Clinical and Diagnostic Laboratory Immunology,* 9**,** 348-351.

Torres-Rodriguez", J. M., Madrenys-Brunet, N., Siddat, M., Lopez-Jodra, O. & Jimenez, T. 1998. *Aspergillus versicolor* as cause of onychomycosis : report of 12 cases and susceptibility testing to antifungal drugs. *Journal of the European Academy of Dermatology and Venereology,* 11**,** 25-31

Trisuwan, K., Rukachaisirikul, V., Borwornwiriyapan, K., Phongpaichit, S. & Sakayaroj, J. 2014. Benzopyranone, benzophenone, and xanthone derivatives from the soil fungus *Penicillium citrinum* PSU-RSPG95. *Tetrahedron Letters,* 55**,** 1336-1338.

Tudek, B., Winczura, A., Janik, J., Siomek, A., Foksinski, M. & Oliński, R. 2010. Involvement of oxidatively damaged DNA and repair in cancer development and aging. *American Journal of Translational Research,* 2**,** 254-284.

Turkez, H., Geyikoglu, F., Yousef, M. I., Celik, K. & Bakir, T. O. 2012. Ameliorative effect of supplementation with l-glutamine on oxidative stress, DNA damage, cell viability and hepatotoxicity induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin in rat hepatocyte cultures. *Cytotechnology,* 64**,** 687-699.

Vahidnia, O. & Chaichi-Nosrati, A. 2014. An assay of citrinin quantity and production patterns in the culture medium of *Aspergillus* isolates of iranian northern states in laboratory condition. *Indian Journal of Fundamental and Applied Life Sciences,* 4**,** 549-559.

van der Straat, L., Vernooij, M., Lammers, M., van den Berg, W., Schonewille, T., Cordewener, J., van der Meer, I., Koops, A. & de Graaff, L. H. 2014. Expression of the *Aspergillus terreus* itaconic acid biosynthesis cluster in Aspergillus niger. *Microbial Cell Factories,* 13.

Varga, J., Rigó, K., Lamper, C., Téren, J. & Szabó, G. 2002. Kinetics of ochratoxin a production in different *Aspergillus* species. *Acta Biologica Hungarica,* 53**,** 381-388.

Varga, J., Rigó, K. & Téren, J. 2000. Degradation of ochratoxin A by *Aspergillus* species. *International Journal of Food Microbiology,* 59**,** 1-7.

Varo, S. D., Martins, C. H., Cardoso, M. J., Sartori, F. G., Montanari, L. B. & Pires-Goncalves, R. H. 2007. [Isolation of filamentous fungi from water used in a hemodialysis unit]. *Revista da Sociedade Brasileira de Medicina Tropical,* 40**,** 326-331.

Verweij, P. E., Varga, J., Houbraken, J., Rijs, A. J. M. M., VerduynLunel, F. M., Blijlevens, N. M. A., Shea, Y. R., Holland, S. M., Warris, A., Melchers, W. J. G. & Samson, R. A.

2008. *Emericella quadrilineata* as Cause of Invasive Aspergillosis. *Emerging Infectious Diseases,* 14**,** 566-572.

Vesper, S. J., Haugland, R. A., Rogers, M. E. & Neely, A. N. 2007. Opportunistic *Aspergillus* pathogens measured in home and hospital water by quantitative PCR. *Journal of Water Health,* 5**,** 427-431.

Viegas, C., Alves, C. l., Carolino, E., Rosado, L. & Santos, C. S. 2010. Prevalence of fungi in indoor air with reference to gymnasiums with swimming pools. *Indoor and Built Environment,* 19**,** 555-561.

Vigier, B., Reid, L. M., Seifert, K. A., Stewart, D. W. & Hamilton, R. I. 1997. Distribution and prediction of *Fusarium* species associated with maize ear rot in Ontario. *Canadian Journal of Plant Pathology,* 19**,** 60-65.

Vismer, H., Snijman, P., Marasas, W. & van Schalkwyk, D. 2004. Production of fumonisins by *Fusarium verticillioides* strains on solid and in a defined liquid medium — effects of L-methionine and inoculum. *Mycopathologia,* 158**,** 99-106.

Vonberg, R. P. & Gastmeier, P. 2006a. Nosocomial aspergillosis in outbreak settings. *Journal of Hospital Infection,* 63**,** 246-254.

Vonberg, R. P. & Gastmeier, P. 2006b. Nosocomial aspergillosis in outbreak settings. *Journal of Hospital Infection,* 63**,** 246-254.

Wagacha, J. M. & Muthomi, J. W. 2008. Mycotoxin problem in Africa: Current status, implications to food safety and health and possible management strategies. *International Journal of Food Microbiology,* 124**,** 1-12.

Wagener, J., Malireddi, R. K. S., Lenardon, M. D., Köberle, M., Vautier, S., MacCallum, D. M., Biedermann, T., Schaller, M., Netea, M. G., Kanneganti, T.-D., Brown, G. D., Brown, A. J. P. & Gow, N. A. R. 2014. Fungal chitin dampens inflammation through IL-10 induction mediated by NOD2 and TLR9 activation. *PLoS Pathogens,* 10**,** e1004050.

Walsh, T. J., Groll, A., Hiemenz, J., Fleming, R., Roilides, E. & Anaissie, E. 2004. Infections due to emerging and uncommon medically important fungal pathogens. *Clinical Microbiology and Infection,* 10**,** 48-66.

Wan, L. Y. M., Turner, P. C. & El-Nezami, H. 2013. Individual and combined cytotoxic effects of *Fusarium* toxins (deoxynivalenol, nivalenol, zearalenone and fumonisins B1) on swine jejunal epithelial cells. *Food and Chemical Toxicology,* 57**,** 276-283.

Wang, D., Vo, N. V., Sowa, G. A., Hartman, R. A., Ngo, K., Choe, S. R., Witt, W. T., Dong, Q., Lee, J. Y., Niedernhofer, L. J. & Kang, J. D. 2011. Bupivacaine decreases cell

viability and matrix protein synthesis in an intervertebral disc organ model system. *The Spine Journal,* 11**,** 139-146.

Wang, H., Dong, H., Qian, H. & Cong, B. 2014. Laboratory assessment of entomopathogenic nematode symbiotic bacteria to control maize pest, *Ostrinia furnacalis*, and fungi diseases, *Bipolaris maydis* and *Curvularia lunata*. *Journal of Asia-Pacific Entomology,* 17**,** 823-827.

Wang, J.-S. & Groopman, J. D. 1999. DNA damage by mycotoxins. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 424**,** 167-181.

Ward, O. P. 2012a. Production of recombinant proteins by filamentous fungi. *Biotechnology Advances,* 30**,** 1119-1139.

Ward, O. P. 2012b. Production of recombinant proteins by filamentous fungi *Journal of Biotechnology Advances* 30**,** 1119-1139

Warris, A., Gaustad, P., Meis, J. F., Voss, A., Verweij, P. E. & Abrahamsen, T. G. 2001. Recovery of filamentous fungi from water in a paediatric bone marrow transplantation unit. *Journal of Hospital Infection,* 47**,** 143-148.

Warris, A., Netea, M. G., Verweij, P. E., Gaustad, P., Kullberg, B.-J., Weemaes, C. M. R. & Abrahamsen, T. G. 2005. Cytokine responses and regulation of interferon-gamma release by human mononuclear cells to *Aspergillus fumigatus* and other filamentous fungi. *Medical Mycology,* 43**,** 613-621.

Warris, A., Voss, A., Abrahamsen, T. G. & Verweij, P. E. 2002. Contamination of Hospital Water with *Aspergillus fumigatus* and Other Molds. *Clinical Infectious Diseases,* 34**,** 1059-1060.

Wartenberg, D., Lapp, K., Jacobsen, I. D., Dahse, H.-M., Kniemeyer, O., Heinekamp, T. & Brakhage, A. A. 2011. Secretome analysis of *Aspergillus fumigatus* reveals Asp-hemolysin as a major secreted protein. *International Journal of Medical Microbiology,* 301**,** 602-611.

Waskiewicz, A., Golinski, P., Karolewski, Z., Irzykowska, L., Bocianowski, J., Kostecki, M. & Weber, Z. 2010. Formation of fumonisins and other secondary metabolites by *Fusarium oxysporum* and *F. proliferatum*: a comparative study. *Food Additives and Contaminants: Part A,* 27**,** 608-615.

Waśkiewicz, A., Irzykowska, L., Karolewski, Z., Bocianowski, J., Goliński, P. & Weber, Z. 2009. Mycotoxins biosynthesis by *Fusarium oxysporum* and *F. proliferatum* isolates of asparagus origin. *Journal of Plant Protection Research,* 49**,** 369-372.

Watanabe, T., Watanabe, Y. & Fukatsu, T. 2001. New species of *Acremonium*, *Cylindrocarpon* and *Verticillium* from soil in the Bonin (Ogasawara) Islands, Japan. *Mycoscience,* 42**,** 591-595.

Wiseman, J. C. D. & Mody, C. H. 2007. Other cells: The role of non-neutrophilic granulocytes, NK and NKT cells in fungal immunology. *In:* Brown, G. D. & Netea, M. G. (eds.) *Immunology of fungal infections.* Springer Science and Business Media.

Wollenberg, G. K., DeForge, L. E., Bolgos, G. & Remick, D. G. 1993. Differential expression of tumor necrosis factor and interleukin-6 by peritoneal macrophages in vivo and in culture. *The American Journal of Pathology,* 143**,** 1121-1130.

Woo, P. C. Y., Chong, K. T. K., Tse, H., Cai, J. J., Lau, C. C. Y., Zhou, A. C., Lau, S. K. P. & Yuen, K.-y. 2006. Genomic and experimental evidence for a potential sexual cycle in the pathogenic thermal dimorphic fungus *Penicillium marneffei*. *FEBS Letters,* 580**,** 3409-3416.

Wu, W., Flannery, B. M., Sugita-Konishi, Y., Watanabe, M., Zhang, H. & Pestka, J. J. 2012. Comparison of murine anorectic responses to the 8-ketotrichothecenes 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, fusarenon X and nivalenol. *Food and Chemical Toxicology,* 50**,** 2056-2061.

Yamaguchi, M. U., Rampazzo, R. d. C. P., Yamada-Ogatta, S. F., Nakamura, C. V., Ueda-Nakamura, T. & Dias Filho, B. P. 2007. Yeasts and filamentous fungi in bottled mineral water and tap water from municipal supplies. *Brazilian Archives of Biology and Technology,* 50**,** 1-9.

Yang, Y.-Y. 2011. Can serum cytokines predict hepatic cytokine expression in liver cirrhosis? *Journal of the Chinese Medical Association,* 74**,** 485-486.

Zain, M. E. 2011. Impact of mycotoxins on humans and animals. *Journal of Saudi Chemical Society,* 15**,** 129-144.

Zalar, P., de Hoog, G. S., Schroers, H. J., Crous, P. W., Groenewald, J. Z. & Gunde-Cimerman, N. 2007. Phylogeny and ecology of the ubiquitous saprobe *Cladosporium sphaerospermum,* with descriptions of seven new species from hypersaline environments. *Studies in Mycology,* 58**,** 157-183.

Zalar, P., Frisvad, J. C., Gunde-Cimerman, N., Varga, J. & Samson, R. A. 2008. Four new species of *Emericella* from the Mediterranean region of Europe. *Mycologia*, 100**,** 779-795.

Zelante, T., Fric, J., Wong, A. Y. W. & Ricciardi-Castagnoli, P. 2012. Interleukin-2 Production by Dendritic Cells and its Immuno-Regulatory Functions. *Frontiers in Immunology*, 3**,** 161.

Zhang, A., Cao, J.-l., Yang, B., Chen, J.-h., Zhang, Z.-t., LI, S.-y., Fu, Q., Hugnes, C. E. & Caterson, B. 2010. Effects of moniliformin and selenium on human articular cartilage metabolism and their potential relationships to the pathogenesis of Kashin-Beck disease. *Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology),* 11**,** 200-208.

Zhang, J.-M. & An, J. 2007. Cytokines, Inflammation, and Pain. *International Anesthesiology Clinics,* 45**,** 27-37.

Zhang, L.-J. & Wang, X.-Z. 2006. Interleukin-10 and chronic liver disease. *World Journal of Gastroenterology,* 12**,** 1681-1685.

Zhang, N., O'Donnell, K. & Geiser, D. M. 2006. Members of the *Fusarium solani* species complex that cause infections in both humans and plants are common in the environment. *Journal of Clinical Microbiology,* 44**,** 2186-2190.

Zhang, Q. Q., Li, L., Zhu, M., Zhang, C. Y. & J., W. J. 2005. Primary cutaneous aspergillosis due to *Aspergillus flavus*: a case

report. *Chinese Medical Journal,* 118**,** 255-257.

Zheng, Y., Yu, X., Zeng, J. & Chen, S. 2012. Feasibility of filamentous fungi for biofuel production using hydrolysate from dilute sulfuric acid pretreatment of wheat straw. *Biotechnology for Biofuels,* 5**,** 1-10.

Zhou, G., Whong, W. Z., Ong, T. & Chen, B. 2000. Development of a fungus-specific PCR assay for detecting low-level fungi in an indoor environment. *Molecular and Cellular Probes,* 14**,** 339-348.

Appendices

APPENDIX I- Raw data on the production of mycotoxins by *Aspergillus* species in µg/g

	Aflatoxin B_1		
	Day 4	**Day 9**	**Day 14**
A. flavus (PDA)	0.966	11.622	0.249
A. flavus (MEA)	1.397	3.412	4.222
	Aflatoxin B_2		
A. flavus (PDA)	0.066	0.246	0.013
A. flavus (MEA)	0.085	0.175	0.274
	Aflatoxin G_1		
A. flavus (PDA)	0.105	0.215	0.723
A. flavus (MEA)	0.066	0.085	0.126
	Aflatoxin G_2		
A. flavus (PDA)	0.000	0.755	0.441
A. flavus (MEA)	0.163	1.126	1.367
	Ochratoxin A		
A. niger (PDA)	$8.63*10^{-6}$	$7.04*10^{-5}$	$3.93*10^{-5}$
A. niger (MEA)	$7.04*10^{-5}$	$4.04*10^{-4}$	$5.08*10^{-4}$

APPENDIX II- Raw data on the production of mycotoxin by *Fusarium* species in µg/g

	Deoxynivalenol		
	Day 4	**Day 9**	**Day 14**
F. verticillioides (PDA)	0.019	0.118	0.103
F. verticillioides (MEA)	0.000	0.020	0.024
F. oxysporum (PDA)	0.004	0.058	0.065
F. oxysporum (MEA)	0.040	0.057	0.145
	Fumonisin B_1		
F. verticillioides (PDA)	21.3	46.15	7.4
F. verticilliodes (MEA)	25.4	31.9	114.4
F. oxysporum (PDA)	5.0	8.2	11.8
F. oxysporum (MEA)	114.6	32.8	70.1
	Nivalenol		
F. verticillioides(PDA)	701.727	829.827	692.961
F. verticillioides(MEA)	1035.270	358.146	808.792

APPENDIX III- Raw data of alteration of hepatocyte viability induced by 4 days old individual filamentous fungi species

	PDA			**MEA**		
	24 hrs	**48 hrs**	**72 hrs**	**24 hrs**	**48 hrs**	**72 hrs**

ve control	100	100	100	100	100	100
pbs control	100	102	99.1	102	98.6	104
MeOH control	98.7	101.6	101.9	99.0	103.1	98.6
A.flavus	98.7	91.04	64.3	98.3	79.4	67.1
A. niger	254.62	218.64	87.2	252.0	207.1	65.9
F. oxysporum	106.03	94.02	86.9	119.3	106.9	70.6
F. verticillioides	106.92	110.59	72.08	104.8	108.5	63.6
P. chrysogenum	105.8	169	67.37	111.2	194.4	68.2
P. expansum	137.06	91.6	70.16	156.1	98.7	61.5

APPENDIX IV- Raw data of alteration of hepatocyte viability induced by 9 days old individual filamentous fungi species

	PDA			**MEA**		
	24 hrs	**48 hrs**	**72 hrs**	**24 hrs**	**48 hrs**	**72 hrs**
ve control	100	100	100	100	100	100
pbs control	100	100.01	103.2	100	101	99.8
MeOH control	97.02	96.23	99.8	96.7	93.2	107.8
A.flavus	97.09	81.03	70.5	96.9	79.9	72.0
A. niger	179	199.04	137.7	185.5	219.4	112.8
F. oxysporum	89.7	68.5	53.03	92.0	90.6	49.9
F. verticillioides	113.8	85.3	133.6	102.4	81.7	119.0
P. chrysogenum	146	157.3	88.16	159.4	162.0	81.5
P. expansum	153.8	114.7	121.08	150.6	115.3	131.9

APPENDIX V- Raw data of alteration of hepatocyte viability induced by 14 days old individual filamentous fungi species

	PDA			**MEA**		
	24 hrs	**48 hrs**	**72 hrs**	**24 hrs**	**48 hrs**	**72 hrs**
ve control	100	100	100	100	100	100
pbs control	102.4	101	102	105.6	100.4	103.1
MeOH control	101.4	103.8	102.7	103.3	106.4	103.9
A. flavus	106.26	136.07	98.2	113.2	128.9	101.3
A. niger	204	153.9	127.3	227.2	171.4	104.8
F. oxysporum	107.2	80.2	42.03	105.9	70.3	39.9
F. verticillioides	89.4	80.2	113.42	82.7	75.0	111.4
P. chrysogenum	139.42	154.7	92.64	139.4	161.5	97.6
P. expansum	123.06	120.9	59.3	121.2	123.8	67.3

APPENDIX VI- Raw data of alteration of hepatocyte viability induced by 4 days old filamentous fungi species combinations

	PDA			MEA		
	24 hrs	**48 hrs**	**72 hrs**	**24 hrs**	**48 hrs**	**72 hrs**
ve control	100	100	100	100	100	100
pbs control	102.4	101	102	102	98.6	104
MeOH control	101.4	103.8	102.7	99.0	103.1	98.6
A. flavus+A. niger+F. oxysporum+F. verticillioides	120.4	171.9	91.23	117.9	186.0	92.9
A. flavus+A. niger+P. chrysogenum+P. expansum	107.21	248.14	69.06	111.8	258.0	71.0
A. flavus+A. niger	62.09	194.07	72.3	59.8	170.4	58.7
A. flavus+ +F. oxysporum	89.01	139.5	83.25	104.0	159.6	96.7
A. flavus+ F. verticillioides	135.4	96.7	73.9	131.6	96.8	71.5
A. flavus+P. chrysogenum	121	169.03	65.2	112.0	171.6	73.2
A. flavus+ P. expansum	123.73	96.1	75.8	134.8	108.0	78.2
A. niger+F. oxysporum	100.16	265.3	83.02	102.5	250.4	87.5
A. niger+ F. verticillioides	97.05	346	85.3	95.0	333.3	64.0
A. niger+P. chrysogenum	104.7	218.3	85.1	101.7	244.7	90.9
A. niger +P. expansum	167.29	348.3	83.14	127.7	355.5	93.5
F. oxysporum+F. verticillioides	100.85	132.84	117	102.1	121.7	106.9
F. oxysporum+P. chrysogenum	106.93	180.5	81.06	123.8	175.9	76.5
F. oxysporum+P. expansum	132.02	154.02	53.9	140.3	175.2	48.1
F. verticillioides+P. chrysogenum	108.8	170.4	60.5	111.4	166.3	68.9
F. verticillioides+P. expansum	123.66	186.05	121.7	105.6	152.3	94.1
P. chrysogenum+P. expansum	120.39	108.5	91.02	144.5	130.4	112.3
F. oxysporum+F. verticillioides+P. chrysogenum+P. expansum	159.2	175.03	130.7	140.2	151.2	101.5
A. flavus+A. niger+F. oxysporum+F. verticillioides+P. chrysogenum+P. expansum	152.61	264.1	121.4	141.7	232.4	109.2

APPENDIX VII- Raw data of alteration of hepatocyte viability induced by 9 days old filamentous fungi species combinations

	PDA			MEA		
	24 hrs	**48 hrs**	**72 hrs**	**24 hrs**	**48 hrs**	**72 hrs**
ve control	100	100	100	100	100	100
pbs control	102.4	101	102	100	101	99.8
MeOH control	101.4	103.8	102.7	96.7	93.2	107.8
A. flavus+A. niger+F. oxysporum+F. verticillioides	132.09	182.3	76.1	134.9	182.1	67.2
A. flavus+A. niger+P. chrysogenum+P. expansum	141.7	166.19	84.4	157.6	178.7	90.1
A. flavus+A. niger	134.26	201.4	104.04	121.7	229.6	119.3
A. flavus+ +F. oxysporum	94.03	82.27	126.1	99.7	88.4	101.7
A. flavus+ F. verticillioides	82.5	98.3	113.06	81.3	82.1	114.3
A. flavus+P. chrysogenum	132.55	120	142.7	123.2	115.4	135.2
A. flavus+ P. expansum	91.27	96.2	97.1	94.4	94.2	97.0
A. niger+F. oxysporum	157.1	171	89	153.7	174.5	91.2
A. niger+ F. verticillioides	130	260.6	112.9	133.1	285.2	109.3
A. niger+P. chrysogenum	198.04	187.6	107.9	214.2	194.3	118.0
A. niger +P. expansum	154.35	195.9	124	172.6	203.7	112.0
F. oxysporum+F. verticillioides	110.2	98.6	123.8	107.4	91.9	122.4
F. oxysporum+P. chrysogenum	133.3	125	86.93	134.7	125.9	77.1
F. oxysporum+P. expansum	138.4	145.2	84.03	147.3	153.5	72.8
F. verticillioides+P. chrysogenum	133	121.9	125.8	141.2	115.9	125.4
F. verticillioides+P. expansum	127.9	139	103.05	115.4	125.2	109.5
P. chrysogenum+P. expansum	145	98.9	122.7	162.4	102.2	130.8
F. oxysporum+F. verticillioides+P. chrysogenum+P. expansum	156.2	134	113.5	148.0	123.1	122.2
A. flavus+A. niger+F. oxysporum+F. verticillioides+P. chrysogenum+P. expansum	132.8	119.7	87.05	158.1	152.2	78.6

APPENDIX VIII- Raw data of alteration of hepatocyte viability induced by 14 days old filamentous fungi species combinations

	PDA			MEA		
	24 hrs	**48 hrs**	**72 hrs**	**24 hrs**	**48 hrs**	**72 hrs**
ve control	100	100	100	100	100	100
pbs control	102.4	101	102	105.6	100.4	103.1
MeOH control	101.4	103.8	102.7	103.3	106.4	103.9
A. flavus+A. niger+F. oxysporum+F. verticillioides	112.9	159	85.09	127.7	161.1	73.9
A. flavus+A. niger+P. chrysogenum+P. expansum	148.57	168.38	85.7	152.6	170.5	87.9
A. flavus+A. niger	136.9	151	74.7	158.2	165.1	76.3
A. flavus+ +F. oxysporum	96.3	132.57	101.32	93.9	111.0	97.0
A. flavus+ F. verticillioides	102	145.97	84.5	115.9	158.3	90.6
A. flavus+P. chrysogenum	123.6	176.21	116.9	141.4	188.5	114.3
A. flavus+ P. expansum	104	110.4	93.58	107.4	108.3	98.7
A. niger+F. oxysporum	143.7	132.94	79.03	134.6	131.8	63.0
A. niger+ F. verticillioides	149.4	237.4	82.49	153.1	253.8	88.3
A. niger+P. chrysogenum	259	201.47	86.43	277.4	222.5	92.7
A. niger +P. expansum	174.9	249	94.8	184.4	241.6	89.4
F. oxysporum+F. verticillioides	97.4	90.58	114.32	92.8	86.6	103.2
F. oxysporum+P. chrysogenum	144.88	119.48	68.91	141.4	123.0	61.1
F. oxysporum+P. expansum	124.97	120	53.09	131.9	122.6	45.7
F. verticillioides+P. chrysogenum	145.8	137.59	104.72	137.9	139.6	114.4
F. verticillioides+P. expansum	97.4	92.6	63	94.4	92.1	51.5
P. chrysogenum+P. expansum	149.6	120.05	129.39	154.2	115.0	126.2
F. oxysporum+F. verticillioides+P. chrysogenum+P. expansum	127.67	136.2	132.93	112.4	143.5	122.6
A. flavus+A. niger+F. oxysporum+F. verticillioides+P. chrysogenum+P. expansum	156.73	163.92	107.38	144.9	167.8	102.9

APPENDIX IX- Raw data of alteration of renal epithelial cells viability induced by 4 days old individual filamentous fungi species

	PDA			MEA		
	24 hrs	**48 hrs**	**72 hrs**	**24 hrs**	**48 hrs**	**72 hrs**
ve control	100	100	100	100	100	100
pbs control	102.7	98.6	100.9	101.7	106.8	101.8
MeOH control	96.1	96.7	96	104.3	95.4	98.3
A.flavus	133.9	113.6	101.4	133.3	131.7	87.8
A. niger	117.8	82.5	77.1	114.6	95.6	66.8
F. oxysporum	134.2	142.5	100.2	130.5	165.2	86.7
F. verticillioides	163.6	145.2	115.5	159.1	168.3	100
P. chrysogenum	98.0	124.2	85.4	90.1	144	73.9
P. expansum	117.1	61.6	97.9	128.9	71.4	84.7

APPENDIX X- Raw data of alteration of renal epithelial cells viability induced by 9 days old individual filamentous fungi species

	PDA			MEA		
	24 hrs	**48 hrs**	**72 hrs**	**24 hrs**	**48 hrs**	**72 hrs**
ve control	100	100	100	100	100	100
pbs control	103.1	100.1	99.9	100.2	101.8	99.8
MeOH control	94.9	102.3	106.6	112	95.4	93.8
A.flavus	117.8	141.2	104.2	121.7	185.9	95
A. niger	94.8	166.9	112.2	98	219.8	102.3
F. oxysporum	156.5	100.1	121.3	161.7	131.9	110.6
F. verticillioides	144.3	105.5	121	149.1	138.9	110.3
P. chrysogenum	196.9	97.8	97.4	203.5	128.8	88.8
P. expansum	84	77.9	110.9	86.8	102.5	101.1

APPENDIX XI- Raw data of alteration of renal epithelial cells viability induced by 14 days old individual filamentous fungi species

	PDA			MEA		
	24 hrs	**48 hrs**	**72 hrs**	**24 hrs**	**48 hrs**	**72 hrs**
ve control	100	100	100	100	100	100
pbs control	106	101.4	99.9	102.2	101.3	100.1
MeOH control	96.1	97.3	103.6	105.2	94.6	93.2
A.flavus	105.4	80.9	61.6	87.8	77.9	54.9
A. niger	80.9	76.1	57.2	67.4	89.9	42.2
F. oxysporum	134.6	100.4	84.1	112.2	118.6	85.8
F. verticillioides	86.4	66.8	57.7	72.1	78.9	44.6
P. chrysogenum	119.5	60.7	57.2	99.6	71.7	44.5
P. expansum	106.9	98.1	75.5	89.1	115.9	77

APPENDIX XII- Raw data of alteration of renal epithelial cells viability induced by 4 days old individual filamentous fungi species combinations

	PDA			MEA		
	24 hrs	**48 hrs**	**72 hrs**	**24 hrs**	**48 hrs**	**72 hrs**
ve control	100	100	100	100	100	100
pbs control	102.7	98.6	100.9	101.7	106.8	101.8
MeOH control	96.1	96.7	96	104.3	95.4	98.3
A. flavus+A. niger+F. oxysporum+F. verticillioides	110.7	193.6	139.9	140.6	224.5	121.1
A. flavus+A. niger+P. chrysogenum+P. expansum	123.7	223.6	171	90.4	259.3	148.1
A. flavus+A. niger	104.6	117.3	105.2	101.8	136	91
A. flavus+ +F. oxysporum	121.5	114.9	123.5	118.1	133.2	106.9
A. flavus+ F. verticillioides	129.4	150.1	125.7	128.4	174	108.8
A. flavus+P. chrysogenum	129.1	360.2	205	133.1	417.7	177.4
A. flavus+ P. expansum	119.7	226.6	129.1	116.5	262.7	111.7
A. niger+F. oxysporum	103.7	121	101.4	100.8	140.2	87.8
A. niger+ F. verticillioides	115.4	303.7	167.1	112.3	352.2	144.6
A. niger+P. chrysogenum	93.5	269.5	246.9	90.9	312.4	213.7
A. niger +P. expansum	106.6	245.1	192.5	103.7	284.1	166.6
F. oxysporum+F. verticillioides	120.3	227.6	126.4	117	263.9	109.4
F. oxysporum+P. chrysogenum	98.4	143.3	90.3	95.7	166.1	78.2
F. oxysporum+P. expansum	151.2	168.9	166.8	147.1	195.8	144.4
F. verticillioides+P. chrysogenum	86.8	138	139.2	84.4	160	120.5
F. verticillioides+P. expansum	114.5	236.3	151.4	111.4	274	131.1
P. chrysogenum+P. expansum	142	222.9	131.8	138.2	258.4	114.1
F. oxysporum+F. verticillioides+P. chrysogenum+P. expansum	154	220.8	163.4	149.8	256	141.4
A. flavus+A. niger+F. oxysporum+F. verticillioides+P. chrysogenum+P. expansum	155.8	314.1	255.4	151.6	364.2	221

APPENDIX XIII- Raw data of alteration of renal epithelial cells viability induced by 9 days old individual filamentous fungi species combinations

	PDA			MEA		
	24 hrs	**48 hrs**	**72 hrs**	**24 hrs**	**48 hrs**	**72 hrs**
ve control	100	100	100	100	100	100
pbs control	103.1	100.1	99.9	100	101.8	104.2
MeOH control	94.9	102.3	106.6	98.6	94.8	91.5
A. flavus+A. niger+F. oxysporum+F. verticillioides	104.1	71.3	104.6	107.6	93.9	95.3
A. flavus+A. niger+P. chrysogenum+P. expansum	97.9	59.1	74.4	101.2	77.8	67.8
A. flavus+A. niger	98.8	110.1	87.1	102.1	145	79.4
A. flavus+ +F. oxysporum	90.7	326.6	125.7	93.8	430.1	114.6
A. flavus+ F. verticillioides	154.6	106.7	118.5	159.8	140.5	108
A. flavus+P. chrysogenum	147.9	151.8	138	152.8	199.9	125.8
A. flavus+ P. expansum	115.9	256.8	207.1	119.8	338.2	188.8
A. niger+F. oxysporum	106.1	175.3	99.8	109.6	230.8	91
A. niger+ F. verticillioides	93.1	86.8	57.5	96.2	114.4	52.4
A. niger+P. chrysogenum	102.4	131.7	104.5	105.8	173.5	95.2
A. niger +P. expansum	110.5	104.1	97	114.2	137.1	88.4
F. oxysporum+F. verticillioides	81.7	148.1	97.5	84.4	195	88.8
F. oxysporum+P. chrysogenum	181.3	134.2	186.9	187.3	176.7	170.3
F. oxysporum+P. expansum	160	142	128.8	165.4	187	117.4
F. verticillioides+P. chrysogenum	87.3	116.3	81.2	90.2	153.2	74
F. verticillioides+P. expansum	133.2	111.6	102.7	137.6	147	93.7
P. chrysogenum+P. expansum	162.9	227.3	162	168.3	299.3	147.7
F. oxysporum+F. verticillioides+P. chrysogenum+P. expansum	152	240.1	207	157	316.2	188.7
A. flavus+A. niger+F. oxysporum+F. verticillioides+P. chrysogenum+P. expansum	76	131.8	138.1	78.5	173.5	125.9

APPENDIX XIV- Raw data of alteration of renal epithelial cells viability induced by 14 days old individual filamentous fungi species combinations

	PDA			MEA		
	24 hrs	**48 hrs**	**72 hrs**	**24 hrs**	**48 hrs**	**72 hrs**
ve control	100	100	100	100	100	100
pbs control	106	101.4	99.9	99.7	102.1	100.1
MeOH control	96.1	97.3	103.6	103.6	91.5	88.2
A. flavus+A. niger+F. oxysporum+F. verticillioides	94.5	209.8	121.2	78.8	313.6	163.4
A. flavus+A. niger+P. chrysogenum+P. expansum	109.2	155.7	92.3	91	216.8	94.1
A. flavus+A. niger	152.3	103.3	71.7	127	122.1	73.1
A. flavus+ +F. oxysporum	127.7	216.8	113.9	106.4	256.1	116.2
A. flavus+ F. verticillioides	117.9	268.9	136.7	98.3	317.6	139.4
A. flavus+P. chrysogenum	108.9	210.9	95.6	90.8	249.2	97.5
A. flavus+ P. expansum	128.1	77.9	43.4	106.8	92.1	35.6
A. niger+F. oxysporum	75.2	65.4	56.3	62.7	77.3	57.4
A. niger+ F. verticillioides	93.8	64.7	57.6	78.2	76.5	58.8
A. niger+P. chrysogenum	77.9	124.7	60.4	65	147.4	61.6
A. niger +P. expansum	118	96.4	67	98.4	113.9	68.3
F. oxysporum+F. verticillioides	117.4	143.5	87.6	97.9	169.6	89.3
F. oxysporum+P. chrysogenum	215	163.3	110.7	179.3	225.8	122.9
F. oxysporum+P. expansum	88.2	118.6	81.6	73.5	173	89.1
F. verticillioides+P. chrysogenum	107.8	88.2	75.8	89.9	104.2	86
F. verticillioides+P. expansum	144.8	141.7	89.4	120.7	190.4	90.6
P. chrysogenum+P. expansum	146.3	117.3	96.9	122	138.5	98.8
F. oxysporum+F. verticillioides+P. chrysogenum+P. expansum	73.5	121.5	90.9	61.3	143.5	92.7
A. flavus+A. niger+F. oxysporum+F. verticillioides+P. chrysogenum+P. expansum	95.2	279.3	184	79.4	330	187.6

APPENDIX XV- Pearson's correlation of hepatocytes viability alteration against individual cytokines expressed when exposed to 4 days old filamentous fungi species for 24 hours

	Cell viability when exposed to filamentous fungi	**IFN**	**TNF**	**IL-10**	**IL-4**	**IL-2**
Cell viability when exposed to filamentous fungi	1	.186	.113	.123	.150	.154
IFN	.186	1	.688**	.452*	.428*	.612**
TNF	.113	.688**	1	.657**	.257	.421*
IL-10	.123	.452*	.657**	1	.417*	.277
IL-4	.150	.428*	.257	.417*	1	.665**
IL-2	.154	.612**	.421*	.277	.665**	1

**- Correlation is significant at the 0.01 level (2 tailed)

*- Correlation is significant at the 0.05 level (2 tailed)

APPENDIX XVI- Pearson's correlation of hepatocytes viability alteration against individual cytokines expressed when exposed to 9 days old filamentous fungi species for 24 hours

	Cell viability when exposed to filamentous fungi	**IFN**	**IL-10**	**TNF**	**IL-4**	**IL-2**
Cell viability when exposed to filamentous fungi	1	0.264	0.153	0.213	0.139	0.276
IFN	0.264	1	0.241	0.560**	0.500*	0.667**
IL-10	0.153	0.241	1	0.430*	0.361	0.241
TNF	0.213	0.560**	0.430*	1	0.490*	0.560**
IL-4	0.139	0.500*	0.361	0.490*	1	0.667**
IL-2	0.276	0.667**	0.241	0.560**	0.667**	1

**- Correlation is significant at the 0.01 level (2 tailed)

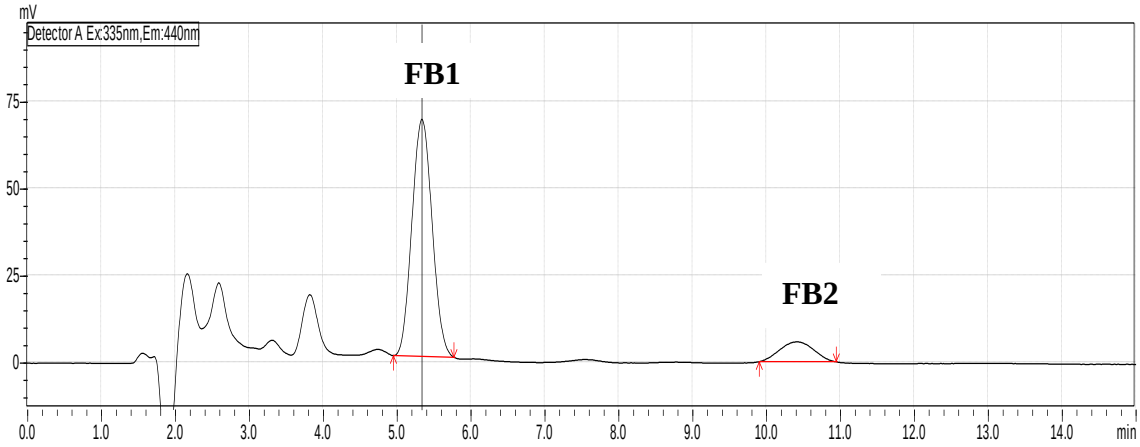
*- Correlation is significant at the 0.05 level (2 tailed)

APPENDIX XVII- Pearson's correlation of hepatocytes viability alteration against individual cytokines expressed when exposed to 14 days old filamentous fungi species for 24 hours

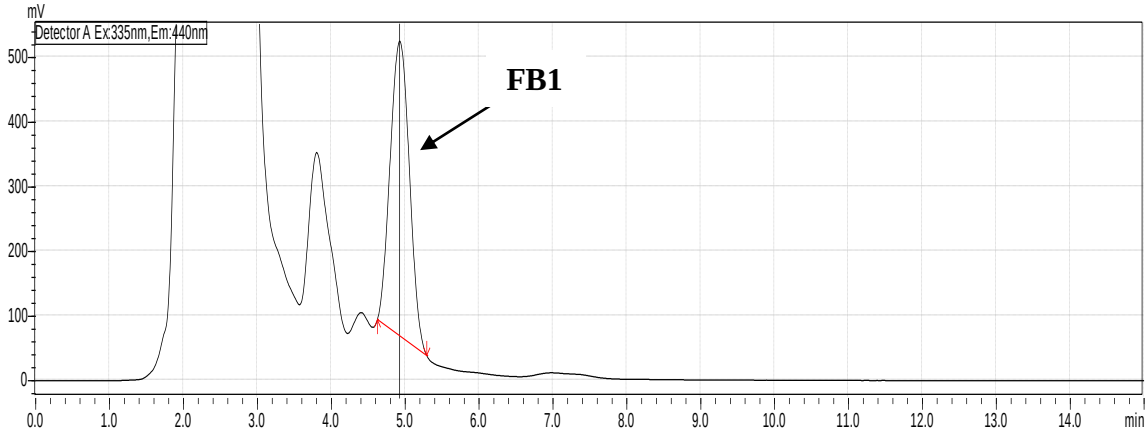
	Cell viability when exposed to filamentous fungi	**IFN**	**TNF**	**IL-10**	**IL-4**	**IL-2**
Cell viability when exposed to filamentous fungi	1	0.525**	0.335	0.271	0.399	0.567**
IFN	0.525**	1	0.553**	0.261	0.724**	0.774**
TNF	0.335	0.553**	1	0.473*	0.764**	0.428*
IL-10	0.271	0.261	0.473*	1	0.361	0.202
IL-4	0.399	0.724**	0.764**	0.361	1	0.560**
IL-2	0.567	0.774**	0.428*	0.202	0.560*	1

**- Correlation is significant at the 0.01 level (2 tailed), *- Correlation is significant at the 0.05 level (2 tailed)

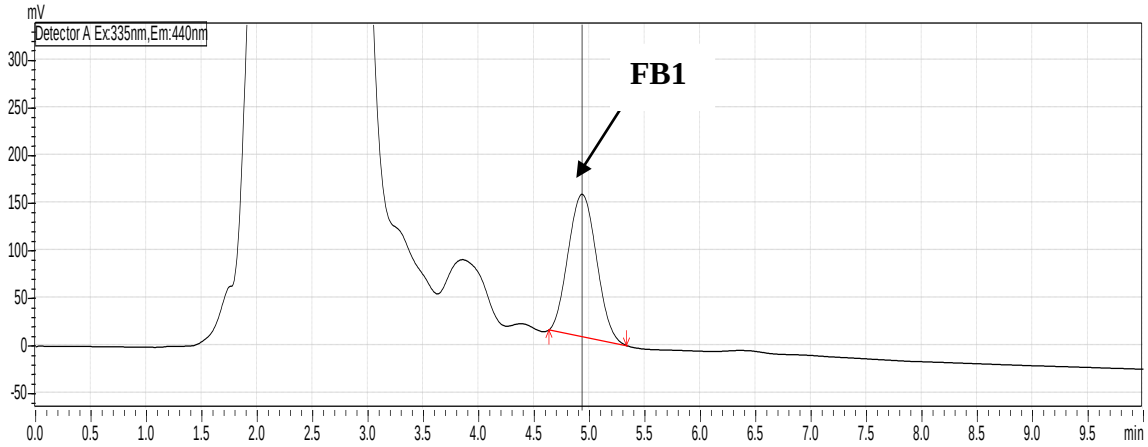
APPENDIX XVIII- High performance liquid chromatographs of standard mycotoxins and detected mycotoxins in filamentous fungi extracts



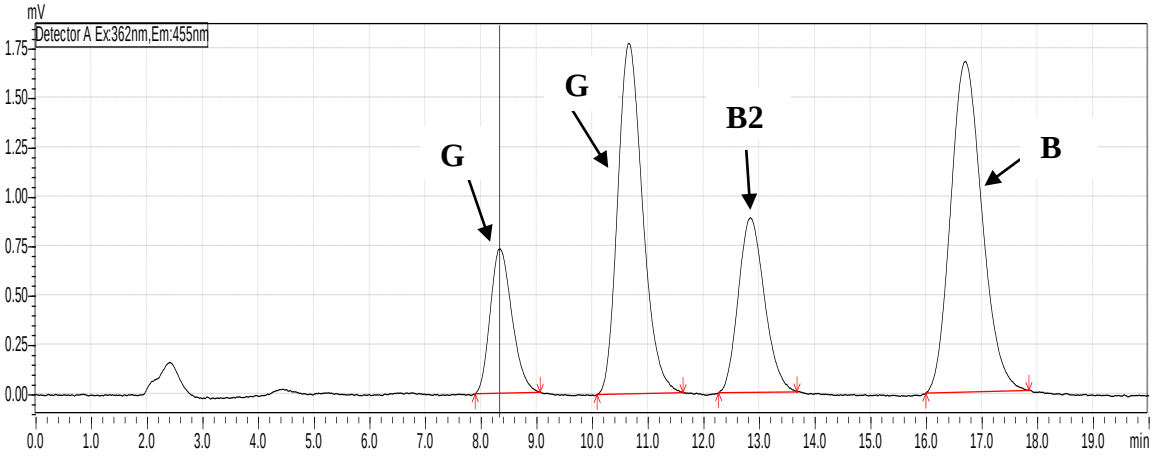
Appendix XVIIIa: Chromatogram of fumonisin standards used for calibration in the experiment



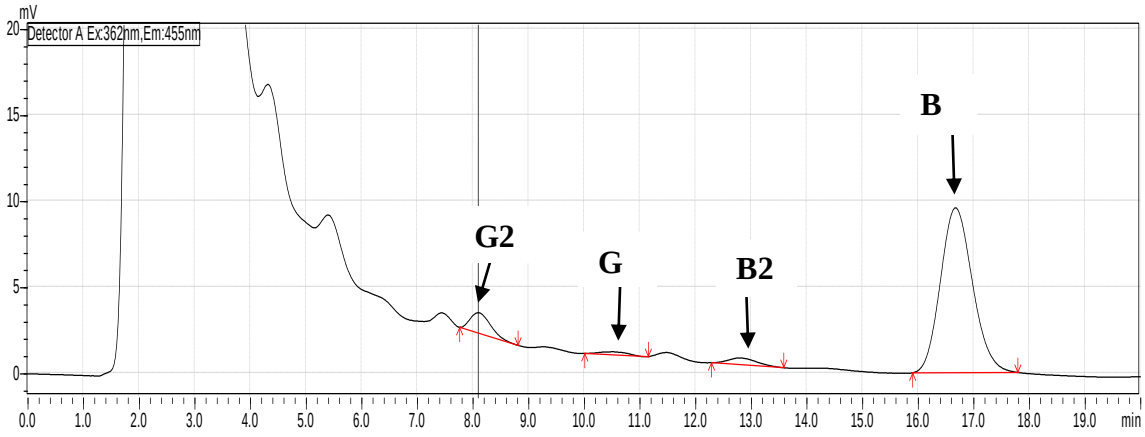
Appendix XVIIIb: Chromatogram of fumonisin B_1 positive extract of 9 days old *F. verticilliodes* isolate cultured on malt extract agar



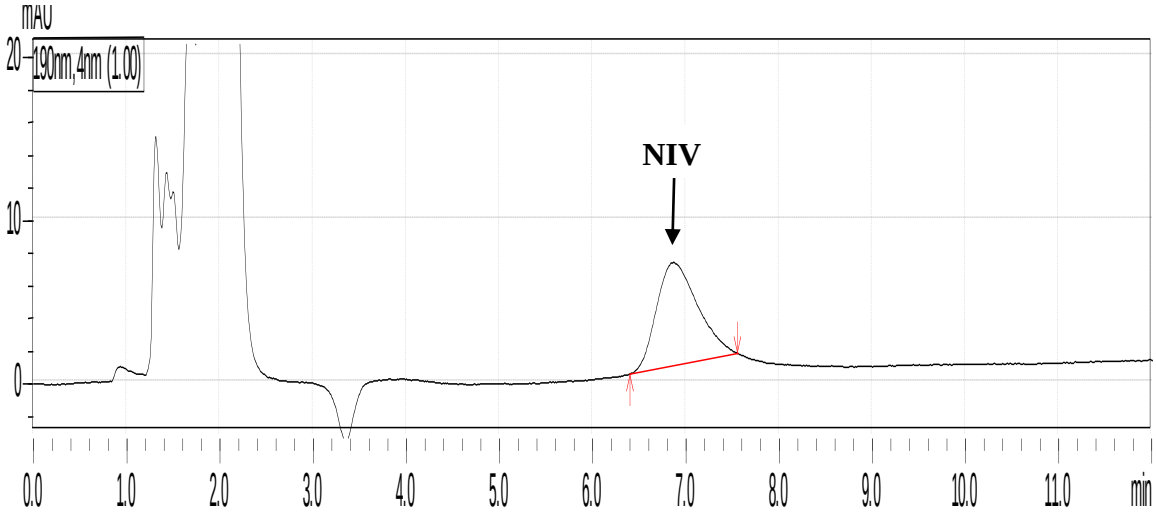
Appendix XVIIIc: Chromatogram of fumonisin B_1 positive extract of 9 days old *F. oxysporum* isolate cultured on potato dextrose agar



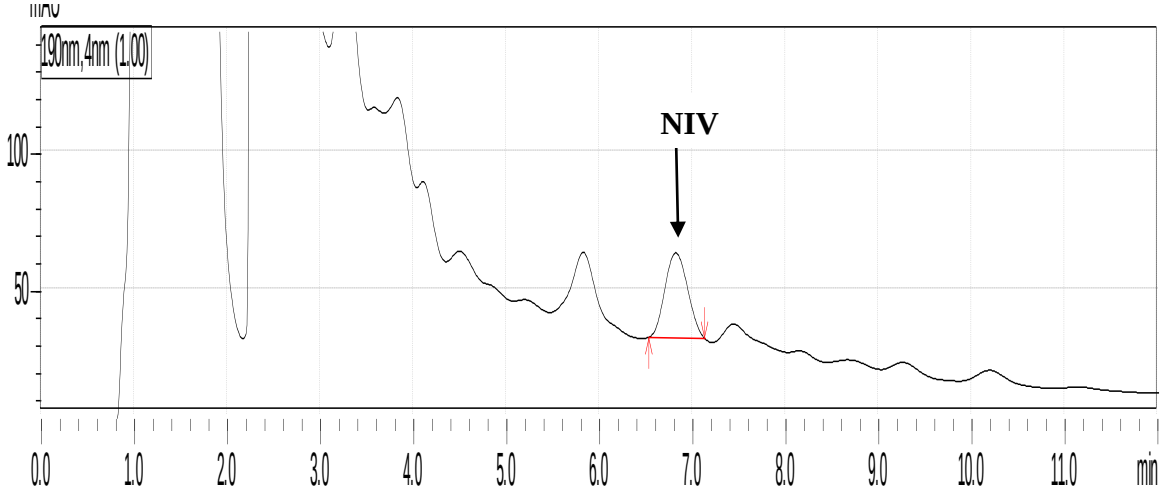
Appendix XVIIId: Chromatogram of aflatoxin B_1, B_2, G_1 and G_2 standards



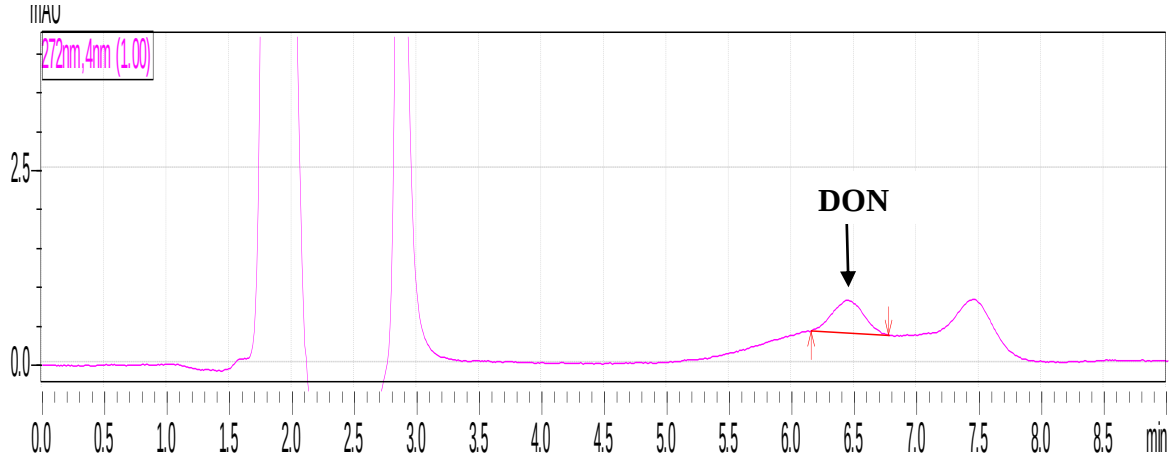
Appendix XVIIIe: Chromatogram of aflatoxins positive extract of *Aspergillus flavus* isolate cultured on potato dextrose agar for 9 days



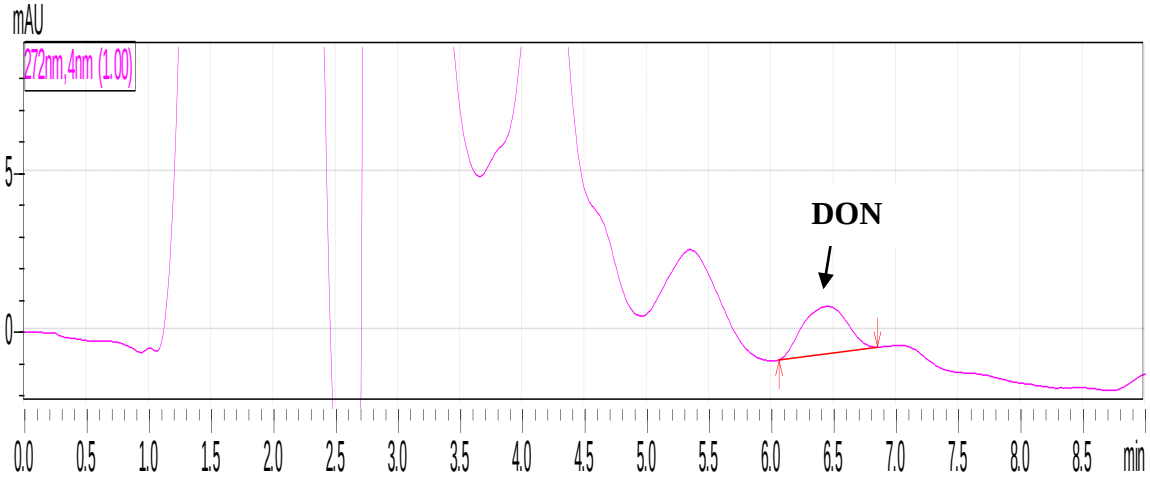
Appendix XVIIIf: Chromatogram of nivalenol standard (200µg/ml)



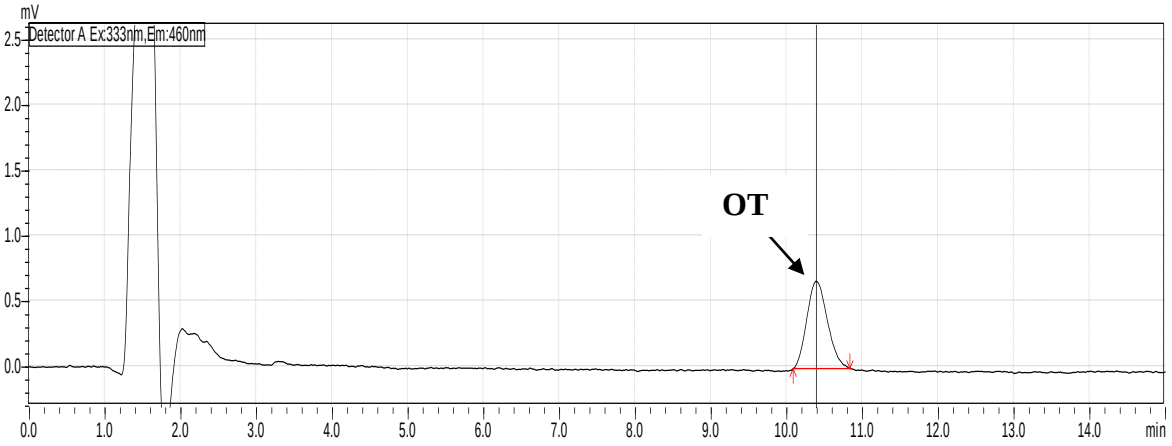
Appendix XVIIIg: Chromatogram of nivalenol positive extract of 4 days old *Fusarium verticillioides* isolate cultured on malt extract agar



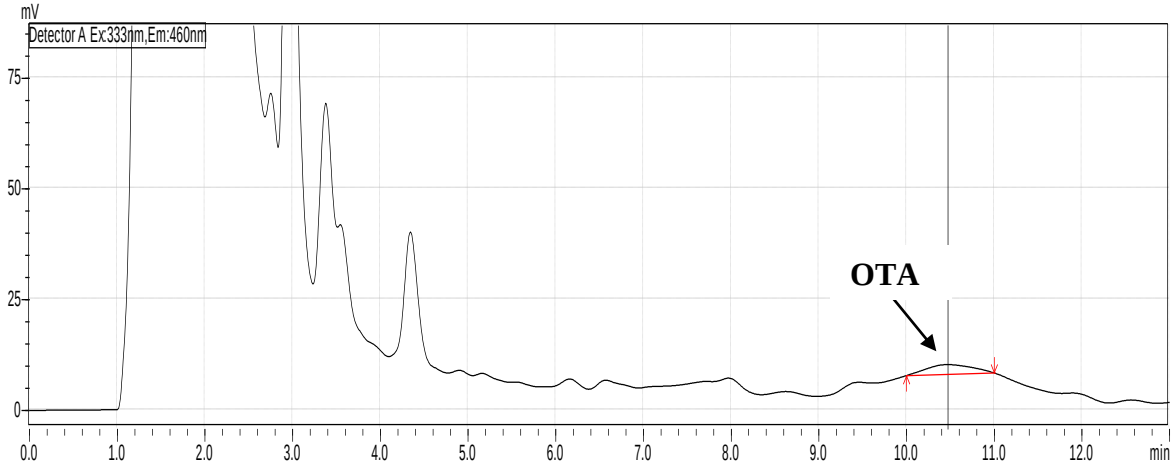
Appendix XVIIIh: Chromatogram of deoxynivalenol standard (20µg/ml)



Appendix XVIIIi: Chromatogram of deoxynivalenol positive extract of 9 days old *Fusarium verticillioides* isolate cultured on potato dextrose agar



Appendix XVIIIj: Chromatogram of ochratoxin A standard (2ng/ml)



Appendix XVIIIk: Chromatogram of ochratoxin A positive extract from 9 days old *Aspergillus niger* cultured on malt extract agar

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2014/05/27

ETHICS APPROVAL OF PROJECT

This is to certify that the next project was approved by the NWU Ethics Committee:

Project title:

Investigating molecular interactions of three filamentous fungal genera in relation to their toxicity **Project leader**:Prof. Babalola **Student:** M Egbuta

Ethics number: NWU-00048-14-A9

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Expiry date: 2019/05/16

The Ethics Committee would like to remain at your service as scientist and researcher, and wishes you well with your project.

Please do not hesitate to contact the Ethics Committee for any further enquiries or requests for assistance.

The formal ethics approval certificate will follow shortly.

Yours sincerely

HM Halgryn

NWU Research Ethics Secratariate