Investigations on the antifungal and cancer modulating properties of extracts from selected species of *Tulbaghia*

by

Zanephyn Keyser

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Supervisor: Prof J A Klaasen

Co-supervisor: Prof J Marnewick

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KEYWORDS

Fusarium verticillioides

Fumonisin

Fungicides

Synergism

Tulbaghia violacea

Tulbaghia alliacea

Tulbaghia. simmleri

Allium sativum

Antioxidant

Phenolic compounds

Modulation

Chemoprevention

ABSTRACT

Fusarium verticillioides (Sacc) Nirenberg a common phytopathogen of maize and maize-based products produces fumonisin B (FB) mycotoxins that have been related to several diseases such as equine leukoencephalomalacia (ELEM), porcine pulmonary edema (PPE), liver toxicity in several animals and esophageal and liver cancer in humans. In one of our studies we hypothesize that aqueous extracts of indigenous South African wild garlic species (*Tulbaghia violacea*, *T. alliacea* and *T. simmleri)* may enhance the efficacy of the fungicides, Sporekill™, Thiram, Itraconazole and Fluconazole against *F. verticillioides* (MRC 826). Data analysis from *in vitro* results indicates that for the 16 different mixtures of each plant extract and fungicide combination, several significantly ($P<0.05$) higher growth inhibition responses were produced. More synergistic interactions were observed for the combinations of sporekill with *T. violacea* (62%) and *T. alliacea* (75%) than for *T. simmleri* (25%).. Mixtures between the azole fungicides and *T. simmleri* produced 94 % synergistic interactions. Combination of fungicides and plant compounds offers the opportunity to find synergistic mixtures and may validate disease control strategies with increased biological activity and low dose rate application. Modulation studies of hepatic drug metabolizing enzymes and oxidative properties of *Allium sativum, Tulbaghia violacea* and *T. alliacea* in male Fischer rats were also evaluated. Due to its complex phytochemical composition a battery of assays were used to evaluate antioxidant potential. The extracts exhibited no adverse effects in the liver and kidneys of the rats. Total plasma iron was not affected showing no evidence for iron catalyzed lipid peroxidation. An increase was noted in hepatic ORAC values for rats consuming *T. violacea* and *T. alliacea.* However, no correlation was observed between the phenolic intake by the rats and the increased hepatic ORAC levels. In this study, pre-treatment with aqueous extracts of *T. violacea, T. alliacea* and *A. sativum* resulted in a significant elevation in GSH levels, induction of GST-μ and UDP-GT and modulation of CAT and SOD. This modulated oxidative status and phase II drug metabolizing enzymes in the liver may protect the liver against the adverse effects related to oxidative damage and mutagenesis. The chemoprotective properties of crude aqueous extracts of *A. sativum, T. violacea* and *T. alliacea* were investigated on preneoplastic foci formation promoted by culture material of *F. verticillioides* MRC 826 utilizing diethylnitrosamine (DEN) as cancer initiator. Clinical chemical parameters related to liver and kidney function and decreased body weight gain suggesting that severe, acute liver injury had been induced in the positive control

(DEN-CMF) rats, while the levels were mostly reduced by the garlic treatments. This study further indicates that *T. alliaceae* (2 % w/v) and *A. sativum* (1% w/v) treatment suppressed GST-P^+ foci formation with the modulation of GST-α phase II detoxification enzymes, as well as the antioxidant enzyme, SOD (*T. alliaceae)* and decreased GSH levels as being possible mechanisms of protection. These results provide new evidence showing the modulation of phase II drug metabolizing enzymes and the oxidative status in the liver of rats by the wild garlic species as well as *A. sativum.*

DECLARATION

I declare that "*Investigations on the antifungal and cancer modulating properties of extracts from selected species of Tulbaghia*" is my own work, that it has not been submitted for any other degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name: Zanephyn Keyser Date: September 2012

Signed: .....

DEDICATION

To Rhoda, Zoë and Roxi for their understanding, love, patience and support

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To God for privileges, mercy and blessings in abundance.

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%	percentage
°C	degrees celsius
µl	microliter
µmole	micromole
2-AAF	2-acetylaminoflourene
6-HD	6-hydroxydopamine
a.i.	active ingredient
AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
ABC	ATP-binding cassette
AFB1	aflatoxin B1
AGE	aqueous garlic extract
AIDS	acquired immunodeficiency syndrome
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANOVA	analysis of variance
As	*Allium sativum*
AST	aspartate aminotransferase
a_w	water activity
BHA	butylated hydroxyanisol
bw	body weight
CAT	catalase
CDNB	1-chloro-2,4-dinitrobenzene
CDs	Conjugated dienes
C_{exp}	expected efficacy
CFU	colony forming unit
CLA	carnation leaf agar
CMF	the fumonisin-containing diet
C_{obs}	observed efficacy
CPUT	Cape Peninsula University of Technology
CYP	cytochrome P450
d	days
DADS	diallyl disulphide
DAS	diallyl sulphide
DATS	diallyltrisulfide

DCNB	3,4-dichloro-nitrobenzene
DEN	diethylnitrosamine
DETAPAC	diethylenetriaminepentaacetic acid
DMSO	dimethyl sulfoxide
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
ECRA	Ethics Committee for Research on Animals
EDTA	ethylenediaminetetraacetic acid
EGCg	epigallocatechin gallate
ELEM	equine leukoencephalomalacia
FB1	fumonisin B1
FB2	fumonisin B2
FB3	fumonisin B3
g	gram
GPx	gluthatione reductase
GSH	glutathione (reduced form)
GSSG	oxidized glutathione
GST	glutathione S-Transferase
GST-μ	glutathione S-Transferase (mu)
GST-P^+	gammaglutamyl transpeptidase-positive foci
GST-α	glutathione S-Transferase (alpha)
HIV	human immunodeficiency virus
hrs	hours
KCl	potassium chloride
l	liters
M	molar
M2VP	1-methyl-2-vinyl-pyridinium
MDA	malondialdehyde
MDG	methyl-α-D-glucopyranoside
MDR	multidrug resistant
MFC	minimum fungicidal concentration
MFS	major facilitator superfamily
mg	milligram
$MgCl_2$	magnesium chloride
min	minute
ml	milliliters
mm	millimeters
mM	milli molar

MRC	Medical Research Council
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NAT	N-acetyltransferases
nm	nanometers
NQO1	quinone oxidoreductase
ORAC	oxygen radical absorbance capacity
OSC	organosulphur compounds
PCA	perchloric acid
PDA	potato dextrose agar
PP	propyl parapen
PPE	porcine pulmonary edema
PROMEC	Programme on Mycotoxins and Experimental Carcinogenesis
ROS	reactive oxygen species
SAMC	S-allylmercaptocysteine
SOD	superoxide dismutase
SR	synergy ratio
ST	sulfotransferases
Ta	*Tulbaghia alliacea*
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TCA	trichloro acetic acid
Ts	*Tulbaghia simmleri*
Tv	*Tulbaghia violacea*
UA	uric acid
UDP-GT	UDP-Glucuronosyltransferase
uM	micro molar
v/v	volume per volume
Vit C	vitamin C
w/v	weight per volume

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

There is an increasing concern worldwide about food safety resulting in an enhanced interest in fungal infection and subsequent production of mycotoxins in food products. In this respect, attention is continuously focused on maize (*Zea mays* L.) because it has been classified as one of the most important dietary staple foods in the world (Boomsma and Vyn 2008). Many South Africans from different cultures consume maize in one form or another (Van Heerden and Schonfeldt 2004). Maize is the third most planted field crop in the world and forms the basis of a number of foods, feed, pharmaceutical and various industrial manufacturers (Flett 2002). The majority of South African livestock are maize fed and consumed by humans as meat, dairy products, cheeses, and eggs.

There is currently overwhelming evidence of contamination of maize, maize-related products and cereals by various fungi and mycotoxins produced by these organisms throughout the world. Several fungi are associated with maize during pre-and postharvest periods, of which the genus *Fusarium* contains important toxigenic species i.e. *Fusarium verticillioides* (Sacc) Nirenberg (formerly known as *Fusarium moniliforme* Sheldon). This fungus is regarded as one of the most economically important species worldwide (Nelson *et al.,* 1981; Munkvold and Desjardins 1997). *F. verticillioides* is known to produce a number of mycotoxins, primarily fumonisins (Marasas 2001). In 1988, Gelderblom *et al* reported that the fumonisins had been identified from cultures of *F. moniliforme* J. Sheld., and that these toxins exhibited cancer promoting activity (Gelderblom *et al.,* 1988b).

During the growth of the maize plant, *F. verticillioides* is associated with disease development at all stages of the plant. Although no symptoms are noticed during infection of the plant, seed-transmitted strains of the fungus can develop systemically to infect the kernels (Kedera *et al.,* 1992; Munkvold *et al.,* 1997). This endophytic relationship is of great concern which may lead to the loss of grain and seed quality as well as the potential incidence of fumonisins and other mycotoxins (Kpodo *et al.,* 2000).

The involvement of mycotoxins in animal and human diseases has been known for more than 2000 years (Marasas *et al.,* 1979). All mycotoxins can be referred to as low-molecular-weight natural products produced as secondary metabolites by filamentous fungi (Sherif *et al.,* 2009). Fungal products that are mainly toxic to bacteria (such as penicillin) are usually referred to as antibiotics while fungal products toxic to plants are referred to as phytotoxins by plant pathologists. Mycotoxins produced by fungi are toxic to vertebrates and other animal groups in low concentrations. The most abundant moulds producing these toxins and causing contamination of human foods and animal feeds are *Fusarium*, *Aspergillus*, and *Penicillium* (Bhatnagar *et al.,* 2004).

These toxic substances of diverse chemical structure are capable of inducing a variety of adverse effects to man and domestic animals. The ingestion of one or a combination of mycotoxins may negatively affect every system of the body leading to various clinical signs and lesions; the expression of which, may vary markedly among animal species (Haschek *et al.,* 2002). However, the target and the concentration of the metabolite in order to cause harm are equally important. Several mycotoxins are known to be carcinogenic and prolonged exposure to these low levels could pose a threat to human and animal health. Human exposure to mycotoxins at sub acute levels may result from consumption of plant-derived foods that are contaminated with toxins, the carry-over of mycotoxins and their metabolites in animal products such as meat and eggs or exposure to air and dust containing toxins (Cast 2003; Bryden 2007).

The most recently described mycotoxins that influence human and animal nutrition are fumonisins. Fumonisins were originally reported in South Africa in 1988 (Bezuidenhout *et al.,* 1988; Gelderblom *et al.,* 1988b). Fumonisins are produced by a number of *Fusarium* species, such as *F. verticilliodides*, *F. proliferatum* and *F. nygamai*, as well as *Alternaria* sp. Fumonisins cause severe animal diseases such as equine leukoencephalomalacia (ELEM) in horses (Marasas *et al.,* 1988), and hydrothorax and porcine pulmonary edema in swine (PPE) (Colvin and Harrison 1992; Halloy 2005). Besides their hepatotoxicity (Gelderblom *et al.,* 2001a) and nephrotoxicity (Edrington *et al.,* 1995) they also affect the immune system (Bhandari *et al.,* 2002; Dombrink-Kurtzman 2003). Shephard *et al* (1996) reported on the lack of evidence of adverse effects of fumonisins on human health. However, studies have reported these toxins to be associated with high incidences of oesophageal cancer in South Africa (Rheeder *et al.,* 1992), China (Wang *et al.,* 2000), Italy

(Franceschi *et al.,* 1990) and Iran (Shephard *et al.,* 2000). It is therefore important to note that the actual involvement of this fungus in the development of oesophageal cancer has, however, not been proven, but cannot be ruled out completely. The implications of fumonisin toxicity in foods and feeds are therefore regarded as being serious (Miller 2008; Shephard 2008). Our knowledge concerning these toxic effects has been obtained from animal toxicity studies.

1.2 The plant pathogen *Fusarium verticilliodes*

Fusarium has been identified as one of the major fungal genera associated with maize in Africa (Rheeder *et al.,* 1992; Schulthess *et al.,* 2002). It is widely distributed in soil and several other agricultural commodities in both tropical and temperate regions. *Fusarium* species are generally considered as field fungi invading in excess of 50% of maize grains before harvest (Keyser *et al.,* 1999; Schulthess *et al.,* 2002; Fandohan *et al.,* 2004). *Fusarium* species is therefore known to be the most important plant pathogen (Booth 1971). This genus consists of a number of toxigenic species including *F. verticillioides* (Sacc.) Nirenburg (= *F. moniliforme*) and *F. proliferatum*, which are able to produce a mycotoxin called fumonisins. *F. verticillioides* is formerly known as *F. moniliforme* and as *Gibberella moniliformis* (= *Gibberella fujikuroi*) in its sexual stage. In addition to being the most prevalent fungus on maize in Africa, its reported existence in other plant species confirm that *F. verticillioides* is not host specific (Gelderblom *et al.,* 2002). Reports of surveys conducted in some African countries showed that *F. verticillioides* is the most prevalent fungus on maize (Kedera *et al.,* 1999; Schulthess *et al.,* 2002).

The *Fusarium* species can effectively compete against other fungal colonisers and, thus, has the ability to sometimes dominate the ecosystem of maize (Marin *et al.,* 1998). The damage caused is not always visibly recognisable, leading to its presence being ignored. Munkvold *et al* (1997) also states that standard washing methods are unable to remove symptom free, infectious kernels from grain.

F. verticillioides is an endophyte of maize, and therefore establishes long-term associations with the plant (Nelson *et al.,* 1991). Only certain strains of *F. verticillioides* produce disease in maize and are able to contaminate maize (Figure 1.1) at all the different stages of plant development. This contamination lead to grain rot during the preharvest and postharvest periods (Munkvold *et al.,* 1997). *F. verticillioides* may sometimes appear as white to salmon pink coloured mould when

grown on maize, but it may not be visible on the corn kernel. This fungus often produces a symptom on the corn kernels referred to as "starburst", which is visible white streaking in the kernel. The visual absence of mould does not mean that the kernels do not contain the toxin (Nelson *et al.,* 1993; Bacon and Hinton 1996).

The *Fusarium* species are also associated with infection of cereals (D'Mello and Macdonald 1997) and are opportunistic pathogens, which destroy the host cells and feeds on the contents, which are digested by coenzymes (Prell and Day 2001). D'Mello and Macdonald (1997) described *F. verticilloides* as a toxigenic fungus while its presence on maize is often overlooked as no visible damage is apparent (Munkvold and Desjardins 1997). There is a strong correlation between insect damage and *Fusarium* kernel rot (Visconti *et al.,* 1999). Visconti *et al.* (1999) showed that a field survey conducted showed that the presence of the European corn borer increased *F. moniliforme*-induced diseases and levels of fumonisins.

Figure 1.1. Visible damage by *Fusarium verticilloides* - ear rot symptoms

Fusarium spp produce colourless spores (conidia), which are canoe-shaped in profile and have a distinct "foot cell" at the lower end, and are divided by several cross-walls (Doyle 1998). Sporodochia are formed when conidiophores cluster and pasty white masses of spores from phialides are produced. There are two different spores forms that can occur, Microconidia (resembles *Acremonium* spores and phialides) and chlamydospores (thick-walled swellings along the filaments) (Doyle 1998).

The presence of *F. verticilloides* and the production of fumonisins in maize are partly influenced by environmental factors in the field, and transportation and storage conditions (Gamanya and Sibanda 2001). *F. verticilloides* occurrence in maize is affected by temperature and relative humidity (Gamanya and Sibanda 2001). The growth and survival of the fungi during storage is affected by tolerance to 'extreme conditions', grain moisture content, temperature, gas composition,

fungal species interactions, fungal interactions with granary insects and mites and seeds active resistance to fungal infection (Wicklow *et al.,* 1998). In isolation, *Fusarium* does not colonize maize grain. However, competition is required from other colonizers for *Fusarium* to become established, with *Aspergillus* and *Penicillium* spp. as possible competitors (Marin *et al.,* 1998). The effectiveness of the competition against the other colonizers depends on environmental conditions, water activity and temperature (Marin *et al.,* 1998).

1.3 Disease Cycle

The complex cycle of infection and disease in the *F. moniliforme*-maize system is shown in Figure 1.2. *F. moniliforme* survives in crop residue (Leslie *et al.,* 1990) where it produces thickened hyphae and not chlamydospores which prolong its survival (Kommedahl and Windels 1981). The fungus is seedborne and seed transmitted (McGee 1988). This phase of the disease cycle is primarily associated with seedling disease (Kedera *et al.,* 1992). Seeds can be infected by *F. moniliforme* without any detrimental effects (Kommedahl and Windels 1981). *F. moniliforme* are known to produce macro- and microconidia, which are airborne (Nelson 1992). Kommedahl and Windels (1981) reported that maize silks can be infected by airborne or water-splashed conidia. Possible infection pathways of *F. verticillioides* include infection from seed to cob leading to grain through systemic movement in stalk; from the root to the grain through the stalk and the cob; from airborne or water-splashed conidia to the silk and further to the grain; through wounds on the maize caused by insects. Insects cause injuries to the maize plant and create sites of infection (Figure 1.3) of maize ears and stalks. *Ostrinia nubilalis,* the European corn borer, is regarded as the most commonly cited associated insect.

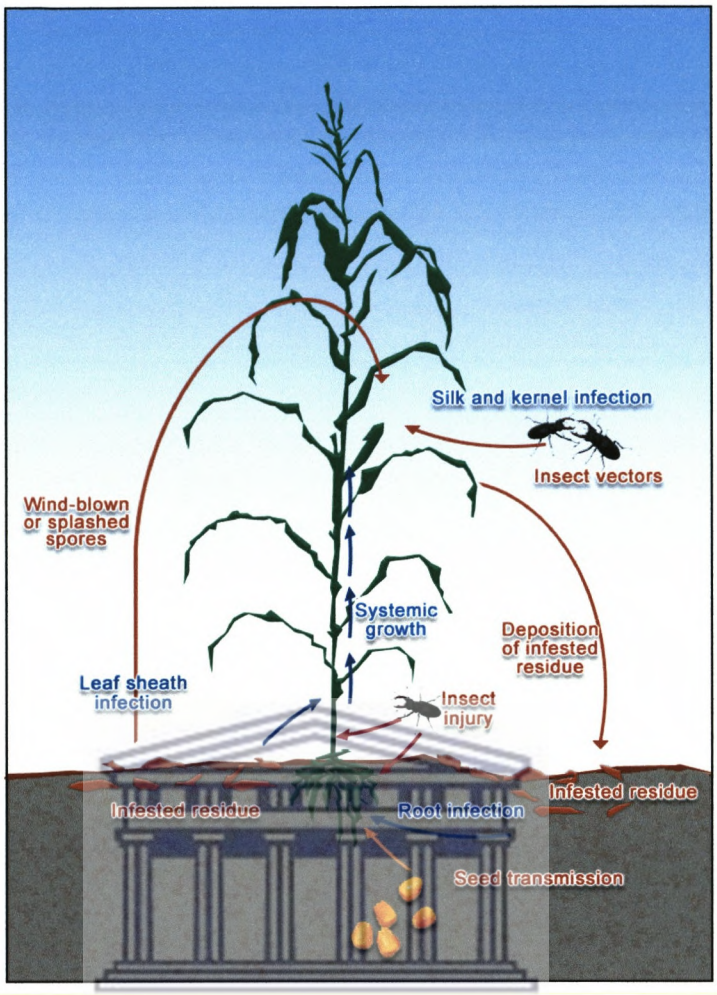


Figure 1.2. Disease cycle of *Fusarium moniliforme* on maize. Various infection pathways are illustrated, but their relative importance is not indicated. The most common pathway to kernel infection is through silks or insect injuries (Munkvold and Desjardins 1997).

Figure 1.3. *Fusarium moniliforme* infection of insect-damaged maize ear (Munkvold and Desjardins 1997).

1.4 Factors affecting the infection of maize with *F. verticillioides*

Several environmental conditions including insect infestation, pre- and- post harvest strategies, generally influence the infection of maize by *Fusarium* species and the subsequent production of fumonisins. The damage to maize is most often a result of complex interactions (Marin *et al.,* 1999; Paterson and Lima 2010; Zain 2011).

1.4.1 *Environmental factors*

High levels of fumonisins are normally associated with warmer and drier climates, but it is not necessarily the same from one year to another (Marasas 2001). The harsh oscillations in rainfall and relative humidity prior to harvesting, may lead to physiological stress in the plant which is likely to create favorable conditions for fumonisin(s) production. Alberts *et al.* (1990) also showed that moisture and temperature conditions during the growing season and storage frequently influence maize infection by *Fusarium* species as well as fumonisins production. Dry weather prior or during pollination of maize is another important parameter for the production of fumonisins in maize (Marasas 1996). Fungal growth is also relient on the level of water activity (a_w) (Jouany 2007). *In vitro* data produced by Alberts *et al.* (1990) indicated a better growth rate of *F. verticillioides* at a temperature of 25°C, whereas at 15°C the growth was much lower. Furthermore, the data also revealed that at a constant temperature, a_w significantly increased the growth rate of *F. verticillioides.*

1.4.2 *Agricultural practices*

Late planting of maize as well as harvesting in wet conditions later in the season has been reported as favourable conditions for disease caused by *F. verticillioides* (Marasas 1996). Furthermore, the repeated planting of maize and other cereal crops on the same land or in close vicinity also support fungal infection by means of an increase in fungal inoculum and insect population (Kedera *et al.,* 1999; Schaafsma *et al.,* 2001). Weed generally harbors a wide range of *Fusarium* species. Therefore, a high weed density may result in an increase infection in wheat crops (Jouany 2007). Weed control strategies can also reduce fungal infection in maize fields as it assists in eliminating non-host weeds harboring infective *Fusarium* species.

1.4.3 *Maize characteristics*

The type of maize cultivar and subsequent grain characteristics, endosperm type chemical composition as well as the developing stage may contribute to fungal infection and fumonisin production. Maize cultivars that mature later in the season result in a decrease in grain moisture content below 30% are most susceptible to *Fusarium* disease (Marasas 1996). Upright cobs, tight husks, thin grain pericarp and an increased propensity for grain splitting make such maize cultivars more vulnerable to *Fusarium* infection (Riley and Norred 1999). Tight husked varieties allows for slow drying and subsequently favouring *Fusarium* infection. The mechanical removal of the outer parts by dehulling can significantly reduce the toxin in maize (Marasas 1996; Jouany 2007) as fumonisins are found to be more concentrated in the pericarp and germ of the grain. The relationship of maize grain colour and fumonisin contamination is unknown but fumonisin levels have been found to be considerably lower in yellow than in white maize in some years but the opposite was observed in other years (Shephard *et al.,* 1996). Fumonisin production in maize may also be influenced by the age of the grain as its production may start early in cob development and as the grains reaches physiological maturity, an increase in toxin levels are noticed with significantly greater levels after physiological maturity (Shephard *et al.,* 1996).

1.4.4 *Post harvest operations*

Damage to maize cobs or grains during mechanical harvesting may assist in entry of the fungal spores. The washing of maize grains by immersion in water and removing the upper floating portion lead to a huge reduction of fumonisins (up to 74 %) (Wilson *et al.,* 2004). Contaminated grains in general have a low density and form part of the floating portion. The addition of salt to the water during the washing process resulted in a more significant (about 86 %) removal of the toxin. Ordinary cooking does not significantly reduce the toxin as fumonisin has been found to be moderately heat-stable. Therefore substantial elimination of fumonisins is more likely to occur only when temperature of more than 150°C is used during cooking (Meister and Springer 2004).

F. verticillioides have the ability to grow inside the maize plant tissues like an asymptomatic endophyte (Oren *et al.,* 2003). The symptomless infection caused by *F. verticillioides* exists throughout the plant in the leaves, stems, roots and grains. Several reports have indicated that fumonisins have been detected in these symptomless infected kernels (Bacon and Hinton 1996; Munkvold and Desjardins

1997; Desjardins *et al.,* 1998; Bacon *et al.,* 2008; Cavaglieri *et al.,* 2005). Therefore, the occurrence of *F. verticillioides* is in several cases ignored since it does not cause noticeable damage to the plant.

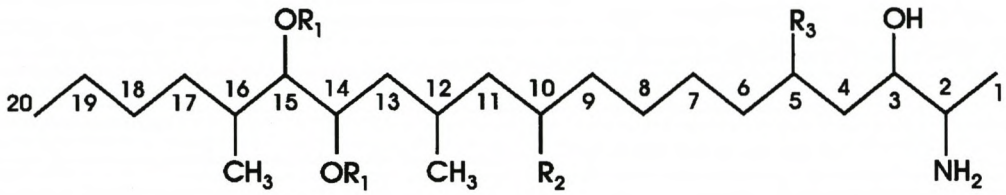
1.5 Fumonisins

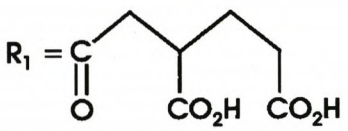
Fumonisins are carcinogenic mycotoxins produced by *F. verticillioides* and were first isolated and characterized in 1988 in South Africa (Gelderblom *et al.,* 1988b; Bolger *et al.,* 2001; Glenn 2007). These mycotoxins are economically important as they are commonly linked to contaminants with maize-based foods and feeds throughout the world (Abbas *et al.,* 1995). Since the isolation and characterisation of fumonisin, more than 28 homologues have been discovered (Rheeder *et al.,* 2002; Humpf and Voss 2004). The most abundantly found fumonisins being fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) found in contaminated feed and foodstuffs are from a toxicological standpoint, the most thoroughly studied (Shephard *et al.,* 1995; Flett 2002; Dilkin *et al.,* 2003; Voss *et al.,* 2007). Fumonisins cause chronic and acute poisoning and cause allergic signs both to animals and humans (Agrios 1996; Bolger *et al.,* 2001; Marasas 2001).

Fumonisins are also found in other commodities (Seefelder *et al.,* 2002; Kritzinger *et al.,* 2003; Binder 2007) other than maize. It is evident that animal and human health problems related to fumonisins are almost completely associated with the consumption of contaminated maize or maize products (Bolger *et al.,* 2001; Marasas 2001). The human health effects of fumonisins are still indecisive (Voss *et al.,* 2007).

1.5.1 Chemical structure

Fumonisins have a structure (Figure 1.4) based on a long hydroxylated hydrocarbon chain containing methyl and amino groups (Dutton 1996). The structural similarity of fumonisins to shinganine, the ceramide biosynthesis it interferes with, is critical to the carcinogenic properties of FB_1 (Dutton 1996; Merrill Jr *et al.,* 2001; Riley *et al.,* 2001). The toxicological impact of FB_1 has been thoroughly studied. Fumonisin B_2, FB_3 and FB_4 are in order less prevalent and differ structurally from FB_1 in the number and placement of hydroxyl groups on the molecule's hydrocarbon "backbone" (Figure 1.4).





	R_2	R_3
Fumonisin B_1	OH	OH
Fumonisin B_2	H	OH
Fumonisin B_3	OH	H
Fumonisin B_4	H	H

Figure 1.4. Chemical structures of the fumonisins (Munkvold and Desjardins 1997)

However, suspected risk factors have been identified linking fumonisins to esophageal and liver cancers as well as cardiovascular problems (Fincham *et al.,* 1992; Ueno *et al.,* 1997; Marasas 2001; Wang *et al.,* 2005; Odhav *et al.,* 2008). There is also alarm that consumption of food containing fumonisins during early pregnancy could result in elevated risk of neural tube defect in the developing fetus (Marasas 1996; Odhav *et al.,* 2008). These findings were based specifically on populations that consume relatively large amounts of food made with contaminated maize.

1.5.2 Toxicological effects and carcinogenesis studies of fumonisin B_1

The toxicological and pathological effects of fumonisins have been extensively studied in laboratory animals. Graham (1912) reported that "Moldy corn poisoning" of farm animals was first described in 1850 in the United States. In 1902, culture material of *F. verticilliodes*, was associated with equine leucoencephalomalacia (ELEM) that was induced by naturally contaminated maize (Butler 1902). The causative agent was identified as *F. moniliforme* by Sheldon (1904) and linked it with an outbreak of moldy corn disease of cattle, horses, hogs, mules, and chickens in Nebraska. However, the scientific involvement of *F. verticilliodes* was only confirmed during the 1970's when pure cultures of the fungus in maize were tested (Wilson and Maronpot 1971; Marasas *et al.,* 1976).

FB_1 is generally regarded as a non-genotoxin, in that it lacks activity in mutagenicity (Gelderblom and Snyman 1991) and genotoxicity assays (Norred *et al.,* 1992; Gelderblom *et al.,* 1994) and appears not to bind directly to DNA. Studies concerning the cancer initiating activity of FB_1 indicated that a cytotoxic/proliferative threshold exists for cancer initiation in rat liver and levels that fail to induce a toxic effect, lack cancer initiating activity (Gelderblom *et al.,* 1994). This was further supported in a long-term study indicating that low dietary levels that cause only mild toxic changes fail to induce hepatocellular cancer in rats (Gelderblom *et al.,* 2001c). A study by Mehta *et al* (1998) conducted with Sprague-Dawley rats, also suggested that compensatory cell proliferation in response to cellular toxicity is a prerequisite for initiation.

ELEM, a fatal brain disease of horses, mules, donkeys, and rabbits, is an example of the most dramatic manifestation of moldy corn disease (Wilson and Maronpot 1971; Leslie *et al.,* 1992; Nelson 1992). Kellerman *et al.* (1990) reported that the consumption of moldy maize has long been a recognized cause of equine ELEM. These findings have demonstrated experimentally that *F. verticillioides*-contaminated feeds and fumonisin B_1 (FB_1) can induce ELEM (Marasas *et al.,* 1988; Wilson *et al.,* 1992; Stankovic *et al.,* 2011).

The primary diagnostic feature of ELEM is softening and liquefying of the cerebral hemispheres of the brain. In combination with ELEM, muscular melting and cardiac failure have also been reported in horses. Horses with these symptoms of ELEM usually die and recovery is very rare (Kriek *et al.,* 1981a; Marasas 1996). Maize contamination with fumonisin up to a level of 10 mg FB per kg could lead to an increased risk for horses to develop ELEM (Viljoen *et al.,* 2003). Although identified as the most dramatic animal disease caused by fumonisins, it is not the only animal disease associated with the consumption of contaminated feed with *F. verticillioides.*

Similarly, *F. verticillioides*-contaminated feeds and FB_1 have been shown to be cardiotoxic and cause pulmonary edema in pigs, a syndrome termed porcine pulmonary edema (PPE) (Harrison *et al.,* 1990) (Haschek *et al.,* 2001). Porcine pulmonary edema was first described during 1988 and 1989 in the US (Harrison *et al.,* 1990; Bane *et al.,* 1992; Osweiler *et al.,* 1992; Smith *et al.,* 2000). Samples of feed were analysed and FB_1 was detected in a range of 20-330 mg/kg. Ross *et al* (1990) also linked PPE with outbreaks of ELEM. Porcine pulmonary edema were reproduced in pigs that were fed naturally contaminated feed, culture material

containing fumonisin and purified FB_1 (Haschek *et al.,* 1992; Colvin *et al.,* 1993; Motelin *et al.,* 1994). Porcine pulmonary edema has the resulting symptoms of enhanced pulmonary artery pressure and decreased heart rate (D'Mello *et al.,* 1999). Bolger *et al* (2001) reported that cattle and poultry showed considerably less sensitivity to fumonisins than horses, pigs, rabbits, or laboratory rodents.

1.6 Medical importance of *F. verticillioides*

F. verticillioides is becoming a major emerging infectious fungal disease, with fusariosis becoming more prominent in the immunocompromised and those with haematological malignancies (Campo *et al.,* ; De Pauw *et al.,* 2008). Humans are frequently exposed to various fungi present in the environment. In the presence of an intact immune system, these interactions rarely result in significant disease. However, a significant increase has been seen in the number of invasive fungal infections during the last decade, especially in the immunocompromised patient (Badiee *et al.,* 2009). This increase is generally attributed to immunosuppressive therapy in cancer patients and also the dramatic rise in human immunodeficiency virus (HIV) infections and acquired immunodeficiency syndrome (AIDS), as these contribute largely to the susceptibility of the host to fungal infection (Venkatesan *et al.,* 2005). Usually opportunistic fungal pathogens, such as *Candida* and *Aspergillus* species are seen, but *Fusarium* infections has emerged as a major threat as these cases are being reported with increasing frequency in high morbidity and mortality, irrespective of antifungal therapy (Gaur and Flynn 2001). There are eight species of *Fusarium* recognized as opportunistic pathogens that cause disseminated infections in the compromised host, with *F. verticillioides* being one of the most common (Guarro *et al.,* 2000; Gaur and Flynn 2001; Ortoneda *et al.,* 2002).

Portals of entry of disseminated infection include the respiratory tract, the gastrointestinal tract, and cutaneous sites (Siegel *et al.,* 2007). They can cause local cutaneous (keratitis and skin infections) infections, including onychomycosis and infections of surgical and burn wounds. The cutaneous lesions observed are indicative of the early stage of the disease and may be painful red or violaceous nodules with an ulcerated centre covered by a black leathery eschar. The multiple necrotizing lesions are often observed on the trunk and the extremities (Siegel *et al.,* 2007).

Most importantly, *Fusarium* species can cause disseminated infections with involvement of multiple organs and numerous skin lesions, causing fusariosis, which involves multiple organs, such as the liver, lung, kidney, heart, spleen, and pancreas, with a high mortality rate (Bodey *et al.,* 2002; Ortoneda *et al.,* 2002). In the treatment of this disease, resistance has been encountered. The use of the standard broad spectrum antifungal agent, amphotericin B, has been seen to be frequently ineffective and often causing adverse side effects (Gómez-López *et al.,* 2003). Fluconazole and miconazole have also shown a low efficacy and new antifungal treatments also have little effect. This resistance encountered is mainly due to the presence of ATP-binding cassette (ABC) transporters, which pump out the active substances, as well as the major facilitator superfamily (MFS) transporters, which are known for the secretion of toxins in pathogenic species (Del Sorbo *et al.,* 2000).

1.7 Control Methods against *Fusarium*

The control *in vivo* is very difficult because of the endophytic habit of *F. verticillioides*. Novel control strategies are being explored to reduce the risk of this pathogen and its toxin. Since *F. verticillioides* are commonly found in soil, seed treatments with an appropriate biocontrol agent are being explored to represent an appropriate method to suppress plant pathogens in the spermosphere and rhizosphere. Many biocontrol agents have been applied in the form of seed coatings against other fungi (Mao *et al.,* 1998; Batson *et al.,* 2000; Kerry 2000). *Bacillus amyloliquefaciens* and *Microbacterium oleovorans* have been tested as biocontrol agents to reduce *F. verticillioides* populations and fumonisin accumulation in the maize agroecosystem. At concentration of 10^7 colony forming unit (CFU) ml^{-1}, these organisms were applied as seed coatings and were found to be effective in reducing *F. verticillioides* counts and fumonisin B_1 and B_2 content from maize grains (Pereira *et al.,* 2007).

1.7.1 Effects of antifungal agents on *F. verticillioides*

Fungicides are toxic substances applied either to prevent the growth of fungi or kill fungi harmful to plants, animals, or humans. Agricultural fungicide are sprayed or dusted onto seeds, leaves or fruit to prevent the spread of rusts, smuts, moulds or mildew. Fungicides are applied to a broad range of plants and fruit trees. The effects of fungicides on the occurrence of *Fusarium* species were also tested in *vitro*. These were done by microbiologically analysing barley and summer wheat

yields of 1993 to 1994 and clarify whether it was possible to reduce occurrence of *Fusarium* species (Lõiveke 2004). The fungicides that were used in this research experiment were Corbel (Fenpropimorph), Sportak 45 EC (prochloraze), Tilt 250 EC (propiconazole), Folicur BT 225 EC (tebuconazole, triadimefon), Rider (fenpropimorph) and Calixin (tridemorph). Tilt and Corbel were effective in 75 – 100% of the tests done. The effects of the other fungicides were unstable, fluctuating from reducing the number of *Fusarium* species to increasing it (Lõiveke 2004). On the basis of the *in vitro* experiments done, it is clear that fungicides are often ineffective in controlling the production of mycotoxins produced by *Fusarium* species and are not effective in restricting the number of the species either.

The sensitivity of *F. verticillioides* conidia to Plantpro-45™, an iodine-based control agent, was also investigated. Plantpro-45™ was demonstrated to inhibited *F. verticillioides* growth from conidia at ≤10 µg active ingredient (a.i.)/ml. However, the lack of interest in using iodine-based agents as fungicides may relate to plant nutritional studies in the first part of the 20th century which reported that iodine-containing materials could be phytotoxic to plants (Cotton 1930; Wynd 1934).

1.7.2 Resistance of *Fusarium* and other fungi to antifungal agents

Based on worldwide surveillance studies, it was found that multi-drug resistance (MDR) in plant pathogenic fungi is increasingly becoming a common phenomenon (Karchmer 2000). Fungal infections in various sectors are becoming more challenging to treat, as a result of the emergence of multi-drug resistance among different fungal strains. The fungal cell wall acts as the border between the fungus and its environment (Marquez 2005). The cell wall has numerous roles which include providing the fungus with its shape and supporting it against osmotic forces and acting as a filter, controlling the secretion and uptake of molecules into the cell. Various families of integral membrane proteins are found in fungi as is the case with all other organisms. These membrane proteins can mediate transport of natural toxic compounds over biological membranes. For these transport processes to occur, the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) of transporters play a pivotal role. ATP-binding cassette transporters are regarded as the largest family of membrane proteins transporting substances across cell membranes (Schmitt and Tampé 2002; Stergiopoulos *et al.,* 2002). During the binding of ABC transporters with mainly ATP, energy is generated to transport solutes across cell membranes. The use of energy makes it possible to move solutes even against an electrochemical gradient and for this reason ABC

transporters are classified as primary active transporter systems. These transporters account for transport of an immense number of endogenous or exogenous toxicants (Higgins 1992). Major facilitator superfamily transporters are not able to hydrolyze ATP and move compounds over membranes driven by the proton-motive force (Hayashi *et al.,* 2002). Therefore MFS transporters are classified as secondary active transport systems (Lewis 1994).

Judelson and Senthil (2006) reported that ABC and, to a lesser extent, MFS transporters are implicated in conferring resistance or tolerance to fungicides (Judelson and Senthil 2006). An overexpression of ABC transporters make it possible for many MDR phenotypes to develop. Furthermore, MFS transporters have been recently implicated in secretion of host-specific and non-host-specific toxins in several species of plant pathogens. These efflux pumps assist in lowering the intracytoplasmic toxin concentration, therefore contributing to self-protection of a pathogen against its own toxin (Hayashi *et al.,* 2002). Micro-organisms produce these toxic compounds against competing organisms while being able to resist the effects of such toxic compounds in their natural environments. The plant pathogenesis of *Fusarium culmorum* can be attributed to two ABC transporters (FcABC1) (Skov *et al.,* 2004).

MDR are shown to play an important role to agricultural azole fungicides in plant pathogens (De Waard 1996; 1997). This finding led to the characterization of drug transporters in various plant pathogens which further assist in generating strategies into effective fungicide resistance management. The virulence factors of plant pathogens are influenced by transporters of natural toxic compounds which can be explored to predict novel disease control strategies. One approach would be to develop modulators that can inhibit the activity of transporters which directly impact on the efflux of host-specific toxins or even plant defense compounds. These modulators act indirectly as disease control agents and are not necessarily fungitoxic themselves (de Waard 1997). The success of these modulators is seen in mixtures of azoles and plant-derived, nontoxic inhibitors of ABC transporters. The result is the lowered inhibitory dosage of these fungicides that can lead to new, safer and effective agents exhibiting synergistic properties (Del Sorbo *et al.,* 1998). Furthermore, the emergence of antifungal resistance is also addressed by these approaches. A common strategy to protect food products from deterioration by various fungi and their toxins is the use of synthetic fungicides. However, the fact that most synthetic fungicides cause residual toxicity in grains which could result in

the development of fungal resistance as well as added consumer pressure to eliminate or reduce chemically synthesized additives has generated attention to the use of natural occurring compounds found in plants (Srivastava *et al.,* 2008). A significant interest was shown in recent years in the scientific community for natural plant extracts as alternatives to synthetic chemicals to control several pathogens in food (Fu *et al.,* 2007; Rota *et al.,* 2008). Plants generally produce an enormous variety of secondary metabolites which serves to protect plants against microbial pathogens (Dixon 2001). Extracts of cinnamon, clove, lemon grass, palmarosa and oregano essential oil were shown to control the growth of *F. verticillioides, F. proliferatum* and *F. graminearum* (Juglal *et al.,* 2002; Velluti *et al.,* 2003; Velluti *et al.,* 2004).

There is however an argument against plant-produced compounds as potential fungicides in that in the natural state, they are generally only weakly active compared to synthetic fungicides. In spite of the fact that these plant-derived anti-microbials are less powerful, plants fight infections successfully. For this reason, it becomes noticeable that plants implement a different paradigm - "synergy" - to combat infections (Hemaiswarya *et al.,* 2008). This can be achieved by the use of combination therapy using existing agents or new, safer and effective agents primarily from plant sources which can exhibit synergy with synthetic agents. The effect of two synthetic drugs namely, mancozeb and carboxin were successfully enhanced against *F. verticillioides* by scopoletin, a hydroxycoumarin from fruits of *Melia azedarach* L (Carpinella *et al.,* 2005).

1.8 Synergistic interactions of fungicides in mixtures

With the increase in resistance in opportunistic organisms, synergistic effects have received a lot of attention in the management of resistance to inhibitory fungicides in these pathogenic organisms (Lorbeer 1996). Synergy is a frequent phenomenon in fungicide mixtures. Its magnitude depends on the ratio of the mixtures and their modes of action. Synergy may reduce the selection process of resistant subpopulation and allow longer duration of the activity. These effects are most pronounced when applied simultaneously (Gisi 1996). The combination is effective because it delays or overcomes resistance in the population of the pathogen to one of the components in the mixture (Lorbeer 1996). Fungicides are combined in mixtures for three main reasons: (i) to widen the spectrum of antifungal activity; (ii) to exploit the synergistic interactions between fungicides, where the overall activity

can be increased or the amounts used can be reduced without loss of activity; and (iii) to delay the selection of resistant strains (Gisi 1996; Kosman and Cohen 1996).

Combinations can be called synergistic when the dosages of one or both of the components can be reduced without the loss of effective control of the pathogen targeted. Most modern fungicides are single-site inhibitors and when these are utilised in two-way mixtures, they can maintain or enhance the level of control of the pathogen. However, when these components are utilised separately a general reduced activity is observed. These combinations reduce the risk of development of resistance of the target pathogen to either component (Gisi 1996; Lorbeer 1996). There are several methods that have been developed to assess the synergistic interactions between fungicides. But to assess the modes of action, two basic approaches are commonly used, namely the Abbot method, which is applied for mixtures that have different modes of action, and the Wadley method, normally used for mixtures with a similar modes of action (Kosman and Cohen 1996).

Fungicides that are systemic and with site-specific activity, have been used frequently over many years. These fungicides were sometimes referred to as modern fungicides. Modern fungicides consist of a relatively low environmental toxicity index in comparison with conventional fungicides. They are also applied at lower concentrations of active ingredients and consequently contributed to environmental safe agriculture. Conversely, modern fungicides have also led to the development of resistance problems (Leroux and Descotes 1996). Modern fungicides are typically single-site inhibitors which made development of resistance possible. In order to reduce the risk of resistance or to prolong the process, the use of fungicide mixtures has been introduced (Hayashi *et al.,* 2003).

1.9 Determination of Synergism

The occurrence of a synergistic effect between pesticides is based on the comparison of observed efficacy of a mixture with its expected efficacy. The Abbott (also known as the Colby) method (Abbott 1925; Colby 1967), have been developed to assess synergistic interaction between pesticides which is usually applied for mixtures whose constituents produce their effects in different modes. The Wadley method (Wadley 1945; Tammes 1964) has been developed for mixtures in which the components have similar modes of action (Kosman and Cohen 1996). The

Abbott formula is used to predict the expected efficacy of a mixture which is expressed as percent control ($\%C_{exp}$) (Levy *et al.,* 1986):

$$\%C_{exp} = (A + B) - (AB/100)$$

in which A and B are the control levels given by the single fungicides. Synergistic interactions are evident when the ratio between the experimentally observed efficacy of the mixture (C_{obs}) and the expected efficacy of the mixture (C_{exp}) is greater than 1. Synergistic interactions at all times reduce rapidly with increasing control levels of the single components (Samoucha and Cohen 1984; Levy *et al.,* 1986; Samouclia and Gisi 1987). It is possible that it may be almost zero at high control levels. Combination therapy of this nature, where any compound, in particular plant based, is able to inhibit the activity of the efflux pumps, results in the increase of intracellular concentration of the other drugs (Ogawa *et al.,* 1998).

1.10 South African Medicinal Plant Species

Indigenous medicinal plants are used by more than 60% of South Africans in their health care needs or cultural practices (Street *et al.,* 2008). Traditional medicine is an integral part of the South African cultural life. It is estimated that between 12 and 15 million South Africans still depend on traditional herbal medicines from as many as 700 indigenous plant species. Approximately 3,000 species are used by an estimated 200 000 indigenous traditional healers (Van Wyk *et al.,* 1997). A number of factors could contribute to this dependence of such a large portion of the population on traditional medicine such as accessibility to the plants, extensive local knowledge and expertise amongst the local communities as well as affordability (Mander *et al.,* 1996).

African traditional medicine is often regarded as the oldest medicinal system (Gurib-Fakim 2006). A review on South African literature reveals research activities where traditional medicinal practices are used to treat a variety of ailments such as skin disorders, tuberculosis, urinary tract infections and gastrointestinal disorders. The majority of these studies, devoted to the antimicrobial activity of South African plants, focus on extracts.

1.10.1 *Allium sativum*

1.10.1.1 *Plant description*

Allium sativum (garlic) belongs to the *Liliaceae* family. It is commonly used as a spice in food (Haciseferogullari *et al.,* 2005). There are currently 500 members of the *Allium* family, all differing in taste, appearance and color, but are similar in biochemical, phytochemical and neutraceutical content (Benkeblia 2004). A typical garlic plant consists of one large bulb which is made up of several small cloves (Figure 1.5). This plant is a extremely well-researched herb with many years of culinary and medicinal uses. This powerful herb is known in the history of numerous cultures. Garlic is generally planted in autumn and harvested about 8 months later in the summer. A variety of cultivars can grow under different environmental conditions such as temperatures, rainfall, and soil types. Widespread research on garlic to test the pharmacological effects resulted in more than 1000 publications over the past decade alone.

Figure 1.5. *Allium sativum* bulbs.

1.10.1.2 *Garlic chemistry*

Garlic is to a certain extent a complex herb with its characteristic odor. Garlic consists of several intrinsic specific enzymes as well as chemical constituents (Figure 1.6) that result in a specific odor by crushing the garlic cloves. *Allium* contains organosulphur-containing compounds (Benkeblia 2004). The majority of the organosulphur-containing compounds are cysteine sulfoxides. When garlic is chopped, an enzyme is released, called allinase (Benkeblia 2004) which converts cysteine sulfoxide into thiosulfinate, which is a reactive, volatile, odour producing and lachrymatory compound (Benkeblia 2004). Allicin is

considered to be the active compound, but is not found in raw whole garlic, but only found in the crushed plant. There may also be secondary metabolites such as alliin, S-allycysteine and methyltrisulfide present (Johnson *et al.,* 2002; Bakri and Douglas 2005). Allicin is converted to diallylsulfide on exposure to air, which has showed antibacterial properties (Jesse *et al.,* 1997).

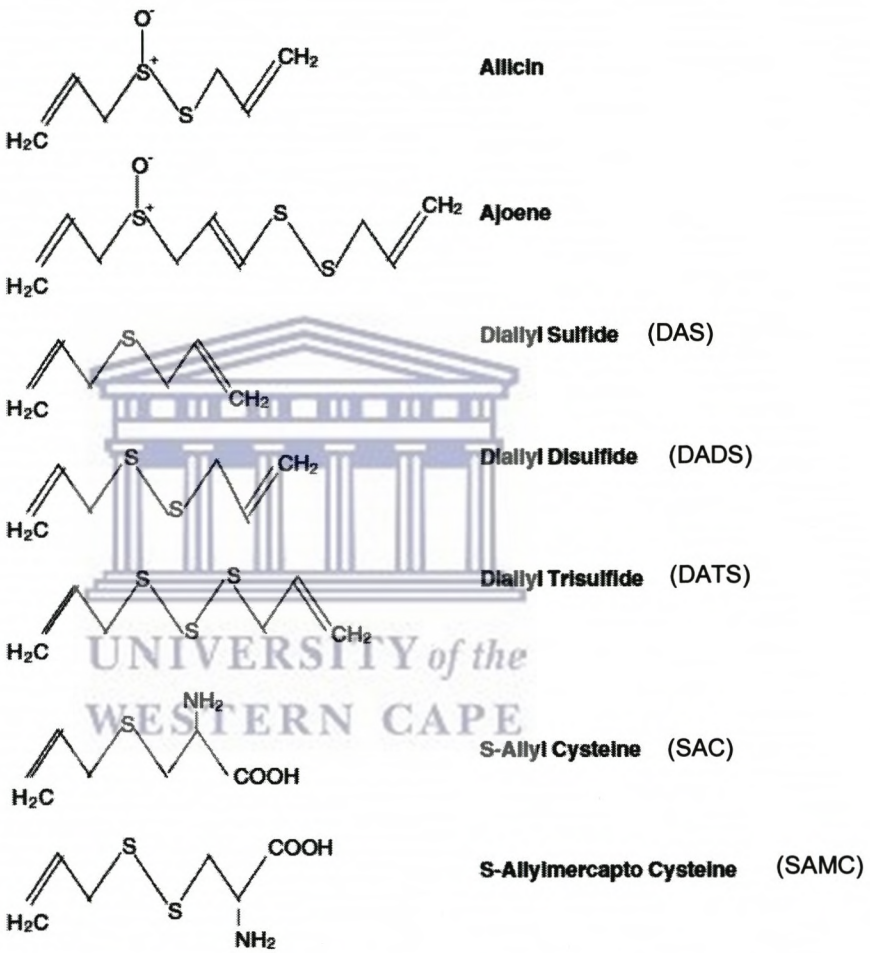


Figure 1.6. Biologically active compounds from garlic (Shukla and Kalra 2007).

1.10.1.3 *Biological activities of A. sativum*

1.10.1.3.1 *Medicinal Uses*

For hundreds of years garlic has been used as a medicine for its antibacterial, antifungal and antiviral properties (Bakri and Douglas 2005). Studies have also shown garlic to stimulate the immune system (Jesse *et al.,* 1997). *Allium sativum* has shown cholesterol-lowering activity; lowering of blood pressure; platelet aggregation inhibition which is closely linked to atherosclerosis, heart disease and

strokes; fibrinogen levels, which may contribute to cardiovascular disease; prevention of Low-density lipoprotein (LDL) (Corzo-Martinez *et al.,* 2007).

1.10.1.3.2 *Antimicrobial activity*

Garlic has been used as folk medicine for centuries in several cultures against parasitic, bacterial, fungal and viral infections. The chemical characterisation of garlic revealed that the sulphur compounds account for their antimicrobial activity. In addition, some proteins, phenolic compounds and saponins may also contribute to this antimicrobial activity (Griffiths *et al.,* 2002). *In vitro* and in vivo studies revealed that garlic and its derivatives are very effective against a broad spectrum of yeasts and fungi, including *Candida, Torulopsis, Trichophyton, Rhodotorula, Aspergillus, Cryptococcus* and *Trichosporon* (Davis and Perrie 2003).

Garlic compounds also showed a synergistic activity with amphotericin B *in vitro*, one of the main antifungal drugs (Shen *et al.,* 1996). The mode of action of these active compounds found in garlic result in the destruction of fungal cells by reducing the oxygen uptake, decreasing cellular growth, inhibiting the synthesis of nucleic acids, proteins and lipids, changing the lipid profile of the cell membrane and by inhibiting the fungal cell wall synthesis (Gupta and Porter 2001).

1.10.1.4 *Anticancer activities*

1.10.1.4.1 *Epidemiological Studies*

Several studies over the years have linked the consumption of garlic to anticancer activity. These findings are supported by epidemiological studies in various human populations who consumed differing levels of plants of the family *Allium*. During the past 30 years, documentation from epidemiological studies indicated a decreased risk for some cancers due to a garlic-rich diet. An early epidemiological study was conducted in China on two big human populations with differing garlic consumption profiles. The one population in the region where a high-garlic diet (about 20 g a day) was consumed, showed that mortality in stomach cancer patients was three times lower compared to the region where people consumed significantly lower levels of garlic (less than 1 g a day) (Xing *et al.,* 1982). From these observations the authors postulated that a decrease in production of carcinogenic nitrosoamines was due to the ability of garlic to reduce nitrates to nitrites with bacterial participation which led to a lowered nitrite concentration in gastric juices. A reduced risk in breast cancer due to an increased consumption of garlic and onion were confirmed in French studies (Challier *et al.,* 1998).

1.10.1.4.2 *Animal Studies*

Documented data on animal studies showed that either individual garlic-derived compounds or fresh garlic macerate were used. In a study where garlic oil was used against skin tumorigenesis initiated by 7,12-dimethyl benz(a)anthracene (DMBA), was the first report on the potential chemopreventive effects of garlic (Belman 1983). Soni *et al* (1997) reported on the protective effect of food additives on aflatoxin-induced mutagenicity and hepatocarcinogenicity in rats. The dietary administration of garlic (0.25%) resulted in a significant reduction in the number of gammaglutamyl transpeptidase-positive foci (GST-P^+) induced by AFB_1 considered to be the precursor of hepatocellular neoplasm. Later studies showed that during diethylnitrosamine-induced liver cancer in Wister rats, fresh garlic treatment (therapeutic dose 20 mg/kg body wt/day) significantly reduced the number (50% reduction, $P < 0.003$) and area (48% reduction, $p < 0.0007$) of GST-P^+ foci, indicating carcinogenic potential, compared with the control group of animals consuming distilled water (Samaranayake *et al.,* 2000). In another study the effects of allyl sulfides e.g., diallyl sulfide (DAS), diallyl trisulfide (DATS) and diallyl disulfide (DADS), on the induction of phase II detoxification enzymes and liver injury by carbon tetrachloride was investigated (Fukao *et al.,* 2004). The phase I enzymes were stimulated by the monosulfide only and the phase II enzymes, strongly by the trisulfide and weakly by the disulfide.

1.10.1.4.3 *Antioxidant effects of aqueous garlic extract*

Several age-related diseases, including cancer have been related to activities of free radicals (Ames *et al.,* 1993). Furthermore, the oxidation of proteins, DNA and lipids by reactive oxygen species (ROS) also contribute to these age related diseases as well as a wide range of other common diseases, including cardiovascular, inflammatory and neurodegenerative diseases, such as Alzheimer's disease and other degenerative conditions (Borek 1997). Between all the garlic-derived products, aqueous garlic extract (AGE) is the preparation resulting in a higher antioxidant activity. This activity is even higher than in other commercial garlic supplements as well as fresh garlic. The increased antioxidant activity can be ascribed to the extraction procedure, which increases stable and highly bioavailable water-soluble organo-sulphur compounds content, including SAC and S-allylmercaptocysteine (SAMC), exhibiting potent antioxidant activity (Thomson and Ali 2003).

It is important to note that phytochemicals in AGE may exhibit a synergistic or additive effect when exercising their antioxidant action by scavenging ROS (Borek 2001). This antioxidant activity is achieved by increasing the activity of the cellular antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and GPx, and by increasing reduced glutathione (GSH) in the cells (Liu *et al.,* 1992), serving as an important defence mechanism in living cells (Borek 1997). Reduced glutathione (GSH) act as a cofactor for GST as well as reductant for glutathione peroxidase (GPX), an enzyme implicated in the natural protection against free radicals, in addition to SOD and CAT.

Banerjee *et al* (2003) reported on the significant increase in the antioxidant activity of cells due to permanent garlic consumption. The effect of AGE on lipid peroxidation and levels of antioxidants were studied during buccal pouch carcinogenesis in male Syrian hamsters by Balasenthil and coworkers (1999) to monitor the chemopreventive potential of garlic. Administration of 250 mg/kg body weight aqueous garlic extract resulted in an increase in GSH level, GPx and GST activity and a reduction in lipid peroxidation in oral tumor tissue (Balasenthil *et al.,* 1999), indicating effective suppressing of DMBA-induced oral carcinogenesis as revealed by the reduced incidence of neoplasms. In vascular cells in culture, garlic extracts caused an increase in SOD (Geng *et al.,* 1997), GPx (Wei and Lau 1998), and CAT (Wei and Lau 1998) activities. Furthermore it was found that S- allyl-cysteine sulfoxide (alliin) prevented a reduction in hepatic SOD and CAT activities in diabetic rats (Augusti and Sheela 1996).

1.10.1.4.4 *Adverse effects*

Garlic also exhibits some adverse effects in humans which can be most common, less frequent and rare (Tattelman 2005). The most common side effects is bad breath resulting from the intake of small amounts of garlic. However, studies also revealed that consumption of moderate amounts of garlic, especially on an empty stomach, can cause undesirable effects, such as gastrointestinal upsets, flatulence and changes in the intestinal flora (Ackermann *et al.,* 2001; Tattelman 2005). Furthermore, garlic preparations employed in complementary medicines with drugs are greatly examined due to their ability to stimulate cytochrome P450 enzymes, responsible for metabolising exogenous chemical compounds in the liver (Corzo-Martínez *et al.,* 2007). The ingestion of large amounts of raw garlic or powdered garlic supplements and Ritonavir as antiretroviral therapy resulted in severe gastrointestinal toxicity (Laroche *et al.,* 1998). Contrasting to garlic powder products,

containing oil-soluble sulphur compounds, AGE neither stimulates P450 enzymes nor produces severe gastrointestinal toxicity. Amagase (2006) also reported that AGE do not cause P450-induced contraindications with drugs. However, the administration of AGE in drinking water also showed a negative effect whereby aspartate aminotransferase levels were significantly elevated which suggest liver injury (Joseph *et al.,* 1989).

In order to minimise the risk of adverse side effects of garlic, the ingestion of garlic and their compounds should be controlled, including ingested dose, long-term medication as well as the safety and effectiveness of a chosen preparation (Davis 2005). In spite of this, the use of garlic as therapeutic agent seems to be very safe, since the adverse effects described appear with an excessive and prolonged consumption.

1.10.2 *Tulbaghia violacea*

1.10.2.1 *Plant description*

Within the family Alliaceae, *Tulbaghia* is the genus that is very closely related to *Allium* and is exclusively indigenous to Southern Africa. In this genus, *T violacea* is the most well-known species that is utilized as medicinal plant particularly in the Eastern Cape and KwaZulu-Natal regions (Burton 1990). The occurrence of *T violacea* elsewhere in the country is due to cultivation in gardens and in the commercial medicinal plant farms (Van Wyk *et al.,* 2009).

Tulbaghia violacea is a fast growing, bulbous plant with a height of 0.5 m. *T. violacea* has evergreen leaves that have a strong garlic smell when damaged or bruised (Kubec *et al.,* 2002). The leaves have been used as a substitute for chives in some cultures and are also frequently used as garnishes (Kubec *et al.,* 2002). *T. violacea* grows from fat tuberous roots which spread to form clumps of plants. The flowers are pinkish purple, tubular clustered into umbels of up to 20 flowers and held above the leaves on a tall flower stalk (Figure 1.7). They appear in a long period in the summer from January to April and also smell like garlic when picked (Duncan *et al.,* 1999). *T. violacea* is also commonly known as, "society garlic"; "wild garlic" and "sweet garlic" (Kubec *et al.,* 2002).

a. b.

Figure 1.7. *Tulbaghia violacea;* a) whole plants, and b) flower

T. violacea is a drought resistant plant and is found in the Eastern Cape, Kwa-Zulu Natal, and Limpopo and as far as Zimbabwe (Kubec *et al.,* 2002). It is adapted for moth pollination and have dull flowers that become sweet scented at night, yet butterflies and bees pollinate it by day. *T. violacea* grows very easily in moist soils and is used as an edging plant. It seldom falls prey to pests and diseases but slugs and snails can cause considerable damage to foliage (Duncan *et al.,* 1999).

1.10.2.2 *Tulbaghia violacea chemistry*

Contrasting to *Allium sativum*, whose chemistry has been studied extensively, only a few scientific articles about the chemical components of *T. violacea* have been published so far. An enzyme similar in action to that found in *A. sativum*, i.e. alliinase, a C–S lyase and three unidentified S-substituted cysteine sulfoxide derivatives has been reported by Jacobsen *et al*., (1968) to be present. The isolation of 2,4,5,7-tetrathiaoctane-2,2-dioxide and 2,4,5,7-tetrathiaoctane from the leaves of *T. violacea* has been conducted by Burton and Kaye (1992). Kubec *et al* (2002) later isolated 2,4,5,7-tetrathiaoctane-4-oxide and subsequently identified the three unknown cysteine derivatives suggested by Jacobsen *et al* (1968) as (*RSR*C)-*S*-(methylthiomethyl) cysteine-4-oxide (marasmin). Important components of *T. violacea* comprise several odour forming compounds (Kubec *et al.,* 2002) and bioflavonoids such as kaempferol and quercitin (Hutchings *et al.,* 1996). Allinase and several sulphur compounds have already been isolated from *T. violacea* (Burton 1990; Kubec *et al.,* 2002). A close genetic relationship between *T. violacea* and *Allium* species has been suggested due to the presence of a C–S lyase in both plants species as well as marasmicin that has been in close analogy to the alliin/allicin system. These similarities are therefore reasonable to assume that a similar mechanism is also operating in *T. violacea* to that of *Allium* species.

1.10.2.3 *Ethnobotany of Tulbaghia species*

Tulbaghia violacea is an ideal plant to be used in an herb garden where the leaves and flowers can be used in salads and other dishes. Crushed leaves are often used to cure sinus headaches and to discourage moles from the garden. According to van Wyk *et al.* (2000), *T. violacea* is used in traditional medicine in the Eastern Cape and KwaZulu Natal to treat problems like fever, colds, asthma, tuberculosis, stomach-ache, and cancer of the oesophagus. The strong smell of crushed leaves placed on the skin is able to repel ticks, fleas and mosquitoes. Fresh bulbs can be boiled in water and decoctions taken to clear colds and coughs. It is also used as a remedy for pulmonary tuberculosis and to destroy intestinal worms (Duncan *et al.,* 1999). The antibacterial and antifungal activities of *T. violacea* are shown to be the same or similar as real garlic. The Zulu people of South Africa use the leaves and flowers and prepare it as spinach and as a hot, peppery seasoning with meat and potatoes (Dold and Cocks 2002). They also use the bulbs to make aphrodisiac medicine. They also use *T. violacea* around their huts because it is a good snake repellent (Duncan *et al.,* 1999; Kubec *et al.,* 2002). Very few studies are available on the biological activities of *T. violacea* (Bungu *et al.,* 2006; Raji *et al.,* 2012). Since *Tulbaghia* belong to the same family and exhibit similar characteristic sulphur smell, it has also been postulated that the plant has similar biological activities and secondary metabolites as commercial garlic (Van Wyk *et al.,* 1997; Van Wyk and Gericke 2000; Bungu *et al.,* 2006).

1.10.2.4 *Bioactivity of Tulbaghia violacea*

1.10.2.4.1 *Antimicrobial activity*

Aqueous *T. violacea* extracts were examined for antifungal activity against *Candida albicans* (ATCC 10231). *T. violacea* showed an inhibitory effect and remained active for 3 and 2 days respectively when stored at 4°C (Motsei *et al.,* 2003). It also showed promising antimicrobial activity against a few medically important pathogenic bacteria as well as fungi that cause opportunistic infections in HIV/AIDS patients (McGaw *et al.,* 2000; Gaidamashvili and Van Staden 2002; Motsei *et al.,* 2003). Gaidamashvili and Van Staden (2006) subsequently reported on the isolation of lectin-like proteins and their prostaglandin inhibitory activity on *Staphylococcus aureus* and *Bacillus subtilis* growth inhibition. Burton (1990) found that the compounds 2,4,5,7-tetrathiaoctane-2,2-dioxide and 2,4,5,7-tetrathiaoctane extracted from the leaves of *T. violacea* exhibited antibacterial activity (Burton and Kaye 1992). More recent studies revealed that extracts from *Tulbaghia* species

successfully control plant fungal pathogens by inhibiting their growth (Lindsey and van Staden 2004; Vries *et al.,* 2005; Nteso and Pretorius 2006).

1.10.2.4.2 *Anticancer activity*

Apoptosis inducing ability was revealed by crude aqueous extracts of *T. violacea* suggesting that the extracts contain potentially anticancer agents (Lyantagaye and Rees, 2003). Lyantagaye *et al* (2005) later reported on the promising anticancer activities of *T. violacea* - derived compounds containing a methyl-α-D-glucopyranoside (MDG) moiety in their structure able to induce reactive oxygen species causing cellular damage and hence apoptotic cell death (Cohen *et al.,* 2002; Pastorino *et al.,* 2002). An 8-year study revealed that three flavonols (kaempferol, quercetin, and myricetin) resulted in a reduced risk of pancreatic cancer by 23 percent (Nöthlings *et al.,* 2008).

1.10.3 *Tulbaghia alliacea*

1.10.3.1 *Plant description*

Tulbaghia alliacea (Figure 1.8) same common name as *T. violacea,* i.e., Wild garlic (English), Wildeknoffel (Afrikaans), Isihaqa (Zulu) and Moelela (Sotho) and is between 15 and 30 cm high and is strongly aromatic (Dlisani and Bhat 1999). The plant is an indigenous species in South Africa and grows predominantly in the Eastern Cape and southern KwaZulu-Natal (Margaret 2001). The plant grows from a cluster of small bulbs attached to a basal plate that is sometimes regarded as a rhizome. It has long, narrow, hairless leaves arising from several white bases. Flowers are 6 – 10 individuals on pedicels and between 15 - 30 mm long. They are brownish to green with an orange corona and scented of coconut and honey at night. The flowering time of *T. alliacea* is between March and May, especially after a fire. The hermaphrodite flowers are pollinated by flies. *T. alliacea* grows in South Africa, Malawi, Botswana, Zimbabwe, Mozambique, Swaziland and Lesotho.

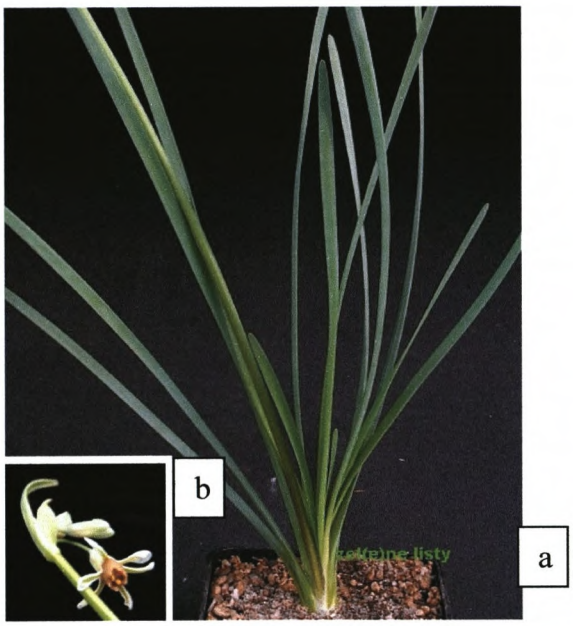


Figure 1.8. *Tulbaghia alliacea;* a) whole plants, and b) flower

1.10.3.2 *Tulbaghia alliacea chemistry*

The secondary chemistry of *Tulbaghia alliacea* is not well known. However, S-alk(en)ylcysteine sulfoxides, thiosulfinates, polysulfides, fructose and glucose compounds have been identified from aqueous extract of *T. alliacea*. Also, a furanoid compound [5-(hydroxymethyl)-2-furfuraldehyde] was identified as an artefact compound generated by the acid hydrolysis step. This compound occurs as a product from the acid-catalyzed dehydration of fructose (Maoela 2005).

1.10.3.3 *Ethnobotany of Tulbaghia alliacea*

Garlic has been used for centuries as herbal medicine against various infections and as an immune booster. Therefore it can be seen that *T. alliacea* has immunostimulation and anti-inflammatory effects (Masika and Afolayan 2003). The bruised rhizome is used locally in bath water as a relief of fever, paralysis and rheumatism as well as in small doses as a laxative (Van Wyk and Gericke 2000). Zulu women also consider the flowers to be a delicacy (Dlisani and Bhat 1999). The plant is used for asthma, pulmonary tuberculosis and stomach problems.

1.10.4 *Tulbaghia simmleri*

1.10.4.1 *Plant description*

Tulbaghia simmleri (syn. *Tulbaghia fragrans*) is a hardy perennial deciduous bulb with light purple flowers. This plant is sometimes referred to as Sweet Garlic or pink Agapanthus. It is sweet smelling with leaves similar to Agapanthus leaves. The flowers develop in early Spring, late Winter and mid Spring. The optimal growing

conditions for this species is in direct sun with good watering. This is a erect bulb and has an ultimate height of 0.6m and a spread of 0.2m. It can take 2-3 years to reach its ultimate height. *T. simmeleri* is often used as alternative for *T. violacea*, where the latter is not available (Burton 1990, van Wyk *et al.* 2000). Very little has been reported in literature about *T. simmleri.*

Tulbaghia simmleri grows in soils ranging from a pH of 6.5 (slightly acidic ranges from 6.1 to 6.5) to 7.5 (neutral ranges from 6.6 to 7.5). It is adapted to clay loam, loam, loamy sand, sandy clay loam and sandy loam soils, and prefers medium fertility.

Figure 1.9. *Tulbaghia simmleri;* a) whole plants, and b) flower

1.11 Carcinogenesis studies with purified fumonisin B_1

1.11.1 *Long-term studies in male BD IX rats*

Subsequent to the outbreaks of ELEM and before the discovery of fumonisins, rats were dosed with feed contaminated with *F. moniliforme* (Wilson *et al.,* 1985; Voss *et al.,* 1989). Experimental rats were used as a model to explore the carcinogenic potential of FB_1, FB_2 or FB_3 Following this, other studies conducted by the PROMEC group (medical Research Council of South Africa) showed that the fumonisins are hepatotoxic and carcinogenic in rats (Gelderblom *et al.,* 1996a). Gelderblom *et al* (1988b; 1991) reported that in general, culture material from *F. moniliforme* fed to experimental rats, produced micro- and macronodular cirrhosis, cholangiofibrosis and primary hepatocellular carcinomas. These findings in rats as a result of feeding contaminated culture material, led the way for further studies exploring the cancer-

initiating capability of purified FB_1, FB_2, and FB_3 (Gelderblom *et al.,* 1992a; Gelderblom *et al.,* 1993; Gelderblom *et al.,* 1994).

The successful isolation and characterisation of fumonisin B_1 mycotoxins from culture material of *F. verticillioides* (referenced as) MRC 826, made it possible to conduct a long-term toxicological study in male BD IX rats (Gelderblom *et al.,* 1988b). The general outcome of long-term and short-term feeding studies in rats showed that the liver and kidneys are the major target organs affected by FB_1 (Voss *et al.,* 1993; Gelderblom *et al.,* 1996c). Furthermore, it was shown that at dietary levels of 50 mg FB1 /kg, liver cancer developed in male BD IX rats (Gelderblom *et al.,* 1991). The diet fed to the experimental rats was semipurified corn meal supplemented with culture material, similar to the diet used by (Jaskiewicz *et al.,* 1987). In addition, the information about the carcinogenic effects of FB_1 obtained from a short-term study suggested that this mycotoxin could affect both cancer initiation and promotion (Gelderblom *et al.,* 1988b), suggesting that FB_1 could act as a complete carcinogen. Cancer initiation and promotion were related to a toxic effect characterized by the proliferation of bile ductules, fibrosis, and nodular regeneration which were similar to those described for *F. verticillioides* (MRC 826) in male BD IX rats (Gelderblom *et al.,* 1992b; Gelderblom *et al.,* 1994). With these investigations it could be concluded that the chemical compound which were responsible for hepatotoxicity and hepatocarcinogenicity in rats were the same compound responsible for ELEM in horses (Kriek *et al.,* 1981b).

A dose–response effect was established using FB_1 at relatively low levels to determine the effect on cancer development in male BD IX rat liver (Gelderblom *et al.,* 2001c). Male BD IX rats received a diet containing levels of 1, 10, and 25 mg FB_1/kg which continued for up to 24 months. It was evident that in order for FB_1 to induce hepatocarcinogenesis, a chronic toxic effect was required. These data therefore suggested that a certain threshold for FB_1 has to be reached. The significance of these investigations and other toxicity and carcinogenicity studies in rats are important to establish risk-assessment parameters for fumonisins in humans (Gelderblom *et al.,* 1996b).

1.12 Carcinogenesis

Carcinogenesis can be seen as the molecular process by which cancer develops. The development of cancer is a very complicated process in which a large number

of factors interact to disrupt normal cell growth and division. Cellular carcinogenesis studies can be seen as the basis for the identification of preventive products, the assessment of their activity, and ultimately the success or failure of a therapy. (Reddy *et al.,* 2003) reported that cancers may be caused in one of three ways, namely incorrect diet, genetic predisposition, and via the environment.

Knowledge regarding carcinogenesis is obtained from initial experimental studies conducted in laboratory animal models. These studies are generally based on chemical compounds which are either able to react with genetic material or are non-reactive compounds tested for their ability to cause cancer. It is believed that the transformation of a normal cell into a cancerous cell can proceed through many stages. The stages of carcinogenesis include initiation, promotion and progression (Kang *et al.,* 2011). Animal models and, if properly controlled by *in vivo* approaches, cell culture experiments can provide valuable information regarding the multistage concept of carcinogenesis. Mouse skin cancer and liver cancer in rats are well defined animal models of multistage carcinogenesis (Ito *et al.,* 1995a). These models allow for systematic investigation and distinction of the individual stages of carcinogenesis which can be used as guidelines in developing cancer chemopreventive measures (Marks and Fürstenberger 2000). In mice skin cancer systems, researchers would paint test chemicals on the skin to observe the growth of tumors. DNA reactive substances only resulted in tumor formation when the animals were further treated with another non-reactive substance.

1.12.1 Carcinogens

The exposure to environmental carcinogens such as natural and manmade chemicals, radiation, and viruses may lead to the greater part of human cancers (Reddy *et al.,* 2003). Chemical carcinogens as well as most toxins are inert substances and generally necessitate metabolic activation by certain cellular enzymes to exert their harmful effects. Carcinogens may be divided into several classes, (1) genotoxic carcinogens which may directly react with nucleic acids and directly affect cellular constituents, (2) procarcinogens which require metabolic activation to induce carcinogenesis and (3) epigenetic carcinogens that are non-genotoxic. Due to the variation in structure and potency of the potential carcinogen it is clear that more than one mechanism is implicated in the process of carcinogenesis (Reddy *et al.,* 2003).

1.12.1 *Multistage process*

In general, cancer development can be viewed as multistage process that is characterized by the cumulative action of a number of altered cellular processes such as replication, angiogenesis, apoptosis, metastasis and others (Ziech *et al.,* 2011). It is therefore of great importance to enhance our understanding about the three main stages of carcinogenesis, namely initiation, promotion and progression, and how they contribute to the complexity of biology (Panayiotidis 2008). Thus, for example, reactive oxygen species (ROS)- mediated DNA damage is involved in the initiation of carcinogenesis (Herath *et al.,* 2006; Fruehauf and Meyskens Jr 2007) along with malignant transformation in carcinogenesis (Lee and Lee 2006).

When a compound reacts with DNA and manages to change the genetic makeup of the cell, it is called a mutagen. The mutagens that predispose cells to develop tumors are called initiators and the non-reactive compounds that stimulate tumor development are called promoters. When a compound is capable of acting as both an initiator and a promoter, it is referred to as a 'complete carcinogen' because tumor development can occur without the application of another compound (Troll and Wiesner 1985).

1.12.1.1 *Initiation*

Initiation (Figure 1.9) is the very first step in the two-stage model leading to cancer development. Initiation is also thought to consist of a single gene mutation which means a reaction between the cancer-producing substance and the DNA of the tissue cells. These mutations are in most cases caused by environmental genotoxic agents such as chemicals, radiation and viruses (Reddy *et al.,* 2003). Initiators can also be altered via drug-metabolizing enzymes in the body enabling them to induce mutations (Troll and Wiesner 1985). The enzymes implicated here, include the phase I and phase II drug metabolizing enzymes and are effectively the same in all experimental animals and man (Autrup 1985).

According to Gelderblom *et al* (1988a) in a 4 week feeding of male BD IX rats with a diet containing 0.1% FB_1, gamma glutamyltranspeptidase positive (GGT+) foci were induced in the liver. When a single or multiple doses (50–200 mg/kg) of FB_1 (and FB_2) were administered by gavage to hepatectomized rats foci were not induced (Gelderblom *et al.,* 1992b; Gelderblom *et al.,* 1993). In a subsequent dose–response study, Gelderblom *et al* (1994) reported that the lowest dietary level to produce cancer initiation (GGT+-foci) was 250 mg FB_1/kg diet.

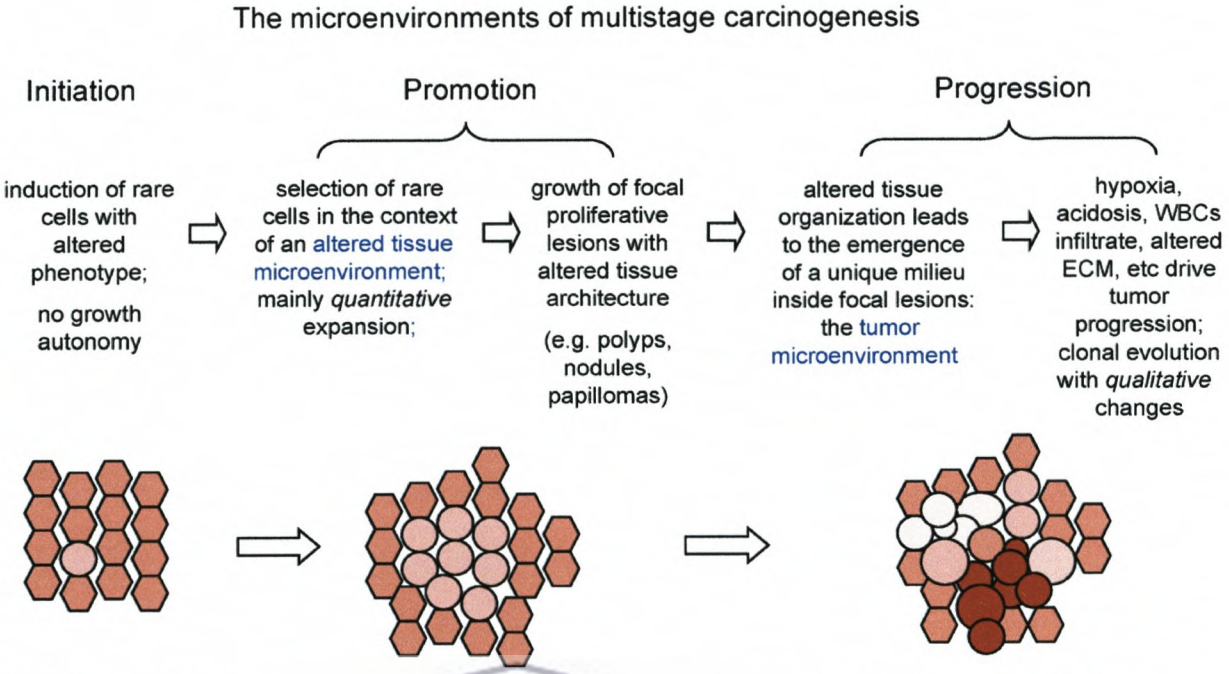


Figure 1.10. Schematic representation of the microenvironmental influences during multistage carcinogenesis; see text for details (Laconi *et al.*, 2008).

1.12.1.2 *Promotion*

After genetic alteration/mutation caused by an initiator, these initiated cells are susceptible to the effects of cancer promoters. Generally, these chemical compounds encourage the proliferation of the initiated cell population (Figure 1.9). The process of proliferation therefore will give rise to a large number of mutated daughter cells (Yamagiwa and Ichikawa 1977). Furthermore, a promoter will have no effect on cells if it has not previously been treated with an initiator (Pitot *et al.*, 1982). It is also evident that promoters do not bind covalently to DNA within the cells like initiators do. Receptors that are found on cell surfaces are targeted by promoters. Thereafter promoters would affect intracellular pathways which may lead to increased cell proliferation (Troll and Wiesner 1985).

Initiation/promoter studies revealed that the risk of tumor growth is generally a dose-dependent process. However, a threshold as well as a maximum effect of promoters is also evident. Alberts *et al* (2004) reported that very low doses of promoters will not lead to tumor development, whereas when extremely high doses were applied the risk for tumor development was the same as with moderate levels of exposure. In a dose response study on the promotion activity of FB_1, administration of increasing levels (10, 50, 100, 250 or 500 mg/kg diet for 21 days) of FB_1 to male Fischer-344 rats at a dose of DEN (200 mg/kg body weight) markedly increased

number and size of the placental glutathione S-transferase-positive ($PGST^+$) foci in the liver were noticed at dietary levels of 50 mg/kg or more (Gelderblom *et al.,* 1996c). It was therefore concluded that the required dose of FB_1 was markedly higher for cancer initiation than that required for cancer promotion (Gelderblom *et al.,* 1996b; Gelderblom *et al.,* 1996c).

1.12.1.3 *Progression*

A third and irreversible step, progression (Figure 1.9), consists of additional damage to the genome comprising the expression of the malignant phenotype. These malignant cells subsequently tend to acquire more aggressive characteristics over time. Furthermore, during metastasis tumor cells may secrete proteases that allow invasion beyond the immediate primary tumor location. Characteristic to the developed malignant phenotype is the tendency for genomic instability and uncontrolled growth (Lengauer *et al.,* 1998). Further genetic and epigenetic changes can occur during this process, which may result in the functional loss of tumor suppressor genes. This phenomenon gives cells a growth advantage as well as the capability for regional invasion, and eventually, distant metastatic spread (Vogelstein *et al.,* 1988; Sidransky 1995). However, the promotion stage, and probably also the progression stage could be regarded as the rate-limiting steps in cancer development making the stages ideal targets for possible intervention (Belitsky and Yakubovskaya 2008).

1.12.2 Liver carcinogenicity studies

1.12.2.1 *Studies using maize culture material.*

The general outcome of both *in vitro* and *in vivo* studies has shown FB_1 to induce DNA damage. These initiation capabilities were most likely caused indirectly and in association with cell toxicity and resultant oxidative damage. Cancer-initiating properties of FB_1 were investigated using male BD IX, Fischer 344 and Sprague Dawley rats (Gelderblom *et al.,* 1988c; Gelderblom *et al.,* 1992b; Gelderblom *et al.,* 1994; Gelderblom *et al.,* 2001b). Over a 2 year period, inbred BD IX rats were fed a commercial/standardized rat chow diet containing freeze-dried or oven-dried culture material containing *F. verticillioides* (MRC 826) (containing fusarin C and later found to produce FB_1 and FB_2). Results from this study reported the incidence of liver tumors (two hepatocellular and eight cholangiocellular carcinomas combined from 30 rats) (Marasas *et al.,* 1984).

1.12.2.2 *Carcinogenesis studies with purified fumonisin B_1*

Fumonisin B_1 (purity of ≥ 90% pure) was isolated from culture material of *F. verticillioides* MRC 826 and added to maize-based diet containing FB_1 at 50 mg/kg. This semi-purified diet was then given to 25 inbred male BD IX rats over a 26 months period (Gelderblom *et al.*, 1991). A control group of 25 rats were also included receiving the same diet, but with the exclusion of FB_1. Over a 26 month period, 5 rats from each group were terminated at 6, 12, 20 and 26 months. It was noticed that the liver was the main target organ in the FB_1-fed rats. These rats suffered from micro- and macronodular cirrhosis with large expansile nodules of cholangiofibrosis at the hilus of the liver. The hepatic lesions increased in severity over time with histological changes being consistent with those of a chronic toxic hepatosis progressing to cirrhosis.

1.12.3 Metabolism of xenobiotic compounds

Living organisms make use of a wide range of enzymatic mechanisms to defend against toxic substances. The enzymes involved are typically responsible for converting lipophilic chemicals into more hydrophilic substances making them easier excretable metabolites (Kumaraguruparan *et al.*, 2006). Enzymatic transformation is also applicable to certain environmental and occupational (pro)-carcinogens (Miller and Miller 1981; Guengerich 2000). In other words, a metabolised carcinogen can be transformed into either non-carcinogenic metabolites (process referred to as detoxication) or to an active metabolite (process referred to as activation). There are several organs that play an important role in the metabolism of various chemicals and include the skin, kidney, and lung (Timbrell 2000). However, the organ that has shown the biggest capability for metabolic transformation is the liver (Timbrell 2000). Enzymes responsible for metabolising ingested xenobiotics have been traditionally categorised into two major categories, i.e. phase I enzymes (functionalisation) and phase II enzymes (conjugation). The xenobiotics, after metabolism, can be absorbed from the digestive system either directly, or after being metabolized by intestinal microflora. The majority of phase II enzymes has the ability to detoxify substances but can also cause metabolic activation. The metabolic activation of carcinogens often results in transformation into electrophilic intermediates. Therefore, when increases in the activity of phase I enzymes are noted, as a result of enzyme induction or stimulation, it may balance on the benefit/risk boundary between detoxification and activation (Kensler 1997). In contrast, when these enzymes are inhibited it might give rise to a buildup of cytotoxic compounds in the body. It could also cause an overdose or loss of the

therapeutic effect of drugs. The level and/or activity of these enzymes have been suggested to play an important role in the susceptibility of an individual for developing cancer (Wilkinson IV and Clapper 1997).

1.12.3.1 *Phase I enzymes*

Belitsky and Yakubovskaya (2008) reported that subsequent to the exposure of a carcinogen, the dominating reactions are mediated by microsomal oxidases encoded by the cytochrome P450 (CYP2) gene superfamily. P450 enzymes comprise 70–80 % of all phase I xenobiotic-metabolizing enzymes (Evans and Relling 1999). P450s are a large superfamily of proteins and important phase I enzymes involved in oxidation reactions of foreign compounds (Nelson *et al.,* 1996). These compounds can also include therapeutically-used drugs. In order to fully understand toxicity mechanisms and chemical carcinogenesis, studies of these enzymes are critical. These mechanisms are also complicated by species differences in the expression and catalytic activities of P450s. Specifically, P450s in the primary CYP2 family involved in drug metabolism, display significant differences in the expression, regulation and catalytic activities between rats, mice and humans (Gonzalez and Nebert 1990; Guengerich 1997). According to Wood *et al.,* (1986) there are numerous naturally occurring flavonoids that have shown to modulate the CYP450 system which can either activate or inhibit these enzymes. Conney (2003) reported that CYP450 enzymes can metabolise many carcinogens to either biologically inactive metabolites or to chemically reactive electrophilic metabolites. These electrophilic metabolites can bind covalently to DNA with a resultant carcinogenic effect. In order to inactivate these reactive compounds/metabolites, they may undergo additional metabolism by other phase I or II enzymes.

1.12.3.2 *Phase II enzymes*

The metabolic step responsible for the detoxification/reduction of these reactive electrophiles can be identified as phase II metabolism. An important mechanism responsible for the chemical protection against carcinogenesis, mutagenesis and other forms of toxicity linked to electrophiles, is the induction of enzymes involved in their deactivation (Hong and Sporn 1997; Talalay 2000; Tan and Spivack 2009). Enzymes in this category include the glutathione S-transferases (GST), N-acetyltransferases (NAT), sulfotransferases (ST), UDP-glucuronosyltransferases (UDP-GT) and NAD(P)H-quinone oxidoreductase (NQO1) (Gonzalez and Kimura 2001). Other examples of xenobiotic-metabolizing enzymes are grouped into the phase II category. Phase II reactions catalyze conjugation by sulfation,

glutathioylation or glucuronidation, and neutralize electrophilic chemicals which reduce the chemical reactivity and the facilitation of elimination of these chemicals (Tan and Spivack 2009).

Several naturally occurring and synthetic compounds are generally capable of inducing the expression of phase II enzymes. This increased activity of the phase II detoxifying or antioxidant enzymes serves as a defense against chemicals (DNA alkylation and DNA adduct formation) and oxidative stress (oxidative DNA base modification). These enzymes usually convert either unchanged xenobiotics or their metabolic products (of the phase I) into more hydrophilic derivatives. By doing this, these hydrophilic derivatives can be easily eliminated through urine or bile (Zamek-Gliszczynski *et al.,* 2006).

Glutathione S-transferases are able to metabolize a diverse group of environmental carcinogens with a large overlap in substrate specificity. The protection of cellular macromolecules against toxic foreign chemicals is done by the conjugation of toxic and carcinogenic electrophilic molecules with glutathione, catalysed by the GST enzymes (Hayes and Strange 2000). Hayes *et al* (2005) have categorized human GSTs into three major families, the cytosolic, mitochondrial and microsomal GSTs when GSTs are found in membrane- and cytosolic fractions they are classified based on their cellular location. The conjugation of xenobiotics with glutathione is predominantly performed by the cytosolic or soluble GSTs. In vertebrates, approximately 20 cytosolic GSTs have been identified and categorised into seven distinct classes: alpha, pi, mu, theta, omega, zeta, and sigma. Cytosolic GSTs exist as hetero- (only alpha and mu) or homo-dimers in the cytoplasm. When cytosolic GSTs are compared with their membrane-bound counterparts, little structural similarities are observed. Subsequent to ingestion of many naturally derived products such as plant phenols, extracts of natural products, and even common herbs, such as garlic, induction of GST by xenobiotics are commonly observed (Ameen *et al.,* 2003; Krajka-Kuzniak *et al.,* 2004). As with phase I enzymes, activation of phase II detoxifying enzymes by flavonoids are able to detoxify carcinogens which represent one possible mechanism of their anticarcinogenic activity. The expression of the GST enzymes can also be impacted by ordinary therapeutic drugs.

The liver is regarded as the most important organ for the process of glucuronidation in the body. During oral absorption the liver is directly exposed to the influx of drugs

from the hepatic portal vein. UDP-GT, a phase II enzyme, recognizes a large number of functional groups and utilizes a common co-substrate, UDP-glucuronic acid, in their conjugating reactions. The location of UDP-GT is principally in the endoplasmic reticulum of the liver and, to a lesser extent, in all other mammalian tissues (Tukey and Strassburg 2000; Kiang *et al.,* 2005). Glucuronidation is regarded as one of the most significant conjugation pathways for the metabolism of drugs and other xenobiotics. This multigenic family of enzymes is responsible for catalyzing the reaction (Mackenzie *et al.,* 1997; Burchell 2003). The organs where glucuronidation occurs are the liver and extrahepatically in the intestine, kidney, lungs, and brain. Glucuronidation of drugs and xenobiotics by UDP-GT enzymes give rise to more hydrophilic compounds that are more easily excreted (Ouzzine *et al.,* 2003). The excretion of hydrophilic glucuronides takes place in the bile or urine.

It is therefore important to note the significance of conjugating enzymes as many drugs and their putative metabolites undergo conjugation reactions (Miners *et al.,* 2004). Effectively, glucuronidation reactions catalysed by UDP-GT are accountable for approximately 35% of all drugs metabolized by phase II enzymes (Evans and Relling 1999). Furthermore, UDP-GT also play an important role in cytoprotection by either preventing the buildup of potentially toxic xenobiotics or by preventing their subsequent bioactivation to even more toxic reactive intermediates (Bock 1994; Vienneau *et al.,* 1995; Green and Tephly 1996). The induction of phase II enzymes is an essential dynamic to consider when studying chemopreventive properties of a compound (Gerhäuser *et al.,* 1997; Hecht 2000).

1.12.4 Chemoprevention

Dr Michael Sporn first introduced the term cancer chemoprevention in 1976. Chemoprevention refers to the use of chemically active compounds in order to reverse, suppress or even prevent the progression of disease. It also may include pre-invasive cancers to malignancy (Sporn *et al.,* 1976). Chemopreventive agents have normally been classified into either blocking or suppressing agents. One of the first approaches is to focus on the reduction in the risk of exposure to environmental carcinogens. Secondly, will be to focus on a protection strategy where exogenous factors such as diet constituents, supplements or drugs and natural plant products are utilized to enhance endogenous mechanisms of tissue repair and defence in order to reduce the risk arising from exposure to environmental carcinogens (Grasso *et al.,* 1991; Ito *et al.,* 1995b; Hodek *et al.,* 2009). By adopting this approach, various stages in cancer development at both molecular and cellular

levels can be affected. Blocking agents assist in preventing the incidence of damage by inhibiting the activation of a carcinogen. These agents play a vital role in inhibiting the interaction between the carcinogen and important cellular macromolecules. It may also induce rapid deactivation and clearance of the carcinogen. The control or the reversal of damage incurred due to carcinogens is done by suppressing agents. Fifteen percent of all cancers are generally shown to be caused by inherited genetic factors (Lichtenstein *et al.,* 2000). The application of these two strategies may significantly reduce the number of cancers originating from the environment and lifestyle factors. Hence, adopting an acceptable lifestyle and diet can therefore assist in preventing 30–40 % of all cancers (Wong *et al.,* 2005; Russo 2007).

Chemoprevention can be categorized into three complementary levels depending on the stage at which the intervention is conducted namely primary, secondary and tertiary (Kelloff *et al.,* 1995; Khuri 2003). Khuri (2003) defined primary prevention approaches as intervention with pharmacological or dietary active compounds in order to prevent the development of preneoplastic lesions in healthy individuals. Individuals with pre-malignant lesions are the aims of secondary chemoprevention targets whereas tertiary chemoprevention targets patients whom are at risk for secondary concerns (van Zandwijk and Hirsch 2003).

It cannot be disputed that chemoprevention has a significant role to play in the war against cancer. The hypothesis that chemoprevention lowers the incidence and mortality rates for a variety of chronic disorders including cancer are strongly supported by preclinical as well as clinical studies. In general, specific cellular defence mechanisms are responsible for eliminating carcinogens but may be counteracted in situations of oxidative stress (Sies 1997; Leverve 2009). Oxidative stress signifies an imbalance between the production and manifestation of reactive oxygen species and the body's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Evidence exists suggesting that oxidative stress forms an obligatory component of carcinogenesis (Sánchez-Pérez *et al.,* 2005).

Numerous studies confirm the fact that certain compounds have the ability to induce oxidative stress. These studies have shown that oxidative stress can be induced by an increase in reactive oxidative/nitrogen species generally generated from endogenous or exogenous sources. It can also be caused by a decrease in endogenous antioxidant capabilities and oxidative DNA repair (Klaunig and

Kamendulis 2004). Oxidative stress may result in tissue injury and subsequent diseases such as atherosclerosis, diabetes, Parkinson's disease and cancers (Finkel and Holbrook 2000; Govindarajan *et al.,* 2005). Oxidative stress can have a direct influence on the multistage process of cancer development, i.e. oxidative DNA damage. This process can have mutagenic effects resulting in development of initiated cells during this process. Furthermore, oxidative stress also influences gene expression in order to promote tumor development during the process of carcinogenesis (Benhar *et al.,* 2002).

In order to evaluate the effect of dietary antioxidants on FB_1-induced hepato- and nephrotoxicity, the protective effect of royal jelly was examined in Sprague-Dawley rats (El-Nekeety *et al.,* 2007). From this study it was evident that royal jelly significantly reduced the toxic effects of FB_1 in the liver and kidneys in a dose-dependent manner. A significant increase in the FB_1-induced reduction of glutathione peroxidase (GPx) and superoxide dismutase (SOD) was observed. Due to the antioxidant properties and the oxygen free radical scavenging properties of royal jelly, lipid peroxidation was also inhibited.

1.13 Free radicals

Normal cellular activities in mammals produce several by-products which may include free radicals. Free radicals are very reactive and as a result are able to attack cells in our body. Environmental factors such as cigarette smoke, radiation, air pollutants and pesticides etc., play a significant role in the generation of free radicals (Li and Trush 1994). In order for our body to combat these free radicals, there needs to be a balance between the amount of free radicals produced and the antioxidant defense system that scavenge these free radicals. The ability of the body to do this prevents the free radicals from causing deleterious effects in the body (Nose 2000). It is therefore important for these free radicals to be within normal physiological levels in order for any antioxidant system to inactivate them. However, when this balance leans towards higher levels of free radicals generally caused by environmental conditions and other compounds produced by the body, it leads to oxidative stress (Finkel and Holbrook 2000; Govindarajan *et al.,* 2005). This in turn may result in serious tissue damage leading into the development of certain diseases (Finkel and Holbrook 2000). It is therefore clear that in order for us to search for radical scavengers or antioxidants to treat any disorders caused by them,

one first needs a proper understanding of the physiological characteristics of free radicals.

A proposal regarding a mechanism for chemically induced carcinogenesis has been made linking the production of reactive oxygen species (ROS) and the subsequent induction of oxidative stress by xenobiotics (Borek 1991; Cerutti 1991). Hydroxylation of DNA during the multistage process gives rise to formation of initiated cells by reactive free radicals for the initiation step in carcinogenesis. However, the mechanism of tumor promotion by ROS producing xenobiotics remains uncertain. Solt and Farber (1976) introduced a model of cancer promotion which was based on the resistant hepatocyte model developed in rat liver. The promotion occurs when initiated hepatocytes are resistant to the toxic effects of xenobiotics giving them a selective growth advantage over normal cells. Another mechanism of ROS-generated carcinogenesis by xenobiotics has been proposed at some stage in cell proliferation or inhibition of apoptosis in initiated cells. The involvement of ROS in hepatocarcinogenesis in the rat is also well documented (Klaunig *et al.,* 2011).

During initiation studies by Nakae *et al* (1997), low doses of N-diethylnitrosamine (DEN) induced liver DNA-8-hydroxydeoxy-guanosine adducts which suggest that oxidative stress participates in hepatocarcinogenesis. It is therefore safe to assume that high doses of DEN may produce oxidative stress as in the case of hepatocarcinogenic models, i.e. Solt and Farber model. In this model, initiation is afforded with DEN promotion with 2-acetylaminoflourene (2-AAF), and partial hepatectomy (PH) as a proliferation stimulus (Solt and Farber 1976).

1.13.1 *Sources of ROS*

There is no doubt about the critical roles of ROS and RNS in tumor development when experimental data are studied (Guyton and Kensler 1993; Petros *et al.,* 2005; Ishikawa *et al.,* 2008; Kumar *et al.,* 2008). Though more information is needed to fully define the exact mechanisms of ROS production-involvement in human cancer development, it is known that ROS can be formed from both endogenous and exogenous sources. Mitochondria, peroxisomes, and inflammatory cell activation are typical examples of endogenous sources (Klaunig and Kamendulis 2004). As mentioned earlier, environmental agents, radiation, therapeutic agents, and tobacco smoke are included in the wide array of exogenous sources. Both endogenous as well as exogenous sources of ROS inflict a direct impact on the cell and if not

handled by the antioxidant defense system can result in an increase in oxidative stress in the cell. As a consequence, oxidative stress may damage critical macromolecules which may result in chromosome instability, genetic mutation and/or modulation of cell growth. All of the above eventually may result in cancer development (Klaunig *et al.,* 2011).

As ROS are produced during normal metabolic processes, the cell has the ability to respond either directly by reducing the ROS via scavenging by enzymatic and non-enzymatic antioxidants or by removing the oxidative damaged by-products. As oxygen is partially reduced during normal metabolism to yield ROS, these ROS may contain an odd number of electrons, such as superoxide (O^{2-}), hydroxyl (OH^{-}), hydroperoxyl (HOO^{-}), peroxyl (ROO^{-}) and alkoxy (RO^{-}) free radicals. ROS may also contain an even number of electrons such as hydrogen peroxide (H_2O_2) and lipid hydroperoxide (ROOH) (Halliwell 1997; Prior and Cao 2000). Due to the odd number of electrons, these free radicals tend to be more reactive than ROS with even numbers of electrons. As a result they are also more biologically toxic. Another characteristic is that ROS are electrophilic in nature which makes them able to attack nucleophilic centers in the cell resulting in lipid peroxidation, protein oxidation and most importantly DNA damage (Halliwell 1997). The least reactive of these are the superoxides. Although superoxides are unable to pass through cellular membranes, they can be spontaneously or enzymatically dismutased to hydrogen peroxide (Radi *et al.,* 1991). Hydrogen peroxide is characteristically present in cells at concentrations of 10^{-7}-10^{-9} M and is able to cross cellular membranes. The Haber-Weiss or the Fenton reactions are normally involved in the formation of the more reactive hydroxyl radical (McCord 1993). Of all the ROS groups the hydroxyl radical is the most reactive and is known to cause cellular damage in the immediate surrounding area where it is produced (Bankson *et al.,* 1993).

1.13.2 *Role of oxidative damage*

Data provided by Abel and Gelderblom (1998) showed a close association between oxidative damage and FB_1-induced cancer initiation *in vivo*. The results illustrate that plasma and microsomal membranes and to some extent the mitochondria and the nuclei emerge to be significantly affected by lipid peroxidation. Oxidative damage as a result of cellular injury induced by FB_1 can modify cellular processes which include cellular membranes, proteins and DNA. All these alterations are possible when free radicals are over produced or when cellular defenses are not functioning optimally.

1.13.3 *Lipid peroxidation*

Lipid peroxidation can be defined as a process whereby polyunsaturated fatty acids (PUFA) become oxidized by ROS and in the process produce lipid peroxyl radicals and lipid hydroperoxides (Rice-Evans and Burdon 1993). Subsequent to the initiation of lipid peroxidation, the process continues as a free radical-mediated chain reaction. The steps usually involve initiation, propagation, and termination (Gago-Dominguez *et al.,* 2005). Gago-Dominguez *et al* (2005) also reported that due to the attack of reactive species on membrane phospholipids, abstraction of hydrogen atoms from the polyunsaturated fatty acid moiety takes place during initiation. During initiation, fatty acid radicals are formed and further react with neighboring lipid molecules. These repeated reactions generate new free radicals. This propagation phase that follows initiation also repeats itself several times until it is terminated by chain breaking antioxidant activity (Rice-Evans and Burdon 1993; Foy 1999; Niki *et al.,* 2005). *In vitro* studies have shown FB_1 to be cytotoxic and pro-apoptotic that can inhibit protein synthesis and causes lipid peroxidation in neural cells *in vitro* (Mobio *et al.,* 2000a; Mobio *et al.,* 2000b; Galvano *et al.,* 2002). In an *in vivo* study it was shown that lipid peroxidation occurred in a dose dependent manner in the liver of rats fed different dietary levels of FB_1 (Abel and Gelderblom 1998). This study also supports the *in vitro* findings where similar effects were observed.

1.13.4 *Oxidative DNA damage*

Mutations observed in living organisms have been greatly attributed to oxidative DNA damage (Lu *et al.,* 2001). Numerous DNA adducts, well over 100, have been recognized (Sonntag 1987; Dizdaroglu 1992; Demple and Harrison 1994; Brown *et al.,* 2009). The building blocks of DNA, both purine and pyrimidine bases can be attacked by free radicals. The damage done to DNA by ROS may include single- or double-strand breakage, deoxyribose modification, base modifications, and DNA cross-links (O'Reilly 2001). The repair of damaged DNA prior to or during replication is of utmost importance in order to prevent cell death, mutation, or induction of transcription, induction of signaling pathways, replication errors, and genomic instability. These potential problems have all been associated with the multistage carcinogenesis process (Marnett 2000; Klaunig and Kamendulis 2004; Valko *et al.,* 2006). Permanent genetic alteration is likely to occur unless a cell containing damaged DNA is repaired before the DNA divide. Unrepaired DNA damage will lead to an early step in the initiation of the carcinogenesis process. During rapid cell

division there exists less opportunity for DNA repair to take place making cells more susceptible to carcinogenesis (Colic and Pavelic 2000).

1.14 Antioxidant defense

Convincing data are available to support the fact that antioxidants and anti-inflammatory compounds are able to influence the redox environment of cancer cells and as a result their behavior (Schafer and Buettner 2001). Furthermore, the data also address the use of antioxidants to reduce genetic instability of cancer cells. In order to combat or to neutralize toxicity of ROS, the mammalian body has an effective system to defend against ROS. This evidence therefore proposes the use of antioxidants in the treatment of instability of cancer cells.

The antioxidant defense systems of the hepatocyte includes enzymes that have detoxifying ability such as superoxide dismutases (SOD), catalases (CAT), glutathione peroxidases (GPx) and glutathione reductases (GR). However, the non-enzymatic antioxidant defenses would include high molecular weight antioxidants such as uric acid (UA), Vit E, vitamin C (Vit C) and glutathione (GSH) which directly act on free radicals (Klaunig *et al.*, 1995). The benefit of the enzymatic defense system is the ability to directly act to eliminate free radicals or act to recycle non-enzymatic molecules. The respective enzyme reactions are shown in Figure 1.10.

$$O_2^- + O_2^- + 2H+ \leftrightarrow H_2O_2 + O_2 \text{ (superoxide dismutase)}$$

$$2H_2O_2 \leftrightarrow O_2 + 2H_2O \text{ (catalase)}$$

$$ROOH + 2GSH \leftrightarrow ROH + H_2O + GSSG \text{ (glutathione peroxidase)}$$

Figure 1.11. Enzyme reactions of superoxide dismutase, catalase and glutathione peroxidase

These free radicals pose a serious hazard to tissues and vital organs, especially membrane lipids, connective tissues, and the nucleic acids of cells. To safeguard against such reactions, special antioxidant enzymes are involved which particularly attack O_2^-, hydrogen, and organic peroxides, in order to repair any damage done to DNA. Reactions with these radicals normally produce products that are less offensive to the cell. It is normal to assume that a moderate level of antioxidant enzyme activity is maintained continuously during normal cellular activity. These levels are not always enough to deal with sudden increases in oxygen metabolites

and therefore needs to be amplified by cells to counter these sudden increases (Klaunig *et al.,* 2011).

Antioxidant enzymes are known to be soluble in water and are therefore present in the plasma, the cytosol, or periplasmic spaces of cells. During spontaneous or catalytic dismutation of O_2^- by SOD, H_20_2 is generated (McCord and Fridovich 1969; Weisiger and Fridovich 1973), which itself is a dangerous oxidant in cells. Therefore with an increase in O_2^- cells cannot solely rely on SOD, but catalase and glutathione peroxidase activity must also be enhanced - the latter to regulate H_2O_2. Studies have also shown that both SOD and GPx decreased in hepatocellular carcinomas (Vo *et al.,* 1988). These changes in cellular enzyme levels may therefore benefit initiated cells by giving them a selective growth advantage.

1.15 Natural products and defense against carcinogenesis

A library of documented evidence points to the fact that many natural products are used as a means of chemoprotection against commonly occurring cancers worldwide (Reddy *et al.,* 2003). The protective agents can be found in vegetables, fruits, plant extracts, and several herbs. Although more studies are needed to shed light on the specific mechanisms of protection by these agents, the incidence of a reduced risk for carcinogenesis by consuming fruits and vegetables broadly support the notion. It is important to note that a variety of plant constituents could be responsible for the protective effects which are influenced under various circumstances. Handelman (2001) reported that although many anticarcinogens have already been detected in plant foods, most attention has been given to the antioxidants vitamins C and E and the provitamin β-carotene.

1.15.1 *Polyphenols – Secondary plant metabolites*

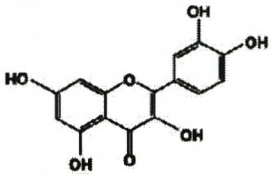
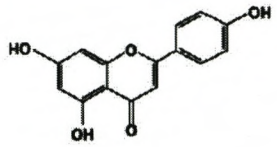
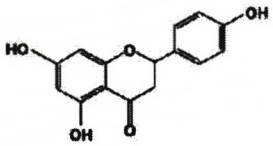
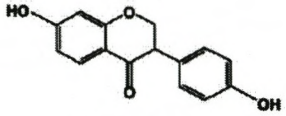
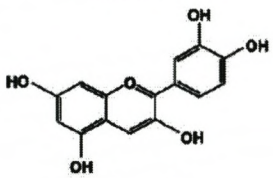
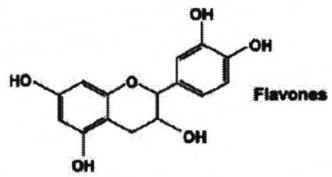
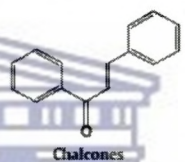
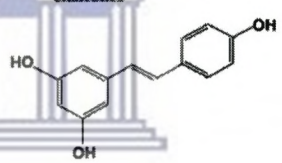
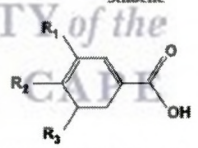
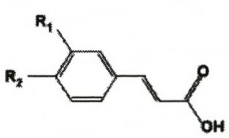
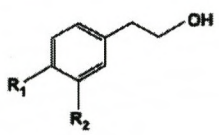
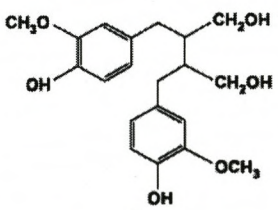
Antioxidants are the compounds that prevent oxidative damage done by free radicals. These molecules/compounds scavenge free radicals, promote their decomposition and/or can prevent their formation (Young and Woodside 2001). According to Doyle *et al* (2002), antioxidants can thus be described as: "substances in foods that significantly decrease the adverse effects of reactive species on normal physiological function in humans." Although antioxidants such as ascorbates, tocopherols and carotenoids are well known, Dimitrios (2006) explained that the phenols of plant origin have not been completely studied because of their

chemical nature being very complex and their extended occurrence in plant materials.

Plant phenolics have been a topic of much attention over the past decade due to their antioxidant properties and are characterised by the presence of more than one phenol ring or building block per molecule. The number of phenol rings and the structural elements that bind these rings to one another play a very important role in classifying polyphenols (Stevenson and Hurst 2007). Although polyphenols appear to have a protective affect against some cancers, Young and Woodside (2001) confirmed that some phenolic compounds can act as carcinogens. All polyphenols are water soluble and can be divided into different groups, including flavonoids, stilbenes, phenolic acids and quinines.

Flavonoids are the best-studied group of the plant phenols and they are widely distributed in plants. The term, flavonoid, is derived from the Latin word "flavus" meaning yellow. Flavonoids are ubiquitous plant secondary metabolites, best known as the characteristic blue, red, and purple anthocyanin pigments of plant tissues (Winkel-Shirley 2001). These pigments were shown to scavenge superoxide, hydroxy, and proxy radicals, as well as breaking lipid peroxide chain reactions (Procházková *et al.,* 2011). The phenolic hydroxyl groups of these compounds have the ability to donate electrons to free radicals and thus preventing oxidative damage. Doyle *et al* (2002) stated that studies reveal a protective effect of flavonoids against cardiovascular disease. It is likely that the presence of other antioxidants and nutrients will increase its effectiveness. According to Young and Woodside (2001), up to 4000 flavonoids have been identified including flavonols, flavones, catechins, flavanones, anthocyanidins, and isoflavones and they are categorised according to their chemical structure (Lotito and Frei 2006)(Table 1.1).

Table 1.1. Classification of polyphenols (Kang *et al.*, 2011)

Subclass	Examples	Base Unit
Flavonoids	Flavonols – quercetin, myricetin kaempferol, isorhamnetin Flavones – apigenin, luteolin, tangeretin Flavanones – naringenin, hesperidin, silybin Isoflavones – genistein, daidzein Anthocyanidins – cyanidin, delphinidin, peonidin, pelagonidin, malvidin Flavanols – catechins (catechin, epigallicatechin, epigallocatechin gallate), procyanidins	 Flavonols  Flavones  Flavanones  Isoflavones  Anthocyanins  Flavones Falvonolsa
Chalcones	Curcumin	 Chalcones
Stilbenes	Resveratrol, piceatannol	 Stilbene
Phenolic acids	Hydroxycinamic acid – chlorogenic acid, caffeic acid, caffeic acid phenethyl ester Hydroxy benzoic acid – gallic acid	 R_1=R_2=R_3=OH : Gallic acid R_1=R_2=OH, R_3=OH : Protocatechuic acid Hydroxybenzoic acid  R_1=OH : Coumaric acid R_1=R_2=OH : Caffeic acid Hydroxycinnamic acid
Phenolic alcohols/lignans	Tyrosol/pinoresinol, podophyllotoxin, steganacin	 R_1=OH,R_2=H:Tyrosol R_1=R_2=OH:Hydroxytyrosol Phenolicalcohols  Lignans

Flavonoids have also been shown to inhibit mutations, to protect cells from X-ray damage, to block progression of cell cycle and to prevent multistage carcinogenesis in experimental animals (Abdulla and Gruber 2000; Procházková *et al.,* 2011). Another mechanism by which flavonoids act is through interaction with various antioxidant enzymes and the ability to induce phase II detoxifying enzymes (e.g. NAD(P)H-quinone oxidoreductase, GST and UDP-GT), the major defense enzymes against electrophilic toxicants and oxidative stress (Zhu and Fahl 2001; Nerland 2007). The chemical structure of flavonoids appear to determine the antitumor effects, with each compound displaying various biological potency and mechanism(s) of action (Leopoldini *et al.,* 2011).

Prior and Cao (2000) reported that flavonoids exhibited much stronger antioxidant capacities than those of vitamins C and E. In addition to their antioxidant properties, flavonoids have been reported to exhibit other multiple biological effects, e.g. antiviral, antibacterial, anti-inflammatory, vasodilatory, anticancer and anti-ischemic (Formica and Regelson 1995; Weber *et al.,* 2003; Calderone *et al.,* 2004; Widlansky *et al.,* 2005; Alvesalo *et al.,* 2006; Mladěnka *et al.,* 2010).

1.15.2 *Plant phenolics and antifungal activity*

Food products that are natural, safe with multi-health benefits and free of chemical preservatives are some of the concerns raised by consumers (Wu *et al.,* 2008). Therefore, the focus is turned to low-molecular-weight phenolic compounds, important components of essential oils exhibiting antimicrobial activity (Davidson *et al.,* 2007). Their chemical structure and concentration are important contributing factors determining the activating or inhibiting effect on microbial growth (Alberto *et al.,* 2002; Vaquero *et al.,* 2007). The content of phenolic compounds of some cultivars of agriculturally important crops have been correlated to their resistance to fungal infection (Assabgui *et al.,* 1993; Carver *et al.,* 1994; Modafar *et al.,* 2000; Siranidou *et al.,* 2002). Additionally, it was found that phenolic compounds are inhibitory to the production of several mycotoxins including fumonisins, tricothecenes and aflatoxins (Bakan *et al.,* 2003; Beekrum *et al.,* 2003). These natural plant secondary metabolites, essentially found in all plant material, provide a satisfactory antifungal agent for application pre-and/or post-harvest (Hammer *et al.,* 1999; Samapundo *et al.,* 2007).

Farnochi *et al* (2005) found that both the growth and fumonisin production by *F. verticillioides* could be controlled by food grade antioxidants, as shown in recent

studies. These studies have been done *in vitro* on culture media and on irradiated maize and it is not affected by different conditions of temperature or water activity. A study carried out by Torres *et al* (2003) and by Farnochi *et al* (2005) included the use of antioxidants, butylated hydroxyanisol (BHA) and propyl parapen (PP), to inhibit the growth of *F. verticillioides* and its fumonisin production. The inoculation was done on natural maize with competing mycoflora being present and stored for 28 days. Depending on dose of BHA or PP, a_w and time, there was a reduction of 10-100 fold in the log colony forming units of *Fusarium*. Both BHA and PP also significantly reduced fumonisin content. Torres *et al* (2003) suggested that there exists a potential for the control of maize pathogens by the synergistic effect obtained from the use of a mixture of antioxidants. A combination of antioxidants was found to be more effective than the use of individual ones. According to Torres *et al* (2003), a variety of mechanisms have been anticipated for the effectiveness of phenolic antioxidants, since there is evidence of their interference with the membrane lipid structure in microorganisms.

In a study determining the inhibiting effect of natural phenolic compounds on FB_1 production revealed that thymol, carvacrol, and isoeugenol followed by eugenol to be the most antifumonisin active compounds (Dambolena *et al.,* 2011). It was also found that the phenolic compounds exhibiting highest antifumonisin activity also showed the highest antifungal activity. This finding led the authors to suggest that the antifumonisin activity of the phenolic compounds may be due to an increase in the lag phase prior to fungal growth. A delay in the lag phase therefore subsequently results in a delay in the onset of the stationary growth phase affecting FB_1 production. This suggestion is in agreement with the finding of Torres *et al* (2003) who reported the synthetic phenolic antioxidant BHA on fungal growth and fumonisin production and concur with Samapundo *et al* (2007), who suggested that the mechanisms described for growth inhibition of the *Fusarium* isolates by phenolic compounds are related to those for inhibition of fumonisin production. The ability of phenolic compounds to penetrate into the plasma membrane and induce changes in the physico-chemical properties of the cell wall, cell membrane, and cellular organelles has been linked to their lipophilic properties and contributes to their antifungal activity (Rasooli and Owlia 2005). Furthermore, (Picot *et al.,* 2010) hypothesised that fungi control secondary metabolite production during stress.

1.16 Research Study Hypothesis

A serious problem against the effective use of synthetic antifungal compounds is the development of resistance by the fungi in various sectors of society (Brent *et al.,* 1998). The application of higher concentrations of chemicals in an attempt to overcome this problem increases the risk of high level toxic residues in end-products. Furthermore, the demand for reduction in the use of pesticides in agriculture increases interest in the possibility of the application of plant extracts to control plant pathogens. The use of synthetic antifungal compounds is considered to be one of the cheapest and most common approaches for the control of filamentous fungi. As a result, the search for more ecofriendly alternative approaches is ongoing to enable safe storage of grains/cereals for human consumption without toxicity problems. Results obtained from laboratory trails using plant extracts of several higher plants have shown these extracts exhibit antibacterial, antifungal and insecticidal properties (Satish *et al.,* 1999; Okigbo and Ogbonnaya 2009). These plant metabolites are known to have minimal impact on the environmental and safe to the consumer compared to synthetic pesticides (Varma and Dubey 1999).

The detection of antifungal compounds from plant origin led us to study the effect of combining plant extracts with known fungicides. The role of *Fusarium* diseases and the subsequent economic and social relevance prompted our interest in developing new and highly effective biofungicides for agricultural or medicinal applications. In order to make their activity more reliable plant extracts are combined with low doses of fungicides. The first part of the study is to indicate that South African medicinal plants contain compounds that can act in a synergistic relationship with synthetic antifungal compounds.

Many plant extracts used in treatment of human health problems have shown antioxidant and free radical scavenging capacities against external and endogenous agents (Zheng and Wang 2001; Raskin *et al.,* 2002). Thus, these diet-derived components may play an important role in the prevention of health problems such as hepatopathies induced by excessive oxidative stress decreasing the cell's antioxidative ability. Antioxidants have the ability to prevent oxidant-induced damage to cells and have been shown to play an important role, at least during cancer initiation and promotion stages of chemical carcinogenesis.

The focus is therefore towards using natural antioxidants, especially plant polyphenolic constituents to alleviate possible health problems. Thus the second part of this study is to investigate the antioxidant properties and consequently the possible cancer modulating properties of these two wild garlic and one commercial garlic species, as cancer chemoprevention is an important emerging area of research. It not only provides a practical approach to identifying potentially useful inhibitors of cancer development, but also gives researchers the opportunity for studying the possible mechanisms involved in carcinogenesis and its modulation. We therefore hypothesise that extracts of *Tulbaghia violacea* and *T. alliacea* will protect against the oxidative stress and cancer promotion by CMF in rat liver. The short-term rat liver cancer model will provide valuable information as to the role of the two South African wild garlic species as possible chemoprotective agents against cancer and the potential mechanisms of protection involved.

1.16.1 Aims of the proposed study is

1.16.1.1 to establish the antifungal effects of selected South African herbal plant extracts, *Tulbaghia alliacea, Tulbaghia simmleri* and *T. violacea,* unaided and their mixtures with fungicides against *F. verticillioides in vitro.*

1.16.1.2 to elucidate the modulation of oxidative stress/status *in vivo* in a feeding study assessing the plant extracts on the oxidative status and activity of drug metabolising enzymes in the liver.

1.16.1.3 to determine whether the oral intake of two South African Wild Garlic species, *Tulbaghia alliacea* and *T. violacea*, will modify the cancer promoting properties of culture material of *F. verticillioides* (CMF), containing fumonisin B mycotoxins, in diethylnitrosamine (DEN)-initiated rat liver.

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CHAPTER 2

In vitro effects of *Fusarium verticillioides* treated with mixtures of fungicides and selected South African herbal plant extracts (*Tulbaghia violacea* and *Tulbaghia alliacea* and *Tulbaghia simmleri*)

2.1 Abstract

Fusarium verticillioides (Sacc. Nirenberg) is a fungus of significant economic importance because of its deleterious effects on plant and animal health and the quality of their products. Maize is the primary host of *F. verticillioides,* which produces the fumonisin mycotoxins. Fumonisins have been shown to be carcinogenic in horses and laboratory rats and mice. Naturally derived compounds with antimicrobial properties to reduce fungal growth or mycotoxin production are required for the agro-food industry. Four fungicides Sporekill™, Thiriam, Itraconazole and Fluconazole at four concentrations each and plant species *Tulbaghia violacea* Harv., *T. alliacea* L.f, *T. simmleri* Beauverd were tested alone and combined in different combinations for their efficacy of radial growth inhibition of *F. verticillioides* on potato dextrose agar. Four plant extract concentrations (62.5, 125, 250, 500, mg.ml^{-1}) for each of the respective plant species were used. A total of 192 combinations between the respective fungicides and wild garlic species were tested against *F. verticillioides*. Data analysis from *in vitro* results indicates that for the 16 different mixtures of each plant extract and fungicide combination, several significantly ($P<0.05$) higher growth inhibition responses were produced. More synergistic interactions were observed for the combinations of sporekill with *T. violacea* (63 mg.ml^{-1}) and *T. alliacea* (75 mg.ml^{-1}) than for *T. simmleri* (25 mg.ml^{-1}). Mixtures between the azole fungicides and *T. simmleri* produced 94% synergistic interactions. These findings suggest that combinations with these compounds from the three *Tulbaghia* species might be useful in the development of antimicrobial strategies against *F. verticillioides*. Combination of fungicides and plant compounds offers the opportunity to find synergistic mixtures and may validate disease control strategies with increased biological activity and low dose rate application.

2.2 Introduction

Fusarium verticillioides (synonym, *Fusarium moniliforme* Sheldon; teleomorph, *Gibberella moniliformis* [synonym, *Gibberella fujikuroi* mating population A]) is the most commonly reported fungal species infecting maize (*Zea mays*) (Nelson *et al.,* 1992; Rheeder *et al.,* 1992; Nelson *et al.,* 1993; Leslie and Marasas 2002). Maize is an important crop grown throughout the world and it plays an important role in human and animal diet (Boomsma and Vyn 2008). *F. verticillioides* is of great concern due to its pathogenic nature and the production of fumonisins (Marasas 2001). Contamination of maize, an important staple of millions of people across the world, by *F. verticillioides* and fumonisins can occur in the field during harvest and under storage conditions.

Fumonisin B_1 (FB_1), which is a mycotoxin produced by *F. verticillioides*, is of concern because of its association with human and animal mycotoxicoses (Marasas 1996; Richard 2007; Raghavender and Reddy 2009). These mycotoxins can cause leucoencephalomalacia in horses, pulmonary edema in pigs, and liver cancer in rats (Gelderblom *et al.,* 1988; Ross *et al.,* 1990; Voss *et al.,* 2007; Voss *et al.,* 2008; Ndube *et al.,* 2009). Fumonisins have also been associated with oesophageal cancer in humans in areas where consumption of maize contaminated with fumonisins is high (Shephard 2008; Van der Westhuizen *et al.,* 2011). *F. verticillioides* is becoming a major emerging infectious fungal disease, with fusariosis becoming more prominent in the immunocompromised patients and those with haematological malignancies (Campo *et al.,* 2009; De Pauw *et al.,* 2008). There are eight species of *Fusarium* recognized as opportunistic pathogens that cause disseminated infections in the compromised host, with *F. verticillioides* being one of the most common fungi (Guarro *et al.,* 2000; Gaur and Flynn 2001; Ortoneda *et al.,* 2002).

It is difficult to control the endophytic *F. verticillioides* with currently available commercial fungicides (Bacon *et al.,* 2008). The risk of repeated application of fungicide over a growing season for disease control may lead to an increase in the number and variety of fungal strains resistant to these fungicides (Bischoff *et al.,* 2005). It is therefore imperative that alternative treatments are needed to suppress the emergence of antifungal resistance. Plant extracts of several higher plants have been reported to demonstrate evidence of antibacterial, antifungal and insecticidal properties under laboratory trails (Satish *et al.,* 1999; Okigbo and Ogbonnaya 2009;

Shariff *et al.,* 2009). These plant based pesticides and plant metabolites appear to be one of the improved alternatives as they are recognized to have negligible environmental impact as well as danger to consumers in contrast to the synthetic pesticides (Varma and Dubey 1999). A problem with plant-produced compounds as potential fungicides is that they are generally only weakly active compared to commercial fungicides. Many aromatic plant essential oils have been tested for their antibacterial and antifungal properties (Rana *et al.,* 1997) and some particularly for their effects against *Fusarium* sp. and their associated mycotoxins (Velluti *et al.,* 2003; Lopez *et al.,* 2004; Velluti *et al.,* 2004). Numerous plant extracts have been used in *in vitro* experiments with other fungi including *Fusarium culmorum*. Recent studies have shown that food grade antioxidants could control both growth and fumonisin production by *F. verticillioides* and *F. proliferatum in vitro* on culture media and on irradiated maize under different conditions of water activity and temperature (Etcheverry *et al.,* 2002; Torres *et al.,* 2003).

Combinations of fungicides and natural plant compounds offers the opportunity to find natural synergistic compounds. Synergism could be defined as the exposure of the pathogen to sublethal concentrations of one fungicide that may affect it to such an extent that sublethal doses of the second fungicide will be more detrimental than in the absence of the first (Samouclia and Gisi 1987). Observations that mixtures persist longer on crops compared with their components alone (Samoucha *et al.,* 1988) provides another possible benefit for synergy (Bashan *et al.,* 1991). This study will determine whether different doses of commercial fungicides and methanolic extracts of wild garlic plant species indigenous to South Africa inhibit the mycelial growth of *F. verticillioides*. The study will also assesses and analyse the dose rates that produce synergistic reactions for combinations of medicinal plant extracts and the fungicides.

Natural defense compounds belonging to various chemical classes that act as constitutive or inducible chemical barriers, such as stilbenes, isoflavonoids, coumarins, and sesquiterpenes have been described (Osbourne 1999). It is commonly accepted that a significant part of phytochemical diversity serves to protect plants against microbial pathogens (Dixon 2001). However, despite a collection of antimicrobial compounds, plant products per se have not been used to any significant extent in the development of pesticides (Duke 1990). A few with simple structures are suitable for use as leads for chemical synthesis, which is

required, since chemical compounds are rather weak when extracted from plants and tested *in vitro* and *in vivo* (Knight, 1997).

To our knowledge, only a few strategies for controlling preharvest *F. verticillioides* infection or fumonisin contamination of maize are currently being pursued. In this respect, we hypothesize that aqueous extracts of selected South African wild garlic species, *Tulbaghia violacea, T. alliacea* and *T. simmleri* may enhance the efficacy of the synthetic fungicides against *F. verticillioides*.

2.3 Materials and Methods

2.3.1 Preparation of the spore suspension

Single-spored lyophilized *Fusarium verticillioides* culture (MRC 826) were used in all experiments, originally isolated from corn in the Transkei region of the Eastern Cape, South Africa and deposited in the culture collection of Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) at the Medical Research Council (MRC) of South Africa. *F. verticillioides* MRC 826 used in the present study have previously been shown to be a high fumonisin producer in nature and on maize grain. Sporulation was achieved by using one freeze-dried vial of MRC 826 which was resuspended in 2 ml sterile distilled water and inoculated onto carnation leaf agar (CLA) slants. The agar slants were incubated at 25°C for 20 days to ensure proper sporulation of *F. verticillioides*. Thereafter, ten ml sterile water were added to each slant and superficially scratched with a sterile bent platinum wire to dislodge the conidia in the water. All inoculations were prepared from standardized conidial suspensions of lyophilized conidia adjusted to 1×10^6 conidia/ml with the aid of a Neubauer hemacytometer.

2.3.2 Fungicides

All fungicides were purchased from pesticide distribution companies in South Africa. Fungicides were suspended in water (concentration is given in milliliters active ingredient per liter) and tested alone or in mixtures with plant extracts in the *in vitro* experiments.

2.3.3 Plant Extraction Method

Tulbaghia violacea (wild garlic) and *T. simmleri* used in the *in vitro* tests were obtained from Cape Peninsula University of Technology's Nursery garden, Cape Town. *T. alliacea* (social garlic) was obtained from local herb traders in Cape Town.

The leaves and bulbs of *T. violacea* were separated and the bulbs were washed to remove the excess debris. The bulbs of *T. violacea, T. simmleri* and *T. alliacea* were cut into smaller pieces. Two solvents were used to prepare the extracts; water and methanol. Homoginized aqueous and methanolic extracts were prepared separately using a Waring blender. Extractions were performed overnight in a closed container at refrigerated temperature in distilled water (1000 ml) to obtain 750 $mg.ml^{-1}$ (75%) stock concentrations. The stock solution was filtered through a muslin cloth and then aseptically pre-filtered through a Whatman (Number 1) filter 11 cm (1.2 µm) and thereafter with a Magna Plain 47 mm filter (0.45 µm). To avoid contamination and prospective chemical alterations, the extracts were used within 3 to 4 days. The filtered stock solution was placed in the freezer at -14°C until further use.

2.3.4 Radial Growth Assay

The inhibitory effect of aqueous plant extracts alone and in mixtures with fungicides on *F. verticillioides* radial growth was carried out in 90 mm petri plates containing 20 ml of solidified potato dextrose agar (PDA). Sterile potato dextrose agar was prepared by suspending 39 g of the medium in one liter of purified water and autoclaved at 121°C for 15 minutes. The aqueous plant extracts were diluted using sterile distilled water to 62.5 mg/ml, 125 mg/ml, 250 mg/ml and 500 mg/ml with triazoles of itraconazole and fluconazole at 62.5 mg/ml, 125 mg/ml, 250 mg/ml and 500 mg/ml. Thiram and Sporekill™ solutions were made up at 4 different concentrations each. Thiram was prepared at its working concentration of 0.008 mg/ml, 0.004 mg/ml, 0.002 mg/ml as well as 0.001. Sporekill™ was prepared at its working concentration of 0.0005 mg/ml, 0.001 mg/ml, 0.00025 mg/ml and 0.000125 mg/ml. A 1-ml suspension of the treatments (as single or mixed concentrations) were spread evenly across the agar surface and allowed to dry in a laminar flow hood. Two 8 mm diameter disc of inoculum of the test *F. verticillioides* MRC 826, cut from an actively growing culture on PDA plates, was placed inverted onto the inoculated agar in each petri plate. The plates were incubated for 5 days at 25°C. During and after the incubation period radial growth was monitored and measured using a vernier caliper.

2.3.5 Statistical Analysis

A statistical difference in the treatment results were indicated when $P<0.05$. A completely randomized experimental design was used for the resulting treatments. The growth of the pathogen was determined by measuring fungal radial growth

(widest axis) with the aid of a vernier caliper. Six replicates were included for each treatment condition. To assess differences in the mycelia growth of *F. verticillioides* among the treatments, the percentage inhibition was calculated from the radial growth as: treatment/control X 100. All analyses were carried out using the statistical software package SPSS. The synergistic ratio for percentage inhibition was based on the Abbott formula (Abbott 1925) as described by (Gisi 1996): expected efficacy of the mixture, Cexp = A + B – (AB/100) in which A and B are the control levels given by the "fungicide" and the "plant extract", respectively. The synergy ratio (SR), between the observed (Cobs) and expected (Cexp) efficacies of the mixture was calculated as SR = Cobs/Cexp. A deviation from the Cexp as calculated from the SR between the expected and the observed response of the two compounds would indicate synergism, additivity, or antagonism. By definition, additive interactions are present if SR=1, synergism occurs if SR>1 and antagonism if SR<1 (Levy *et al.,* 1986; De Waard and Gisi 1995).

2.4 Results

Results from a preliminary experiment indicated that aqueous and methanol extracts prepared from fresh *Tulbaghia* plant material were very efficient in inhibiting *F. verticillioides* (MRC 826) growth (Figure 2.1 and Figure 2.2). Results from preliminary experiments showed that the inhibition zones were increased with increasing concentration of aqueous and methanolic extracts of *T. violacea, T. alliacea* and *T. simmleri,* respectively. The lower concentration levels of the plant extracts resulted in a reduced inhibition, while at higher concentrations (250 and 500 $mg.ml^{-1}$), the plant extracts exhibited stronger inhibition activity against MRC 826 for both methanol and aqueous extracts. When considering methanolic and aqueous garlic extracts, *T. violacea* exhibited the strongest inhibitory effect against MRC 826 compared to the other garlic extracts, while *T. simmleri* was the least effective.

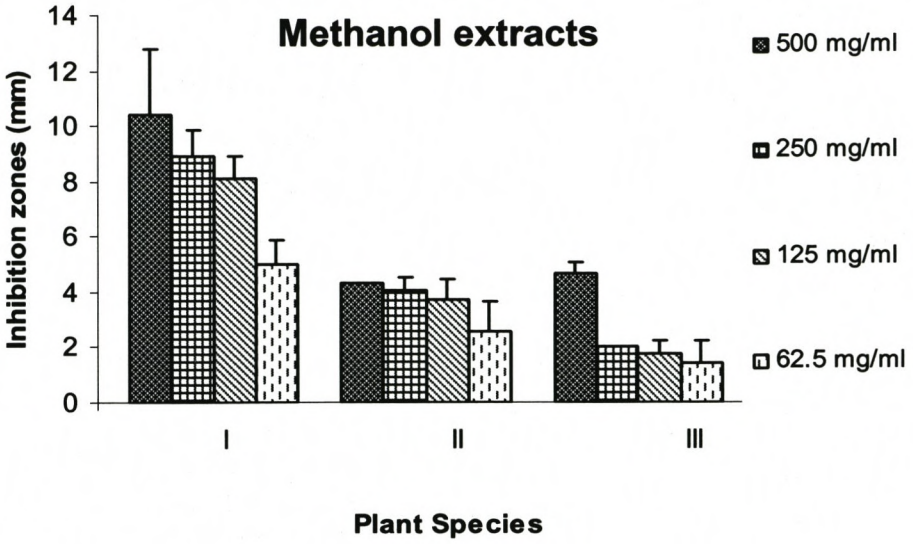

Figure 2.1. Effect of methanolic *Tulbaghia violacea* (I), *T. alliacea* (II) and *T. simmleri* (III) extracts on the growth, in solid medium, of *Fusarium verticillioides* MRC 826.

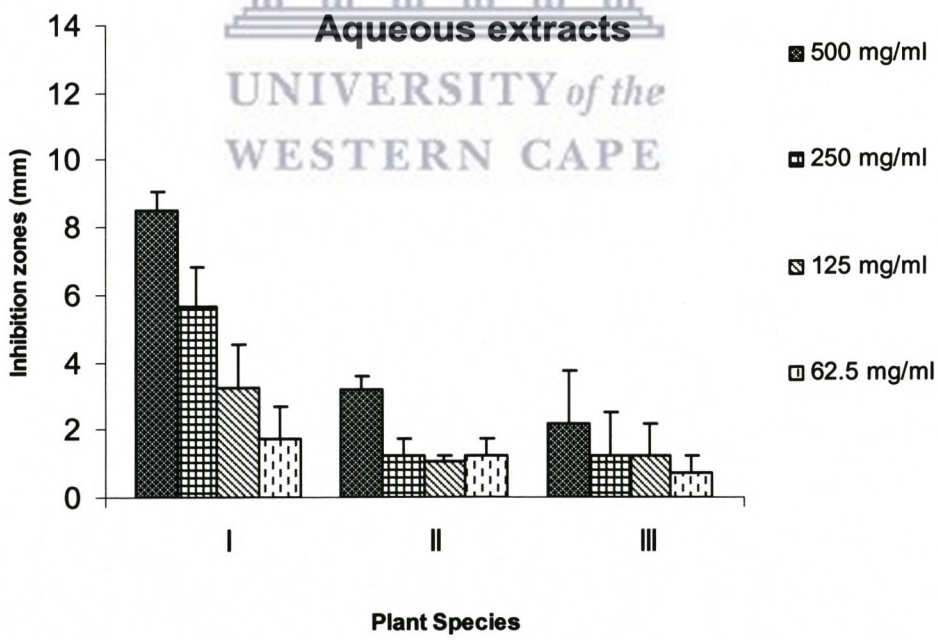

Figure 2.2. Effect of aqueous *Tulbaghia violacea* (I), *T. alliacea* (II) and *T. simmleri* (III) extracts on the growth, in solid medium, of *Fusarium verticillioides* MRC 826.

The relative efficacies of single and combinations of fungicides and plant extract concentrations were tested on radial growth inhibition of *F. verticillioides* (Table 2.1 to Table 2.4). A total of four plant extract doses were used for each of the respective plant species as well as four fungicide doses for each respective fungicide. Therefore, a total of 192 (4x4x3x4) combinations were tested for each fungicide and plant extracts. Analysis of variance (ANOVA) on radial growth inhibition of *F. verticillioides* MRC 826 indicated highly significant ($P<0.0001$) interaction among plant type, extract and fungicide doses (Table 2.1 to 2.4). Our findings are showing significant interactions in radial growth inhibition of the specific *F. verticillioides* strain between plant extract doses and fungicide (Tables 2.1 to 2.4). On the basis of these significant interactions, data were summarized for fungicides and respective plant extracts in tables to show significant differences of growth inhibition and synergistic interactions.

Of the 16 mixtures of Sporekill™ and *T. violacea* tested, 2 (13%) exhibited significantly ($P<0.05$) higher inhibitory responses against *F. verticillioides* compared to *T. violacea* and Sporekill™ alone (controls). The inhibitory activity of Sporekill™ alone against *F. verticillioides* showed reduced inhibition with lower concentrations compared to higher concentration doses (Table 2.1). A marked increase in inhibitory response was noticed against *F. verticillioides* when combinations were evaluated. Comparatively, 12 (56%) of the 16 mixtures tested were significantly higher with regard to inhibition levels than Sporekill™ controls. Furthermore, our results also indicate that 10 of the 16 mixtures showed synergistic interaction (SR>1.0) while only 6 were antagonistic (SR<1.0). For mixtures between Sporekill™ and extracts of *T. alliacea,* 10 (63%) of the mixtures resulted in higher inhibitory activity compared to *T. alliacea* and Sporekill™ controls. Also, 10 (94%) produced significantly higher inhibitory activity than the fungicide controls. Combined extracts of Sporekill™ and *T. alliacea* indicate that 69% resulted in a synergistic (SR>1.0) interaction while 3 mixtures exhibited antagonistic (SR<1.0) interactions in this case. Combinations of Sporekill™ and *T. simmleri* reported varying inhibitory activity. These combinations resulted in a significant ($P<0.05$) improved inhibitory activity of only 4 (25%) mixtures against *F. verticillioides* when compared to the controls. Similarly, 6 (38%) mixtures were successful in increasing the inhibition against *F. verticillioides* when compared to the fungicide alone. Also, at lower concentrations (0.000125, 0.00025 mg/ml) of Sporekill™, reduced inhibitory activity to *F. verticillioides* was noticed, irrespective of the concentration of *T. simmleri.* Moreover, this reduced effect was

also observed for the synergistic interaction in that 4 (25%) exhibited synergistic (SR>1.0) interactions, while 12 (75%) was antagonistic (SR<1.0).

When different concentrations of *T. violacea* were combined with Thiram™, only 2 (13%) responded with significantly (P<0.05) higher levels compared to the controls, while 12 (75%) significantly increased the inhibitory activity when compared to Thiram™ alone. Also, when considering synergistic interactions, 12 (75%) of the mixtures exhibited synergism. With regards to the mixtures of Thiram™ with *T. alliacea,* 11 (69%) significantly increased the radial growth inhibition of *F. verticillioides* compared to the controls. For the combinations of Thiram™ with *T. alliacea,* 13 (81%) exhibited significantly (P<0.05) higher radial growth inhibition levels compared to Thiram™ alone. Also, 11 (69%) of the mixtures showed synergistic (SR>1.0) interactions, while 5 (31%) reported antagonistic (SR<1.0) interactions. Radial growth inhibition was significantly affected when Thiram™ was combined with *T. simmleri.* In this instance, 10 (63%) of mixtures of Thiram™ and *T. simmleri* performed significantly (P<0.05) higher inhibitory activity against *F. verticillioides* compared to the controls. We also found that 10 (63%) of the combinations were very efficient when this was compared to Thiram™ alone. However, these combination resulted in 8 (50%) to be synergistic (SR>1.0) and 8 (50%) being antagonistic.

Table 2.1. Inhibition of mycelium growth and synergy ratio (SR) between fungicide Sporekill™ and *Tulbaghia violacea* (Tv), *Tulbaghia alliacea* (Ta) and *T. simmleri* (Ts) plant extract against *Fusarium verticillioides* MRC 826 *in vitro*.

Synergy Ratio[a]	Plant Species	Plant Extract Dose (mg.ml^{-1})	Mycelium Growth Inhibition (%) Sporekill™ Dose (mg.ml^{-1}) 0	0.000125	0.00025	0.0005	0.001
	Control		0	-23	1	-3	22
	Tv	62.5	23	20^{l}	23^{l}	43^{kl}	42^{kl}
SR			-	0.7	0.91	1.64^{+}	1.83^{+}
	Tv	125	38	19^{l}	38^{l}	41^{l}	23
SR			-	0.7	1.61^{+}	1.70^{+}	1.13^{+}
	Tv	250	42	36^{l}	36^{l}	40^{l}	22
SR			-	1.36^{+}	1.56^{+}	1.68^{+}	1.11^{+}
	Tv	500	27	20^{l}	31^{l}	19	20
SR			-	0.71	1.25^{+}	0.72	0.91
	Ta	62.5	16	9^{l}	9	39^{kl}	42^{kl}
SR			-	-2.31	0.54	2.93^{+}	1.19^{+}
	Ta	125	14	9^{l}	24^{kl}	30^{kl}	45^{kl}
SR			-	-1.36	1.62^{+}	2.70^{+}	1.35^{+}
	Ta	250	21	14^{l}	34^{kl}	37^{kl}	51^{kl}
SR			-	4.74^{+}	1.54^{+}	1.97^{+}	1.31^{+}
	Ta	500	28	10^{l}	36^{kl}	38^{l}	66^{kl}
SR			-	0.89	1.23^{+}	1.50^{+}	1.5^{+}
	Ts	62.5	-8	-32	-27	-10	-32
SR			-	-1.13	-0.91	-0.33	-1.18
	Ts	125	-7	-6	-17	32^{kl}	-6
SR			-	-0.23	-0.58	1.09^{+}	-0.24
	Ts	250	-14	19^{kl}	0	45^{kl}	19
SR			-	0.66	0.00	1.49^{+}	0.69
	Ts	500	17	17^{l}	30^{l}	50^{kl}	17
SR			-	0.69	1.15^{+}	1.93^{+}	0.72

^{k}Mycelium growth inhibition values of the plant extract and fungicide mixture differ significantly ($p<0.05$) from the value of the fungicide and the value of the plant extract doses. ^{l}Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the fungicide dose in the column. ^{a}Synergy ratio between expected and observed mycelium growth (SR=Exp/Obs; SR>1=Synergistic ; SR=1=Additive; SR<1=antagonistic). $^{+}$ Synergistic interaction.

Table 2.2. Inhibition of mycelium growth and synergy ratio (SR) between fungicide Thiram™ and *Tulbaghia violacea* (Tv), *Tulbaghia alliacea* (Ta) and *T. simmleri* (Ts) plant extract against *Fusarium verticillioides* MRC 826 *in vitro*.

Synergy Ratio[a]	Plant Species	Plant Extract Dose ($mg.ml^{-1}$)	Mycelium Growth Inhibition (%) Thiram™ Dose ($mg.ml^{-1}$) 0	0.001	0.002	0.004	0.008
	Control		0	11	14	5	5
	Tv	62.5	23	10	18	36^{kl}	30^{l}
SR			-	0.39	0.76	1.44^{+}	1.19^{+}
	Tv	125	38	18	30^{l}	32^{l}	36^{l}
SR			-	0.81	1.36^{+}	1.36^{+}	1.56^{+}
	Tv	250	42	37^{l}	38^{l}	26^{l}	39^{l}
SR			-	1.71^{+}	1.77^{+}	1.13^{+}	1.74^{+}
	Tv	500	27	31^{l}	17	27^{l}	46^{kl}
SR			-	1.29^{+}	0.70	1.08^{+}	1.86^{+}
	Ta	62.5	16	30^{kl}	25^{l}	19	11
SR			-	1.2^{+}	0.95	0.91	0.52
	Ta	125	14	10	22^{l}	30^{kl}	38^{kl}
SR			-	0.42	0.85	1.64^{+}	2.08^{+}
	Ta	250	21	38^{kl}	35^{kl}	31^{kl}	36^{kl}
SR			-	1.29^{+}	1.07^{+}	1.23^{+}	1.42^{+}
	Ta	500	28	47^{kl}	40^{kl}	46^{kl}	53^{kl}
SR			-	1.32^{+}	1.07^{+}	1.42^{+}	1.69^{+}
	Ts	62.5	-8	15	11	3	6
SR			-	0.52	0.38	0.12	0.22
	Ts	125	-7	13	9	22^{kl}	30^{kl}
SR			-	0.45	0.30	0.82	1.02^{+}
	Ts	250	-14	31^{kl}	36^{kl}	35^{kl}	30^{kl}
SR			-	1.05^{+}	1.25^{+}	1.25^{+}	0.99
	Ts	500	17	49^{kl}	31^{kl}	48^{kl}	44^{kl}
SR			-	1.94^{+}	1.25^{+}	2.01^{+}	1.68^{+}

[k]Mycelium growth inhibition values of the plant extract and fungicide mixture differ significantly ($p<0.05$) from the value of the fungicide and the value of the plant extract doses. [l]Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the fungicide dose in the column. [a]Synergy ratio between expected and observed mycelium growth (SR=Exp/Obs; SR>1=Synergistic ; SR=1=Additive; SR<1=antagonistic). [+] Synergistic interaction.

Our findings show that only 4 (25%) of the mixtures of Itraconazole with *T. violacea* resulted in significantly higher inhibition levels compared to Itraconazole and *T. violacea* controls (Table 2.3), whereas 9 (56%) of the mixtures exhibited significantly ($P<0.05$) higher inhibition levels against *F. verticillioides* compared to Itraconazole alone. Furthermore, our results indicate that 16 (100%) of the mixtures exhibited synergistic interactions (SR>1). When Itraconazole was combined with *T. alliacea*, 11 (69%) of the combinations reported significantly ($P<0.05$) higher inhibitory activity when compared to the controls. Of the 16 mixtures, 12 (75%) showed good efficacy against *F. verticillioides* compared to the fungicide alone. Moreover, only 5 (31%) mixtures showed synergistic (SR>1) interactions, while 69% was antagonistic (SR<1). Combinations of Itraconazole with *T. simmleri* resulted in 15 (94%) synergistic interaction with 11 (69%) exhibited significantly ($P<0.05$) higher inhibition compared to the plant extract and Itraconazole alone. In this instance 11 (69%) of the mixtures resulted in higher radial growth inhibition compared to Itraconazole alone.

The data pertaining to the antifungal potential of Fluconazole mixtures with *T. violacea, T. alliacea* and *T. simmleri* respectively are presented in Table 2.4. For interactions with *T. violacea*, 2 (13%) produced significantly higher inhibitory response compared to plant extract and Fluconazole alone. The combinations of Fluconazole with *T. violacea* resulted in a significant ($P<0.05$) higher radial growth inhibition of 2 (13%) compared to Fluconazole. When considering synergistic interactions, combinations resulted in 3 (19%) for *T. violacea,* while 13 (81%) was antagonistic. Among the 16 mixtures with *T. alliacea*, 4 (25%) showed significantly higher inhibitory activity compared to Fluconazole and *T. alliacea* alone. Similarly, 4 (25%) of the mixtures resulted in an increased inhibition against *F. verticillioides* compared to Fluconazole alone. When considering mixtures of Fluconazole and *T. simmleri,* 14 (88%) significantly increased the inhibitory response to *F. verticillioides* compared to the controls and 14 (88%) significantly increased the response compared to Fluconazole alone. Almost all (94%) mixtures showed synergistic interactions in this case.

Table 2.3. Inhibition of mycelium growth and synergy ratio (SR) between fungicide Itraconazole and *Tulbaghia violacea* (Tv), *Tulbaghia alliacea* (Ta) and *T. simmleri* (Ts) plant extract against *Fusarium verticillioides* MRC 826 *in vitro*.

Synergy Ratio[a]	Plant Species	Plant Extract Dose ($mg.ml^{-1}$)	Mycelium Growth Inhibition (%) Itraconazole Dose ($mg.ml^{-1}$) 0	6.25	12.5	25	50
	Control		0	21	30	32	43
	Tv	62.5	23	38^{kl}	26	41^{kl}	52
SR			-	1.65^{+}	1.18^{+}	1.95^{+}	2.63^{+}
	Tv	125	38	40^{l}	37	40	50
SR			-	1.90^{+}	1.89^{+}	2.10^{+}	2.81^{+}
	Tv	250	42	43^{l}	41^{l}	47^{l}	54
SR			-	2.09^{+}	2.15^{+}	2.53^{+}	3.13^{+}
	Tv	500	27	39^{kl}	41^{kl}	36	36^{l}
SR			-	1.73^{+}	1.93^{+}	1.71^{+}	1.87^{+}
	Ta	62.5	16	38^{kl}	43^{kl}	41^{kl}	46
SR			-	1.15^{+}	1.05^{+}	0.95	0.88
	Ta	125	14	42^{kl}	40^{kl}	43^{kl}	51^{kl}
SR			-	1.33^{+}	1.01^{+}	1.03^{+}	0.99
	Ta	250	21	32	35	46^{kl}	38
SR			-	0.85	0.78	0.98	0.69
	Ta	500	28	34^{l}	43^{kl}	50^{kl}	48^{kl}
SR			-	0.79	0.86	0.98	0.82
	Ts	62.5	-8	31	38^{kl}	45^{kl}	44
SR			-	1.15^{+}	1.45^{+}	1.74^{+}	1.79^{+}
	Ts	125	-7	36^{kl}	37^{kl}	45^{kl}	48
SR			-	1.33^{+}	1.43^{+}	1.74^{+}	1.97^{+}
	Ts	250	-14	22	40^{kl}	49^{kl}	43
SR			-	0.80	1.50^{+}	1.85^{+}	1.69^{+}
	Ts	500	17	47^{kl}	49^{kl}	51^{kl}	56^{kl}
SR			-	1.97^{+}	2.19^{+}	2.30^{+}	2.72^{+}

[k]Mycelium growth inhibition values of the plant extract and fungicide mixture differ significantly ($p<0.05$) from the value of the fungicide and the value of the plant extract doses. [l]Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the fungicide dose in the column. [a]Synergy ratio between expected and observed mycelium growth (SR=Exp/Obs; SR>1=Synergistic ; SR=1=Additive; SR<1=antagonistic). [+] Synergistic interaction.

Table 2.4. Inhibition of mycelium growth and synergy ratio (SR) between fungicide Fluconazole and *Tulbaghia violacea* (Tv), *Tulbaghia alliacea* (Ta) and *T. simmleri* (Ts) plant extract against *Fusarium verticillioides* MRC 826 *in vitro*.

Synergy Ratio[a]	Plant Species	Plant Extract Dose ($mg.ml^{-1}$)	Mycelium Growth Inhibition (%) Fluconazole Dose ($mg.ml^{-1}$) 0	6.25	12.5	25	50
	Control		0	9	20	33	27
	Tv	62.5	3	19[kl]	16	19	6
SR			-	1.68[+]	0.71	0.56	0.19
	Tv	125	3	21	19	11	19
SR			-	1.81[+]	0.84	0.32	0.67
	Tv	250	13	15	29	23	29
SR			-	0.67	0.88	0.53	0.75
	Tv	500	15	24[kl]	20	19	22
SR			-	1.06[+]	0.62	0.44	0.58
	Ta	62.5	-2	16[k]	16[k]	12[k]	10[k]
SR			-	2.25[+]	0.86	0.38	0.41
	Ta	125	5	13	19	17[k]	17[k]
SR			-	0.98	0.78	0.46	0.54
	Ta	250	13	24[k]	29[k]	25[k]	22[k]
SR			-	1.12[+]	0.93	0.60	0.61
	Ta	500	8	20[k]	20	20[k]	26
SR			-	1.22[+]	0.75	0.53	0.77
	Ts	62.5	-8	33[kl]	36[kl]	32	27
SR			-	1.15[+]	1.45[+]	1.74[+]	1.79[+]
	Ts	125	-7	34[kl]	40[kl]	42[kl]	37[kl]
SR			-	1.33[+]	1.43[+]	1.74[+]	1.97[+]
	Ts	250	-14	44[kl]	44[kl]	45[kl]	45[kl]
SR			-	0.80	1.50[+]	1.85[+]	1.69[+]
	Ts	500	17	47[kl]	43[kl]	54[kl]	61[kl]
SR			-	1.97[+]	2.19[+]	2.30[+]	2.72[+]

[k]Mycelium growth inhibition values of the plant extract and fungicide mixture differ significantly ($p<0.05$) from the value of the fungicide and the value of the plant extract doses. [l]Mycelium growth inhibition values of the plant extract and fungicide mixtures differ significantly ($p<0.05$) from the value of the fungicide dose in the column. [a]Synergy ratio between expected and observed mycelium growth (SR=Exp/Obs; SR>1=Synergistic ; SR=1=Additive; SR<1=antagonistic). [+] Synergistic interaction.

2.5 Discussion

In our study, more effective inhibitory responses were observed with methanol extracts compared to aqueous extracts. Utilization of aqueous formulations for control of *Fusarium* infections is relevant since they could be easily used for soil and root disinfection. The mycelial growth inhibition data of fungicide and wild garlic extract controls showed that the doses used produce relatively low antifungal activity against *F. verticillioides*. These doses could therefore be described as sublethal. Data analysis indicates that doses of the fungicide and plant extracts and fungicide combinations showed significantly higher growth inhibition responses than their respective single fungicide and plant extract treatments. Fungicide doses that give low or non-detectable residues in the crop are actively sought in research programs (Knight 1997). Compounds to be selected should rapidly degrade on plant surfaces, metabolize quickly in the plant, require use at very low rates, or act indirectly by promoting the plant's defence mechanism. However, balancing fungicidal potency and improved performance with low environmental impact remains a challenge for fungicide research.

More synergistic interactions were observed for the combinations of sporekill with *T. violacea* (62%) and *T. alliacea* (75%) than for *T. simmleri* (25%). Synergistic interactions for Sporekill™, Thiram™ and the wild garlic extracts did not result in the best possible inhibitory response against *F. verticillioides* in this study. Stronger synergistic interactions as well as improved inhibitory activity were achieved at the lower doses of Itraconazole and Fluconazole. Mixtures between the azole fungicides and *T. simmleri* produced 94% synergistic interactions. Our findings regarding synergism are in agreement with other studies where fungicides were enhanced by combination with other compounds. Carpinella *et al* (2005) reported that Scopoletin, a hydroxycoumarin, isolated from the fruits of *Melia azedarach* enhanced the effect of two synthetic fungicides namely, mancozeb and carboxin against *F. verticillioides*. Synergistic interaction between Epigallocatechin gallate (EGCG) and antimycotics such as amphotericin B and fluconazole has been reported against *Candida albicans*. EGCG possibly attacks the cell membrane and causes cell lysis (Toyoshima *et al.,* 1994). Amphotericin B below the minimum fungicidal concentration (MFC) is known to enhance the permeability of catechin through the fungal membrane, thereby increasing its uptake into the cell (Hirasawa and Takada 2004). Synergistic interactions between components in a mixture can relate to one of the following mechanisms (De Waard, 1985): (i) non-mediated

diffusion across the plasma membrane, (ii) carrier-mediated transport to the target site, (iii) activation, (iv) detoxification, (v) affinity for the target site, (vi) circumvention of the target site, and (vii) compensation of the target site.

Several laboratory trials have reported on the antibacterial, antifungal and insecticidal properties of plant extracts (Satish *et al.,* 1999; Ergene *et al.,* 2006; Okigbo and Ogbonnaya 2009; Shariff *et al.,* 2009). *T. alliacea* and *T. violacea* are two indigenous South African garlic species, which are traditionally used as remedies for a variety of infections and ailments (van Wyk 2008). *Tulbaghia violacea* has also shown effective antimicrobial activity against a few medically important pathogenic bacteria and fungi in HIV/AIDS patients (McGaw *et al.,* 2000; Gaidamashvili and Van Staden 2002; Motsei *et al.,* 2003). *Fusarium* species have also shown resistance to most antifungal agents, leading to mortality rates which exceed 70% in immunocompromised patients with fusarial infections (Nucci and Anaissie 2002; Raad *et al.,* 2006). Commercial garlic extracts and its derivatives were shown to be very effective against a broad spectrum of yeasts and fungi, including *Torulopsis*, *Candida*, *Aspergillus*, *Trichophyton*, *Rhodotorula*, *Cryptococcus* and *Trichosporon* (Davis and Perrie 2003, Davis *et al*, 2003). It has been reported that the chemical characterisation of garlic, in particular their sulphur compounds, some proteins, phenolic compounds and saponins account for their antimicrobial activity (Griffiths *et al.,* 2002).

A common storage technology to protect food products from deterioration caused by various fungi and their mycotoxins contamination is the utilisation of synthetic fungicides. However, the use of synthetic fungicides has caused residual toxicity in grains which may contribute to increased fungal resistance (Srivastava *et al.,* 2008). *Fusarium* species have shown also resistance to most antifungal agents, leading to mortality rates which exceed 70% in immunocompromised patients with fusarial infections (Nucci and Anaissie 2002; Raad *et al.,* 2006). This phenomenon and consumer pressure to reduce or eliminate chemically synthesized fungicides has generated attention in the use of natural occurring compounds (Dambolena *et al.,* 2011). Furthermore, natural substances including herbal extracts have attracted huge attention of scientific interest mainly because of their natural antioxidants and biologically active compounds (Wannissorn *et al.,* 2005). Hamburger and Hostettmann (1991) reported that more than 10 000 secondary metabolites may be produced by plants whose primary role in plants is defensive in nature. Contrary to the increasing research in this field, knowledge about these natural antifungal

compounds of plant origin is still scarce, and practically non-existent for the control of the diseases caused by *Fusarium*.

In conclusion, considering that *F. verticillioides*-maize interactions are of great importance to economic, social and health issues, environmentally compatible strategy such as natural defence compounds represent another alternative to cope with the problem of maize contamination and possibly fumonisin contamination. Effective control of growth does not necessarily mean that secondary metabolite production is also inhibited (Reynoso *et al.,* 2002). Furthermore, the results obtained in this study on culture media cannot necessarily be extrapolated to natural ecosystems and should therefore be corroborated in *in vivo* experiments. Combinations of fungicides and natural plant compounds offers the opportunity to find natural synergistic compounds and may validate disease control strategies with high biological activity, low dose rate application, and a low risk of pathogen resistance development to fungicides. Further research needs to be carried out to examine the efficacy of mixtures of these plant species and fungicides for control of *Fusarium* species in natural maize grain to examine effects on growth as well as mycotoxin production.

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CHAPTER 3

Modulation of hepatic drug metabolizing enzymes and oxidative status by *Allium sativum*, *Tulbaghia violacea* and *Tulbaghia alliacea* aqueous extracts in rats

3.1 Abstract

Many previous studies have identified garlic as a potent chemopreventive agent. This protective effect has been attributed to the presence of organosulphur compounds (OSC) and other antioxidants which include phenolic compounds. Therefore, the objective of this study was to evaluate the modulation properties of a commercially available garlic specie, *Allium sativum* and South African wild garlic species, *Tulbaghia violacea* and *T. alliacea* in male fischer rats. The rats were randomly divided into 7 diet groups of ten, receiving various aqueous garlic extracts as their sole source of drinking fluid. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) levels were determined to assess liver functions and tissue damage, respectively. Liver tissues were taken for determination of various oxidative stress parameters, detoxifying and antioxidant enzymes. The various liver enzymes and plasma total iron levels did not differ significantly from the control. Creatinine levels, as a marker for kidney function, were also not altered. The activity of Glutathione S-Transferase-(mu) (GST-μ) was significantly ($P<0.05$) enhanced by *T. violacea* (2% w/v) and *T. alliacea* (1% w/v) when compared to control rats while a non-significant increase in the activity of GST-α (alpha) in rat liver was also noted for all the garlic species. Similarly, the UDP-Glucuronosyltransferase (UDP-GT) activity was significantly ($P<0.05$) increased subsequent to treatment of the rats with *A. sativum* (2% w/v), while *T. alliacea* (1%, 2% w/v) and *A. sativum* (1% w/v) non-significantly increased. Treatment with *A. sativum* (2% w/v) exhibited a significantly ($P<0.05$) higher hepatic GSH level while *T. alliacea* (1%, 2% w/v) and *A. sativum* (1% w/v) treatment groups were similar to that of the control group. The GSH/GSSG ratio increased significantly for animals that consumed *T. alliacea* (1% w/v) whereas this ratio was significantly reduced in rats consuming *T. alliacea* (2% w/v) and *A. sativum* (2% w/v). Catalase (CAT) and superoxide dismutase (SOD) activity in the liver was also found to be increased by *T. alliacea* (1% w/v and 2% w/v) and *T. violacea* (1% w/v) and *A. sativum* (2 % w/v), respectively. Garlic administration to male Fischer rats

modulated the oxidative status as well as phase II drug metabolizing enzymes in the liver and may be crucial to protect the liver against the adverse effects related to oxidative damage and mutagenesis.

3.2 Introduction

Several treatments of many diseases have been associated with the invaluable role that garlic consumption, but evidence is mostly anecdotal (Budoff *et al.,* 2004; Macan *et al.,* 2006; Takasu *et al.,* 2006). The pharmacological potentials of garlic (*Allium sativum*) have been revealed through bactericidal (Stoll and Seebeck 1951), antibiotic (Cavallito *et al.,* 1944) and fungicidal (Moore and Atkins 1977) studies, while certain inhibitory effects of garlic may also be shown during tumor cell initiation and promotion (Takeyama *et al.,* 1993; Singh *et al.,* 1998). Many substances are found in aqueous solution of fresh garlic responsible for the induction of several antioxidant and phase II enzymes in the body which act in concert to prevent disease and age related conditions (Das and Saha 2009). These complement of bioactive components are water-soluble allyl amino acid derivatives which account for most of its organosulfur content (OSCs), or oil-soluble, such as diallyl sulphide (DAS), diallyl disulphide (DADS), and diallyltrisulfide (DATS) and essential macro- and micronutrients that demonstrate antioxidant activity (Amagase *et al.,* 1996). Allicin is believed to be the main biologically active component in garlic (Stoll and Seebeck 1951; Cho *et al.,* 2006). Garlic also consists of other antioxidants which include phenolic compounds, notably alexin, whose phenolic hydroxyl group confers antioxidant activity, N-fructosyl glutamate, N-fructosyl arginine, and selenium (Ide and Lau 1997).

Two indigenous South African garlic species, *Tulbaghia alliacea* and *T. violacea* are traditionally used as remedies for a variety of infections and ailments (Van Wyk *et al.,* 1997). The bulbs and leaves of *T. violacea* are used traditionally for the treatment of gastrointestinal ailments, asthma, fever, and tuberculosis (Hutchings *et al.,* 1996; Van Wyk and Wink 2004). *T. violacea* have also shown antimicrobial activity against some medically important pathogenic bacteria and fungi linked to opportunistic infections in HIV/AIDS patients (McGaw *et al.,* 2000; Gaidamashvili and Van Staden 2002; Motsei *et al.,* 2003). It has also been reported that a variety of undesirable symptoms, such as abdominal pain, inflammation, and gastroenteritis are caused by extensive consumption of this plant (Kubec *et al.,* 2002). *T. alliacea* exhibited immunostimulation and anti-inflammatory effects (Masika and Afolayan

2003). Extracts of *T. alliacea* in chloroform was more potent than the aqueous and methanol extracts in inhibiting the growth of *Saccharomyces cereviseae*, *Candida albicans*, *C. glabrata*, *C. krusei* and *Cryptococcus neoformans* (Thamburan *et al.,* 2006).

Oxidative stress induced by an overproduction of reactive oxygen species (ROS) and inflammation are reported to play an important role in many cancers (Winzer *et al.,* 2002). ROS are produced during several intracellular pathways(Das and Saha 2009). During incidences such as infection, inflammation, exposure to pollutants, radiation, and sunlight, an increase is noticed in ROS production. The increased production of these oxygen radicals eventually lead to the damage of cellular DNA and protein which subsequently foster tumor growth in many types of cancer (Sander *et al.,* 2003; Valko *et al.,* 2006; Ozben 2007). The use of dietary agents as a means of chemoprevention has emerged as a cost-effective method for preventing oral cancer and generally acts by multiple pathways in order to block tumorigenesis (Flora *et al.,* 2001; Tuba and Gülçin 2008; Ganie *et al.,* 2010b; a)

The modulation of carcinogen-induced genotoxicity and inhibition of carcinogen activation by modifying the activities of phase I and II enzymes and scavenging ROS via the antioxidant defence systems are significant chemoprevention pathways (Chandra Mohan *et al.,* 2006). Decrease lipid peroxidation and maintenance of the redox status of intracellular enzymes needed for homeostasis of the cells, have been demonstrated by several phytochemicals (Chandra Mohan *et al.,* 2006; Oarada *et al.,* 2008). Phase I enzymes are known to activate chemicals to a toxic or mutagenic product, including aflatoxins and polycyclic aromatic hydrocarbons. Furthermore, they also produce ROS that occurs as a result of cytochrome P450 activity (Percival 1997). In order for potential chemoprotective agents to function as anticarcinogenic agents, a balance is needed between their abilities to inhibit P450s and to induce phase II detoxification enzymes (Krajka-Kuzniak and Baer-Dubowska 2003). The biotransformation of toxic xenobiotics and body wastes into less harmful and more readily excreted substances by drug-metabolizing systems, serves as an important defence mechanism composed of phase I and phase II enzymes (Guengerich and Shimada 1991; Kensler *et al.,* 2003). The conjugation of phase I metabolites to various water-soluble molecules in order to accelerate the rate of metabolite excretion, are catalysed by phase II enzymes (Talalay *et al.,* 1995). Some reports have indicated that DAS and DADS do not only have

chemopreventive effect, but may also act as cancer promoters in rat hepatocarcinogenesis (Haber Mignard *et al.,* 1996; Ip and Lisk 1997).

The modulation of Glutathione S-Transferase (GST) activity and cytochromes P450 (CYP), a family of enzymes that activate many chemical carcinogens in experimental animals, have been reported on organosulfur compounds (Omar and Al-Wabel 2010). The addition of garlic powder to the diet of rats increased the activity of GST in the liver. However, it was found that the maximum activity of GST did not coincide with the maximum inhibition of carcinogenesis (Omar and Al-Wabel 2010). Diallyl disulphide in the diet did not only increase the activity of GST but also that of other detoxifying enzymes which include reduced nicotinamideadenine dinucleotide phosphate [NAD(P)H]–dependent quinoneoxidoreductase and of uridinediphosphate glucuronosyl transferase (UDP-GT) in rat tissues (Munday and Munday 1999).

An important antioxidant enzyme, catalase (CAT), is present in most aerobic cells. The detoxification of hydrogen peroxide (H_2O_2) a toxic product of normal aerobic metabolism and pathogenic ROS production, is mainly controlled by CAT (Ichikawa *et al.,* 2003). Superoxide dismutase (SOD) is another important enzyme involved in the prevention of several diseases linked to oxidative stress. Superoxide dismutase catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen. It is also important to note that many variables including species, organ, age, sex and several environmental factors may influence the expression of above mentioned enzymes (Prohaska and Sunde 1993; Holovska *et al.,* 2005).

The present study investigates the modulation of phase II drug metabolizing enzymes as well as the antioxidant status in the liver of rats that consumed *A. sativum*, *T. alliacea* and *T. violacea*.

3.3 Matrerials and Methods

3.3.1 Chemicals

Butylated hydroxytoluene/Ethanol; butanol; perchloric acid (PCA); triton X-100 and dimethyl sulfoxide (DMSO) was purchased from Merck (Pty) Ltd (Cape Town, South Africa). Sodium pentobarbital; KCl; thiobarbituric acid (TBA); NaCl; fluorescein; 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH); trichloro acetic acid (TCA); ethylenediaminetetraacetic acid (EDTA); 3 mM 1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate (M2VP); glutathione reductase; 5,5'-dithiobis-2-

nitrobenzoic acid (DTNB); nicotinamide adenine dinucleotide phosphate (NADPH); 1-chloro-2,4-dinitrobenzene (CDNB); 3,4-dichloro-nitrobenzene (DCNB) *p*-nitrophenol; uridine diphosphate-glucuronic acid; Tris-HCl; $MgCl_2$ and NaOH were purchased from Sigma (Sigma–Aldrich, Cape Town, South Africa).

3.3.2 Plant material and preparation

Bulbs of *A. sativum* (commercial garlic) were obtained from a retail outlet in Cape Town, South Africa. *T. violacea* (wild garlic) was collected from the Cape Peninsula University of Technology's Nursery garden (Cape Town), while *T. alliacea* (social garlic) was supplied by herb traders in Cape Town. The various garlic bulbs were cut into smaller pieces and aqueous extracts were prepared by adding freshly boiled tap water to the plant material, to final concentrations of 1 g/100 ml (1%) and at 2 g/100 ml (2%) for each plant, respectively. Homogenization was done in a Waring blender. The aqueous extracts were cooled to room temperature, filtered (Whatman no. 4) and stored at -20 °C until used. Aqueous garlic extracts were freshly prepared every second day and dispensed into water bottles of the experimental rats.

3.3.3 Treatment of animals

All animal experiments were performed in accordance with the guidelines set out by the Ethics Committee for Research on Animals (ECRA) of the Medical Research Council who approved this study (Ref 02/07). Male Fischer rats (150–170 g) were obtained from the Primate Unit, MRC (Tygerberg, South Africa). Rats were randomly divided into seven treatment groups of ten each and housed separately in wired top and bottom cages fitted with Perspex™ houses. The animals were maintained at controlled conditions of temperature (24-25 °C), a 12 h light/dark cycle while keeping the humidity at 50%. The rats of the control group were fed Epol rat mash and had free access to drinking water. Rats treated with garlic, had free access to the various aqueous garlic extracts as their sole source of drinking fluid and Epol rat mash for the duration of the experiment (90 days). Body weights of the rats were monitored weekly and the average weekly feed intake was calculated using the mean daily rat feed intake as a function of the body weight and expressed as gram feed. Upon termination, fasting (16 hr) animals were euthanized by i.p. injection of sodium pentobarbital (0.15 ml /100g bw). Blood was collected at room temperature to obtain serum for clinical chemical analyses while livers were excised and immediately frozen in liquid nitrogen and stored at -80°C for biochemical analyses.

3.3.4 Total polyphenolic and soluble solid analyses

The soluble solid content of each garlic extract was determined by drying 1ml aliquots at 90 °C for 12 h. The phenolic content of the garlic extracts was determined using the Folin- Ciocalteu method with gallic acid as standard (Waterhouse, 2005). Gallic acid standard was added to the designated wells in a clear well plate, while the garlic samples were added in triplicate to respective wells and the absorbance was read using a microplate reader (Multiskan Spectrum Thermo Electron Corporation).

3.3.5 Clinical chemistry

Hepatotoxicity was assessed by the quantification of serum, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and total iron. Activities of these serum enzymes were measured using a Technicon RA 1000 automated analyzer. The levels of creatinine were also determined as a marker for kidney function.

3.3.6 Antioxidant status parameters

3.3.6.1 *Lipid peroxidation*

Lipid peroxidation was estimated by determining malondialdehyde (MDA) concentration [measured as thiobarbituric acid reacting substances (TBARS)] in the liver homogenates by the modified method of Esterbauer and Cheeseman (1990). For the assay, livers (0.1 g) were homogenized on ice in 500 µl of KCl (0.15M) and 0.01 M phosphate buffer (pH 7.4). Following centrifugation, the resultant supernatant (50 µl) was mixed with 6.25 µl of butylated hydroxytoluene/ethanol (0.01%) and 50 µl ortho-phosphoric acid. Thiobarbituric acid (TBA) (6.25µl of 0.67%) solution was added to the homogenate and incubated at 90 °C for 45 min. After allowing the mixture to cool on ice, 1000 µl butanol and 50 µl NaCl (saturated solution) was added and mixed. All samples were centrifuged at 12000 *g* for 2 min at 4 °C and the absorbance measured at 532 nm. According to Esterbauer and Cheeseman (1990), lipid peroxidation was expressed as nmole MDA per mg protein using a molar extinction coefficient of 1.56 $x10^5$ M^{-1} cm^{-1} for MDA. Conjugated dienes (CDs) were also measured, according to the method of Recknagel and Glende (1984) to assess lipid peroxidation in the liver homogenates. A chloroform and methanol mixture (2:1) was added (400 µl) to 50 µl of tissue homogenate and vortexed for 60 seconds. The top aqueous layer was removed and discarded after centrifugation at 8000 *g* for 15 min at 4 °C. With a glass Pasteur pipette along the wall of the tube through the protein layer, the lipid layer was removed and

transferred into a clean eppendorf tube. These samples were dried under liquid nitrogen for 30 min. The dried samples were vortexed with 1 ml cyclohexane for 60 seconds. The samples (300 µl) were transferred in duplicate into a clear 96-well plate and the absorbance read at 234 nm using a Multiscan spectrophotometer. The results were expressed as umole/g liver using a molar extinction coefficient of 26550 M^{-1} cm^{-1}.

3.3.6.2 *Oxygen radical absorbance capacity (ORAC)*

The ORAC assay was conducted according to the method of Cao and Prior (1999) using a Fluoroskan Ascent (Thermo Electron Corporation) fluorescence spectrophotometer. Frozen liver samples (0.1 g) were thawed and then homogenized (10 strokes) using a Potter-Elvehjem Teflon pestle and glass tube tissue homogenizer, in 500 µl phosphate buffer (75 mM, pH 7.0). After centrifugation at 12000 *g* for 15 min at 4 °C, the supernatant was deproteinised with 0.25 M perchloric acid (PCA) and centrifuged at 14000 *g* for 15 min. Fluorescein (138 µl, 95 nM), which was used as a target for free radical attack, was added to 12 µl of the diluted sample (1:4) in a 96 well microtiter plate. The reaction was initiated by adding 50 µl (92 mM) 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) to the sample mixture. Fluorescence (emission 538 nm, excitation 485 nm) was recorded with 5 min intervals and declined to a reading less than 5 % of the initial reading. A regression equation ($Y = a + bx + cx^2$) between Trolox concentration (Y) (uM) and the net area under the fluorescence decay curve (x) was used to calculate the ORAC values for each sample. The calculated ORAC values were expressed as µmole/l Trolox equivalents/g wet liver weight.

3.3.6.3 *Glutathione analysis*

The modified method of Tietze (1969) was used to determine total glutathione (GSH and GSSG) levels. For GSH determination, the liver samples were homogenized (1:10) on ice in 15% (w/v) trichloro acetic acid (TCA) containing 1 mM ethylenediaminetetraacetic acid (EDTA). In the determination of GSSG, 6% (v/v) perchloric acid (PCA) containing freshly prepared 3 mM 1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate (M2VP) and 1 mM EDTA was added to the liver samples and homogenized on ice. The homogenate was centrifuged at 10 000 *g* for 10 min and 50 µl of supernatant was added to glutathione reductase (1U) and 0.3 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). The addition of 1.0 mM nicotinamide adenine dinucleotide phosphate (NADPH) to a final volume of 200 µl initiated the reaction. Pure GSH and GSSG were used as standards and changes in absorbance were

monitored at 410 nm for 5 min. The GSH/GSSG ratio was also calculated (Ratio = GSH ÷ GSSG).

3.3.6.4 *Activity of antioxidant enzymes*

The Catalase (CAT) and superoxide dismutase (SOD) activities were determined according to the modified method of Ellerby and Bredesen (2000), for a microplate reader (Multiskan Spectrum Thermo Electron Corporation). A Potter-Elvehjam homogeniser was used to homogenise the liver tissue samples (200 mg) in 2 ml phosphate buffer for five strokes on ice. Following sonication for two 15 second bursts on ice, the homogenate was centrifuged for 10 min at 15 000 *g* at 4ºC and stored at -80 ºC for CAT and SOD activity determination. To determine CAT activity, homogenates were diluted to a concentration of 0.1 µg/µl protein with phosphate buffer. The homogenates (10 µl) were added to a 96-well plate containing 75 µl of H_2O_2 and 170 µl of phosphate buffer (0.1M) and mixed. For CAT activity, the decomposition of H_2O_2 was determined spectrophotometrically at 240 nm and expressed as µmole/min/mg protein using the millimolar extinction coefficient of 0.000394. To determine SOD activity, stored homogenised liver tissue samples were diluted to a final concentration of 0.1 µg/µl protein with SOD assay buffer. Duplicate samples (12 µl) were added to a 96-well plate followed by 15 µl (1.6 mM) of 6-hydroxydopamine (6-HD). To ensure sufficient mixing, the diethylenetriaminepentaacetic acid (DETAPAC) (173 µl, 0.1 mM) solution was added last. SOD activity was expressed as the amount of protein (ng) required to produce a 50% inhibition of auto-oxidation of 6-HD and recorded at 490 nm for 4 min in 1 min intervals.

3.3.7 Preparation of microsomal and cytosolic liver fractions

For sub cellular fractions, frozen liver samples (300 mg) were homogenized in 900 µl of ice-cold KCl (0.15 M) solution using a Potter-Elvehjem tissue homogeniser for 10 seconds. Double layer cheesecloth was used to filter the homogenates and homogenized with a glass dounce (10 strokes) using a loose pestle. The resultant homogenates were centrifuged at 9000 *g* for 10 min at 4 °C. The supernatant was centrifuged at 100000 *g* for 60 min at 4 °C and the cytosolic preparations were collected. A Pasteur pipette was used to wash (10 times) the microsome pellet and subsequently resuspended in 1 ml 0.15 M KCl using a glass dounce. The homogenate was centrifuged at 100 000 *g* for 60 min at 4 °C and pellet resuspended in 0.15 M KCl. These microsome samples were stored with the cytosolic fractions at -80 °C. The Bradford (1976) method was used for protein

determination of the microsomal and cytosolic fractions using BSA as standard protein.

3.3.8 Phase II enzyme assays

3.3.8.1 *Glutathione S-Transferase (GST) assay*

The GST-α and GST-μ activities were determined according to the method of Habig *et al* (1974). GST-α activity was determined from the rate of increase in the conjugate formation between GSH and 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. A reaction mixture consisting of 200 mM GSH, 0.1 M potassium phosphate buffer (pH 6.5) and 100mM CDNB was prepared. The reaction was initiated following the addition of 2 μl hepatic cytosolic preparation (10 mg protein/ml). Due to the formation of CDNB-GSH conjugates (Multiskan Spectrum, Thermo Electron Corporation) an absorbance was recorded at 340 nm for 3 min at 25 °C. The specific activity (μmole/ml/min) was calculated using a millimolar extinction coefficient of 9.6 for CDNB-GSH conjugate. The determination of GST-μ activity was achieved using 60 mM 3,4-dichloro-nitrobenzene (DCNB) as substrate. The hepatic cytosolic preparation (0.3 g/ml) was added to a reaction mixture containing 0.1 M potassium phosphate buffer (pH7.5), 86.1 mM GSH, and 60 mM DCNB. A Multiskan Spectrum (Thermo Electron Corporation) was used to measure the absorbance at 344 nm for 3 min at 25 °C. The specific activity for GST-μ was expressed as μmole/ml/min protein using a millimolar extinction coefficient of 8.5 for the DCNB-GSH conjugate.

3.3.8.2 *UDP-Glucuronosyltransferase (UDP-GT) assay*

The activity of microsomal UDP-GT was determined according to the method of Bock *et al* (1983). This activity was measured spectrophotometrically in a Multiskan Spectrum (Thermo Electron Corporation) using *p*-nitrophenol and UDP-glucuronic acid as substrates. The reaction mixture consisted of hepatic microsome (100 μl) preparation (1 mg of protein/ml) which was activated with 20 μl (0.25% w/v) Triton X-100 and subsequently incubated with 50 μl (0.1 M) Tris-HCl (pH 7.4), 50 μl (50 mM) $MgCl_2$, and 50 μl (5 mM) *p*-nitrophenol for 2 min at 37 °C. The reaction was initiated by the addition of 50 μl UDP-glucuronic acid (30 mM) and terminated after 10 min by the addition of ice cold 500 μl (0.5 M) trichloro acetic acid (TCA) and kept on ice. The mixture was centrifuged at 3000 *g* for 10 min. the absorbance was determined at 405 nm following the addition of 100 μl (2 M) NaOH to 0.4 ml supernatant. The specific activity for UDP-GT was expressed as nmole/min/mg protein using the millimolar extinction coefficient of 18.1.

3.3.9 Statistical analysis

The SPSS statistical package with the analysis of variance and Duncan's multiple range test was used for all statistical analyses. The statistical comparisons were carried out between the negative control rats consuming only water and the various garlic treated groups and among the different garlic treated groups in so-called inter garlic comparisons. Statistical significance was at the 5% level ($P < 0.05$).

3.4 Results

3.4.1 Liquid intake profiles, body weight and relative liver weight gains.

No significant differences were recorded for fluid intake of rats consuming *T. violacea* (1% and 2% w/v) and *T. alliacea* (1% w/v) when compared to the negative control group consuming tap water as drinking fluid (Table 3.1). The average *T. alliacea* (2% w/v) consumption was significantly ($P<0.05$) less when compared to the negative control group and consumption of *A. sativum and T. violacea. T. alliacea* (2% w/v) treated rats also recorded the lowest consumption of the aqueous extract when all the garlic treatment groups were compared. In contrast, animals receiving *A. sativum* (1 and 2% w/v) recorded a significantly ($P<0.05$) higher consumption level of garlic compared to all the other garlic treatments and the negative control group. In this study, rats consuming aqueous extracts of *A. sativum* (1% w/v) had a significantly ($P<0.05$) higher body weight gain compared to the control rats receiving only water as drinking fluid. No significant differences were noted in the body weight gains of all the other groups when compared to the negative control group. With regards to the relative liver weight, none of the garlic treated groups reported any significant increase or decrease when compared to the control group. When considering the garlic treated groups, *T. alliacea* (1% w/v) showed a significant ($P<0.05$) increase in relative liver weight when compared to *A. sativum* (2% w/v).

3.4.2 Serum biochemical parameters

Serum biochemical parameters associated with liver and kidney functions were examined in the serum collected from each garlic treated group as well as the negative control group and the results are summarized in Table 3.1. The activities of the liver function enzymes AST and ALT, after treatment with aqueous extracts of commercial and wild garlic, exhibited no significant differences when compared to the control rats. However, the ALP levels were significantly ($P<0.05$) reduced in rats

consuming *T. violacea* (2% w/v) and *A. sativum* (1 and 2% w/v), respectively when compared to the control rats. The remaining garlic treatment groups had no significant effect on the ALP levels compared to the negative control. As a marker for kidney function, the creatinine level for rats consuming *T. violacea* (2% w/v) was significantly ($P<0.05$) higher, compared to the control group, while no differences were noticed with the remaining garlic treated groups when compared to the control. The total plasma iron levels were also similar to that of the control rats.

3.4.3 Soluble solid, total phenolic and total phenolic intake.

A significantly ($P<0.05$) higher soluble solids content was noted between the 1% and 2% plant extracts, which is in agreement with the larger amount of garlic used. The soluble solids obtained from *T. violacea* (1 and 2% w/v) were similar when compared to *T. alliacea* at the same concentrations (Table 3.2). *A. sativum,* at both concentrations, contained significantly ($P<0.05$) higher soluble solids when compared to the two African wild garlic species, *T. violacea* and *T. alliacea,* for the same concentrations.

The total phenolic content of *T. violacea* (1% w/v) did not differ significantly when compared to its 2% (w/v) extract. Similarly, the total phenolic content of *A. sativum* exhibited no significant difference for both concentration (1 and 2% w/v) levels, while the 1% (w/v) extract of *T. alliacea* contained significantly more soluble solids when compared to the 2% (w/v) extract. The total phenolic content of the soluble solids of *T. alliacea* (1% w/v) was significantly higher than *T. alliacea* (2% w/v).

No significant differences were noted in the phenolic intake per day between rats that consumed the *T. alliacea* (1%, 2% w/v) and *A. sativum* (1% w/v), while rats that consumed *A. sativum* (2% w/v) had the highest phenolic intake, differing significantly ($P<0.05$) from the other rats. Rats consuming *T. violacea* (1%, 2% w/v) consumed significantly ($P<0.05$) less phenolic intake per day compared to all other garlic treated groups.

3.4.4 Hepatic antioxidant status

3.4.4.1 Oxygen radical absorbance capacity (ORAC)

Our results showed that ORAC values were significantly ($P<0.05$) increased in rats treated with *T. violacea* (1% w/v and 2% w/v) and *T. alliacea* (2% w/v) compared to the control group (Table 3.3). Rats consuming *A. sativum* (1% w/v and 2% w/v) and

T. alliacea (1% w/v) as drinking fluid showed no difference in ORAC values when compared to the control group.

3.4.4.2 Hepatic glutathione

The glutathione level in the livers of the *T. violacea* (2% w/v) treated group was significantly ($P<0.05$) lower than that of the control group and other garlic treated groups, while *T. violacea* (1% w/v) had no effect on the GSH level (Table 3.3) when compared to the negative control group. The GSH values for the *T. alliacea* (1% w/v and 2% w/v) and *A. sativum* (1% w/v) treatment groups were significantly ($P<0.05$) higher when compared to the negative control group, while the GSH value for rats receiving *A. sativum* (2% w/v) was of the same order as the control group. Animals exposed to *A. sativum* (1% w/v) had significantly ($P<0.05$) higher GSSG levels when compared to the negative control rats, while *T. violacea* (1% and 2% w/v) and *T. alliacea* (1 % w/v) non-significantly reduced the activity of GSSG compared to the control group. Rats consuming *T. alliacea* (2% w/v) and *A. sativum* (2% w/v) exhibited a non-significant increase in the GSSG level compared to the control rats. As a result of changes in the GSH and GSSG levels, the GSH/GSSG ratio increased significantly for animals that received *T. alliacea* (1% w/v) compared to the control group, whereas no significant difference were observed for any of the remaining treatment groups compared to the control rats.

3.4.4.3 Antioxidant enzyme activity

Catalase activity in rats consuming *T. alliacea* (1% w/v) and *A. sativum* (1% w/v) in comparison with control animals, was significantly ($P<0.05$) increased (Table 3.3). However, the CAT values obtained for the *T. alliacea* (2% w/v) fed group was not statistically different compared to the control group. None of the other garlic treated groups showed any significant differences when compared to the negative control rats. When considering all the garlic treated groups, *A. sativum* (1% w/v) exhibited the highest CAT activity. The specific activity for SOD in the liver was significantly ($P<0.05$) increased in the liver of rats consuming *A. sativum* (1% w/v) when compared to the control and other garlic treated rats. Furthermore, the differences in the specific activities for SOD in the remaining garlic treated groups were not significantly different when compared to the control group.

3.4.5 Lipid peroxidation

Lipid peroxidation in the liver, measured as TBARS, was increased in certain garlic-fed rats compared to the control group. *T. violacea* (1%, 2% w/v) and *A. sativum* (1% w/v) significantly ($P<0.05$) increased the TBARS level compared to rats

consuming tap water as drinking fluid. However, rats receiving *T. alliacea* (1%, 2% w/v) and *A. sativum* (2% w/v) non-significantly increased the TBARS levels compared to the control group. The conjugated dienes (CDs) in the liver of rats treated with the various garlic extracts showed no significant difference when compared to the negative control group.

3.4.6 Cytosolic glutathione S-transferases (GST) and microsomal UDP-glucuronosyl transferases (UDP-GT)

Aqueous extract of *T. violacea* (2% w/v) and *T. alliacea* (1% w/v) significantly ($P<0.05$) enhanced the activity of GST-μ (mu) when compared to the control rats (Table 3.4). Although rats consuming *A. sativum* (1% w/v), *T. violacea* (1% w/v) and *T. alliacea* (2% w/v) also showed increased activities of GST-μ when compared to the negative control rats, it was not significant. No significant ($P>0.05$) difference could also be shown for rats consuming the various garlic extracts when considering the cytosolic GST-α (alpha) activities.

Animals consuming *T. alliacea* (1% w/v) and *A. sativum* (2% w/v) showed a significantly ($P<0.05$) increased activity of the microsomal UDP-GT when compared to the negative control animals. All other garlic groups, except for *T. violacea* (2% w/v) showed a tendancy to also increase UDP-GT activity, although not significant. Rats consuming *T. violacea* (2% w/v) had significantly lower activity of UDP-GT when compared to the control rats.

Table 3.1. Liquid intake and effect of garlic treatments on the body weight gain, relative liver weight, and selected blood clinical chemical parameters related to liver and kidney function.

treatment	liquid intake/ day/ 100 g BW (mL)	body weight gain (g)	relative liver weight (g)	AST (U/L)	ALT (U/L)	ALP (U/L)	creatinine (µmole/L)	total iron (µmole/L)
control	13.47 ± 0.12a	117.20 ± 17.01a	8.35 ± 0.74ab	22.44 ± 8.99a	37.18 ± 37.18a	99.88 ± 99.80b	69.96 ± 2.52ab	75.64 ± 7.32a
Tv 1%	13.98 ± 0.18a	115.33 ± 12.00a	8.70 ± 1.03ab	17.83 ± 9.58a	35.17 ± 35.17a	89.67 ± 89.67ab	69.33 ± 8.02ab	83.40 ± 8.21a
Tv 2%	12.63 ± 2.12a	111.00 ± 7.67a	8.46 ± 0.77ab	19.33 ± 6.59a	32.33 ± 32.33a	87.17 ± 87.17a	72.08 ± 7.81b	72.60 ± 6.81a
Ta 1%	12.88 ± 1.51a	126.83 ± 9.72ab	9.28 ± 0.88b	20.67 ± 7.34a	34.17 ± 34.17a	93.00 ± 93.10ab	63.80 ± 2.21ab	70.80 ± 6.63a
Ta 2%	6.39 ± 2.13b	115.66 ± 14.46a	8.40 ± 0.41ab	22.00 ± 7.98a	34.83 ± 34.83a	92.00 ± 92.01ab	69.45 ± 7.61ab	84.40 ± 6.12a
As 1%	15.31 ± 4.01c	134.60 ± 14.71b	8.22 ± 0.46ab	18.40 ± 8.79a	26.80 ± 26.81a	84.80 ± 84.80a	61.24 ± 3.37a	83.28 ± 7.22a
As 2%	16.08 ± 1.23c	116.33 ± 17.60a	7.82 ± 0.72a	19.00 ± 4.15a	26.50 ± 26.50a	85.17 ± 85.17a	68.28 ± 7.24ab	78.00 ± 9.51a

Values in columns represent the mean ± STD.
Values in columns represent the mean ± STD.
Abbreviations: Tv 1% = *Tulbaghia violacea* at 1% (w/v), Tv 2% = *T. violacea* at 2%, Ta 1% = *T. alliacea* at 1% (w/v), Ta 2% = *T. alliacea* at 2% (w/v), As 1% = *Allium sativum* at 1% (w/v) and As 2% = *Allium sativum* at 2% (w/v), AST = aspartate aminotransferase, ALT = alanine aminotransferase, ALP = alkaline phosphatase . Values followed by the same letters do not differ significantly. When letters differ then $P < 0.05$. N = 7 rats per group.

Table 3.2. Different intake parameters of male Fischer 344 rats fed various garlic preparations over a 90 day period

treatment	soluble solids (mg/mL)	total phenolic content (mg gallic acid equivs/ g soluble solids)	total phenolic intake (mg gallic acid equivs/ day/100 g BW)
control	none	none	none
Tv 1%	2.63 ± 1.94a	0.47 ± 1.11a	1.74 ± 0.52a
Tv 2%	10.20 ± 2.65b	0.33 ± 0.13a	4.30 ± 0.11a
Ta 1%	2.23 ± 0.60a	3.57 ± 1.11c	10.27 ± 0.31b
Ta 2%	9.90 ± 1.56b	1.39 ± 0.21b	8.79 ± 0.10b
As 1%	13.37 ± 0.68c	0.75 ± 0.10a	15.33 ± 0.01b
As 2%	31.60 ± 1.59d	0.42 ± 0.02a	21.19 ± 0.01c

Values in columns represent the mean ± STD.
Abbreviations: Tv 1% = *Tulbaghia violacea* at 1% (w/v), Tv 2% = *T. violacea* at 2%, Ta 1% = *T. alliacea* at 1% (w/v), Ta 2% = *T. alliacea* at 2% (w/v), As 1% = *Allium sativum* at 1% (w/v) and As 2% = *Allium sativum* at 2% (w/v). Values followed by the same letters do not differ significantly. When letters differ then $P < 0.05$. N = 7 rats per group.

Table 3.3. Effect of *Tulbaghia violacea*, *Tulbaghia alliacea* and *Allium sativum* on reduced glutathione (GSH), oxidized glutathione (GSSG), the Ratio GSH/GSSG, oxidative capacity (ORAC), oxidative enzyme parameters and lipid peroxidation in livers of rats

treatment	ORAC (uMTroloxequivs/ mg liver)	GSH (uM/mg protein)	GSSG (uM/mg protein)	GSH/GSSG ratio	CAT (umole/min/ mg protein)	SOD U/ng protein	TBARS mmole/g liver	CD umole/g liver
control	6.61 ± 0.51a	820.21 ± 151.11a	26.21 ± 1.61ab	32.21 ± 7.52ab	18.31 ± 4.32a	72.41 ± 20.71a	20.11 ± 1.21a	851.91 ± 95.25a
Tv 1%	8.22 ± 0.61c	803.32 ± 145.12a	21.22 ± 5.11ab	37.81 ± 9.21ab	23.01 ± 3.52ab	77.71 ± 27.51a	41.12 ± 12.22c	708.51 ± 63.42a
Tv 2%	7.54 ± 0.52bc	487.82 ± 63.12b	17.52 ± 1.82a	29.11 ± 3.92a	23.12 ± 2.92ab	68.62 ± 24.53a	43.11 ± 3.23c	864.82 ± 56.42a
Ta 1%	7.44 ± 0.63abc	1101.11 ± 104.23c	18.91 ± 3.82a	59.22 ± 9.11c	27.11 ± 3.71b	55.72 ± 13.41a	27.92 ± 3.11ab	827.61 ± 80.14a
Ta 2%	7.64 ± 0.64bc	1171.22 ± 122.23c	35.23 ± 8.32bc	39.71 ± 7.71ab	26.42 ± 3.71ab	76.81 ± 23.92a	28.81 ± 6.81ab	736.12 ± 93.12a
As 1%	7.11 ± 0.55ab	1421.32 ± 125.33c	42.24 ± 2.91c	34.13 ± 9.92ab	38.12 ± 2.52b	125.23 ± 25.01b	34.02 ± 3.01bc	772.21 ± 94.23a
As 2%	6.62 ± 0.64a	988.71 ± 139.11a	38.02 ± 7.12bc	29.81 ± 7.93a	18.11 ± 1.83a	77.03 ± 18.91a	25.22 ± 4.31ab	840.01 ± 83.23a

Abbreviations: Tv 1% = *Tulbaghia violacea* at 1% (w/v), Tv 2% = *T. violacea* at 2%, Ta 1% = *T. alliacea* at 1% (w/v), Ta 2% = *T. alliacea* at 2% (w/v), As 1% = *Allium sativum* at 1% (w/v) and As 2% = *Allium sativum* at 2% (w/v).
Values in columns represent average of 6 values per group ± STD. Means followed by the same letter do not differ significantly, when letters differ then $P < 0.05$. CAT = catalase; SOD = superoxide dismutase, TBARS = thiobarbituric acid reactive substances, CD = Conjugated dienes.

Table 3.4. Effect of *Tulbaghia violacea*, *Tulbaghia alliacea* and *Allium sativum* treatments on the activities of the cytosolic Glutathione S-Transferases (GST) and the microsomal UDP-Glucuronosyl Transferases (UDP) in livers of rats.

treatment	GST-μ (mu) (μmole/min/mg protein)	GST-α (alpha) (μmole/min/mg protein)	UDP-GT (nmole/min/mg protein)
control	0.25 ± 0.02a	5.86 ± 0.34a	34.51 ± 3.77a
Tv 1%	0.38 ± 0.09a	5.61 ± 0.69a	38.47 ± 7.46a
Tv 2%	0.42 ± 0.12b	6.73 ± 1.33a	20.44 ± 1.26b
Ta 1%	0.46 ± 0.10b	6.52 ± 0.75a	40.60 ± 1.31c
Ta 2%	0.34 ± 0.15ab	6.07 ± 0.35a	36.03 ± 1.40ac
As 1%	0.38 ± 0.10ab	6.55 ± 0.62a	38.35 ± 2.33ac
As 2%	0.29 ± 0.07a	6.11 ± 1.32a	42.99 ± 3.80c

Abbreviations: Tv 1% = *Tulbaghia violacea* at 1% (w/v), Tv 2% = *T. violacea* at 2%, Ta 1% = *T. alliacea* at 1% (w/v), Ta 2% = *T. alliacea* at 2% (w/v), As 1% = *Allium sativum* at 1% (w/v) and As 2% = *Allium sativum* at 2% (w/v).
Values in columns are means ± STD of 7 rats per group. Means followed by the same letter are not significantly different ($P > 0.05$). If letters differ, then $P < 0.05$. DCNB was used as substrate for GST-*μ* determination and CDNB for GST-α.

3.5 Discussion

Several environmental pollutants are able to cause oxidative damage to biological systems. Metabolism of xenobiotics (exogenous chemical substances that enter the body) is generally divided into phase I and phase II metabolism (Hardman and Limbird 2001), which primarily occurs in the liver. Documented evidence has shown several phytochemicals to enhance or inhibit phase I or phase II metabolic enzymes (Block and Gyllenhaal 2002). The activation of the expression of these enzymes results in induction. In contrast, inhibition of these enzymes is often as a result of direct inhibition of the metabolic activity of the enzyme (Block and Gyllenhaal 2002). Furthermore, the modulation of phase I or phase II enzyme activity by valuable phytochemicals can result in protecting or having detrimental effects, depending on the nature of the chemical carcinogen as well as the type of modulation. Previous studies have shown that consuming vegetables rich in antioxidant phytochemicals as well as organosulfur compounds, such as garlic, could prevent tumorigenesis (Kumar and Berwal 1998; Milner 2001). The antioxidant ability of garlic is well established (Banerjee *et al.,* 2003; Rahman and Lowe 2006) and its association with the protective effect in a number of experimental models (Ip *et al.,* 1992; Liu *et al.,* 1992; Reuter *et al.,* 1996; Pedraza-Chaverri *et al.,* 2000; Thabrew *et al.,* 2000; Gedik *et al.,* 2005; Sener *et al.,* 2005; Pal *et al.,* 2006). The purpose of this study was to examine the modulation of the enzyme systems which are responsible for the metabolic activation/detoxifying of chemical carcinogens in the cell and the antioxidant status (Kellen 1999; Nishino *et al.,* 2005).

Consumption of aqueous extracts of *A. sativum, T. violacea* and *T. alliacea* as sole drinking fluid did not have any adverse effect on the body weight and relative liver weights of rats aside from *A. sativum* (1% w/v) which significantly increased the body weights compared to controls and the other garlic treated rats. Several other studies also reported similar findings with regards to *A. sativum* or its compounds such as DAS and DADS with no significant reduction or increase in dietary intakes and body weight of experimental animals (Le Bon *et al.,* 1997; Schaffer *et al.,* 1997; Pedraza-Chaverri *et al.,* 1998). Also, no adverse effect was noticed in the liver and kidneys of the rats treated with garlic. The liver function enzymes showed no adverse effects with consumption of the aqueous extracts of the various garlic species in this study. The observed maintenance and/or decrease in these enzyme levels suggests that these aqueous garlic extracts may preserve the structural integrity of the tissues. The creatinine levels, as marker for kidney function, were

also not altered compared to the control rats. Subsequently, total plasma iron was similarly affected with no significant differences compared to control rats irrespective of the difference in phenolic composition and uptake. Therefore no interference with iron uptake was seen in the present study. It is important to note the importance of iron in several metabolic disorders as cardiovascular (Alpert 2004), neurodegenerative diseases (Castellani *et al.,* 2007) infection (Bullen *et al.,* 2005) and cancers (Boult *et al.,* 2008). The maintenance of iron in a strict narrow range is therefore crucial (Fleming and Sly 2002) and also show no evidence for iron catalyzed lipid peroxidation (Saravanan and Prakash 2004).

The antioxidant potential of the two African wild garlic species and *A. sativum* was evaluated by a battery of assays, as a single method is not recommended due to the complex nature of their phytochemical composition. Oxygen radical absorbance capacity (ORAC) is one of several analyses that measure the total antioxidant activity of foods and other chemicals *in vitro.* In the present study the treatment of rats with aqueous extracts of the wild garlic resulted in increased ORAC values compared to controls. A significant ($P<0.05$) increase was shown with rats consuming aqueous extracts of *T. violacea* (1%, 2% w/v) and *T. alliacea* (2% w/v). None of the other extracts resulted in a significant increased ORAC capacity, although showing a tendency to increase the ORAC capacity. Under the present experimental conditions, it is difficult to conclude whether a relationship exist between the total phenolic intake and the increased hepatic ORAC levels. Rats exhibiting the highest phenolic intake per day did not necessarily also exhibit the highest hepatic ORAC levels. It seems that the type of phenolic compound as well as possible synergistic effects could play an important role here. The high soluble solids obtained for each plant (Table 3.2) could also account for their organosulphur compounds contributing to the antioxidant activity (Bozin *et al.,* 2008) of the plant species.

The products of lipid peroxidation from the polyunsaturated fatty acids, which include malondialdehyde, have been repeatedly regarded as a cancer promotive substances (Vaca *et al.,* 1988). Additionally, it is also evident that the TBARS contents, an important marker for oxidative stress, appeared to be partly affected by GSH or GSH-dependent detoxifying enzymes (Thomas and Girotti 1989). It has been reported in some studies that the organosulphur compounds from garlic or garlic extracts led to the reduction of TBARS contents (Takada *et al.,* 1994; Balasenthil *et al.,* 2000). However, in our study the TBARS content was increased in

certain garlic-fed rats. The *T. violacea* (1% w/v and 2% w/v), and *A. sativum* (1% w/v) treatment of rats caused a significant increase in the TBARS level. In contrast, the conjugated dienes in the liver of rats treated with garlic showed a marginal (non-significant) reduction. It should be kept in mind that the TBA test is non-specific for MDA and other compounds present in the cells and plant extracts could interfere, distorting the results. Results from the present study could imply, the treatment of garlic at these concentrations, could make these cells more susceptible to undergo lipid peroxidation and therefore cellular damage. However, another study by (Nahdi *et al.,* 2010) showed pre-treatment of rats with garlic (1g/kg) for 5 weeks to reduce the MDA levels in the liver and colon.

An important defence mechanism to protect cells against free radicals is seen in the form of GSH (Awad and Bradford 2006). Supporting reports suggest that its presence in excess *in vivo* could assist in scavenging the electrophilic moieties formed by xenobiotics resulting in conjugation to less toxic products (Younes and Siegers 1980). The present study showed that *T. alliacea* (1%, 2% w/v) and *A. sativum* (1% w/v) significantly increased the hepatic GSH content. *A. sativum* also caused a significant increase in hepatic GSSG content. A subsequent significant increase was noticed for the GSH/GSSG ratio for *T. alliacea* (1% w/v). This increase in GSH observed for the respective garlic treatments could also be as a result of the modulation of several enzyme systems. Therefore, by maintaining the intracellular GSH levels, the treatment of rats with aqueous extracts of garlic can prevent the most damaging effects of oxidative stress. In addition, the significant increase in GSH/GSSG ratio in the liver of the *T. alliacea* treated rats, may be indicative of an increased antioxidant capacity in the cell or a possible reduced oxidative stress. This could translate into a reduced susceptibility to oxidative damage. Our findings are in agreement with similar increases observed for GSH content in both bovine pulmonary artery endothelial cells after 24 hr in culture with aqueous garlic extracts (Geng and Lau 1997) and in human prostate carcinoma cells exposed for 3 h to *S*-allyl cysteine and *S*-allylmercaptocysteinein culture (Pinto *et al.,* 1997). Stout and Becker (1986) reported that by inducing the GSH content it could be a defence system against the increase of oxidative stress, and by supplementing GSH has shown the inhibitory effect on malondialdehyde formation (Mohamed *et al.,* 2000). Another study reported that in rats treated with N-methyl-NV-nitro-N-nitrosoguanidine, aqueous garlic extract (0.25 g/kg body weight) caused an increase in GSH level (Arivazhagan *et al.,* 2000). GSH has the ability to bind to a potential carcinogen metabolite, with subsequent excretion from the host system.

Therefore, an increase in GSH and GSH/GSSG ratio by the aqueous extract of garlic could partly protect the cells from active carcinogens.

An antioxidant defence system is an important tool to create a balance between the production of oxidants and the scavenging of these oxidants by antioxidants is an important tool that determines the extent of lipid peroxidation. Therefore another important responsibility to protect cells against oxygen mediated toxicity relies on glutathione peroxidase/reductase redox cycle enzymes, CAT and SOD (Cross *et al.,* 1987; Reed 1990). Chronic garlic intake has been shown to significantly decrease lipid peroxidation and at the same time increase endogenous antioxidants and enzymes, such as SOD, CAT, GSH and GPX (Banerjee *et al.,* 2002). Superoxide dismutase plays a very important role in scavenging of superoxide anion, an initial free radical among the oxygen radicals. Catalase averts oxidative hazard by catalyzing the formation of water and oxygen from hydrogen peroxide. In the present study, the activities of SOD, CAT were increased by *T. alliacea* (1%, 2% w/v) and *A. sativum* (1% w/v), respectively. Therefore, oxidative stress-induced tissue damage can be prevented by assisting the balance towards a lower oxidative status.

Many chemopreventive agents can also apply their action via the induction of phase II detoxifying enzymes (Shureiqi *et al.,* 2000). The fact that phase II enzymes are inducible during carcinogenesis, of importance are their expressions which are regarded as significant determinants of cancer susceptibility and reliable biomarkers of tumorigenesis (Hu *et al.,* 1997). The GST and UDP-GT enzymatic reactions are identified as important in the detoxification process of many xenobiotics (Yao *et al.,* 2012). Feeding of garlic in the present study has increased the enzyme GST-μ while no adverse effect was noticed with GST-α. The activity of GST-μ was significantly increased by *T. violacea* (2% w/v) and *T. alliacea* (1% w/v) while a marginal enhancement was noticed with *T. alliacea* (2% w/v) and *A. sativum* (1% w/v). The high specificity of GST for glutathione provides an enzyme-bound glutathione in the liver (Siess *et al.,* 2000). Many reactive chemicals normally categorised as hydrophilic electrophiles are successfully sequestered by GSTs. The increased stimulation of GST activity exhibited an increased ability for detoxification of some carcinogens (Stavroc 1994; Kondraganti *et al.,* 2008; Yao *et al.,* 2012). Furthermore, several studies have shown commercial garlic or its compounds to increase GST activity in some tissues, such as liver and small intestine (Maurya and Singh 1991; Hatono *et al.,* 1996; Ip and Lisk 1997).

In the current study, a similar trend could be seen in the UDP-GT activity. A significant increase ($P<0.05$) in the UDP-GT activity was evident subsequent to treatment of the rats with *A. sativum* (2% w/v) and *T. alliacea* (1% w/v). In contrast, the activity of UDP-GT was significantly reduced by *T. violacea* at 2 % (w/v). Glucuronidation of drugs and xenobiotics by UDP-GT enzymes results into more hydrophilic compounds that are more easily excreted (Ouzzine *et al.,* 2003). Effectively, glucuronidation reactions catalysed by UDP-GT are accountable for approximately 35 % of all drugs metabolized by phase II enzymes (Evans and Relling 1999). Thus, incorporation of garlic at these levels in the diet elicited a beneficial effect based on phase II enzymes. It can be confirmed that the two wild garlic species, specifically garlic extracts have enhanced the ability of the animal to detoxify potential chemical carcinogens, although the effects are variable and multidimensional.

The presence of organosulfur compounds and phenolic compounds in garlic extract can serve as an additional source of available electrons for the removal of electrophiles. Therefore, the net effect of the administration of organosulfur compounds in garlic is to protect GSH pools, induce antioxidant enzymes and to increase phase II detoxifying enzymes. We have shown in our study that cells pre-treated with aqueous garlic extracts showed a significant elevation in GSH levels, induction of GST-μ and UDP-GT and modulation of CAT and SOD. This was similar in other studies. Sener *et al* (2003) reported that aqueous garlic extracts in one study using rats as a model, was shown to restore GSH levels during thermal injury. The presence of specific sulfur and/or phenolic compounds in garlic could not alone be accountable for their antioxidant activity. This phenomenon is more likely the result of a synergistic relationship of different compounds. Findings from the present study show that the garlic extracts have therefore some useful biological properties, as indicated by the significant changes observed. Further studies are required for the selection of appropriate dose, duration of treatment and their possible effects on chemoprevention against other cancer. Phytochemical analyses, of the garlic extracts used in this study, did not fall within the scope of this study, but future studies will focus on this important aspect, to assist in the elucidation of possible mechanisms involved

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CHAPTER 4

Chemoprotective properties of *Allium sativum*, *Tulbaghia violacea* and *Tulbaghia alliacea* against cancer promotion induced by culture material of *Fusarium verticillioides* MRC 826 in rat liver

4.1 Abstract

This study was conducted to examine chemoprotective properties of commercially available garlic species, *Allium sativum* and South African wild garlic species, *Tulbaghia violacea* and *T. alliacea* at different levels on preneoplastic foci formation promoted by culture material of *Fusarium verticillioides* MRC 826 (CMF) in rat liver using diethylnitrosamine (DEN) as cancer initiator. Clinical chemical parameters linked to liver and kidney damage were significantly enhanced by DEN-CMF treatment while the levels were mostly reduced by various garlic treatments. Activities of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) status were differently affected by the various garlic treatments. Glutathione levels were decreased by *T. alliacea* and *A. sativum*. The glutathione-S-transferase placental form positive (GST-P$^+$) foci were significantly (P<0.05) promoted by the DEN-CMF treated rats while a significant (P<0.05) reduction occurred with aqueous garlic extract preparations of *T. violacea* (2%), *T. alliacea* (2%) as well as *A. sativum* (1% and 2%). Glutathione S-Transferase-α (GST-α) enzymes were found to be significantly increased in all garlic-treatment groups compared to the DEN-CMF control rats. The present study indicates that *T. alliaceae* (2 % w/v) and *A. sativum* (1% w/v) treatments not only suppressed GST-P$^+$ foci formation but also modulated phase II detoxification enzymes and in the case of *T. alliaceae* (2 % w/v) other antioxidant enzymes (i.e. SOD) in support of the reduced GST-P$^+$ foci formation. It is also important to acknowledge that other possible mechanisms of protection due to the organosulfur compounds in garlic may also be involved in reducing GST-P$^+$ foci formation. It can therefore be concluded that garlic treatments increased the inhibition of cancer promotion effected by the CMF (250 mg fumonisin/kg) diet which therefore imply that the dose dependent chemopreventive properties of wild garlic and commercial garlic could be further evaluated.

4.2 Introduction

Garlic (*Allium sativum*) is a member of the *Alliaceae* family and has been considered as one of the most important plants used for decades by the Egyptians in therapeutic formulas for the treatment of a variety of diseases. Garlic has also been utilised as a folk remedy for a variety of ailments and therefore widely consumed as a therapeutic medicinal agent. Its long-term usage has shown to have several positive effects on human health (Dausch and Nixon 1990; Shaarawy *et al.,* 2009). The wealth of scientific literature strongly supports the suggestion that garlic and its preparations can aid in preventing or reducing the risk of cardiovascular complications, stroke, and cancer (Fleischauer and Arab 2001; Banerjee *et al.,* 2003; Shaarawy *et al.,* 2009). Several reports indicate that the health/therapeutic properties of garlic can be ascribed to the bioactive water-and lipid-soluble organosulfur compounds (OSC) and phenolic compounds (Ichikawa *et al.,* 2003; Tepe *et al.,* 2005), but many other constituents (i.e. essential oil, flavonoids, anthocyanins, lectins, prostaglandins, fructan, pectin, adenosine, vitamins B1, 2, 6, C, E, biotin, nicotinic acid, fatty acids, glycolipids, phospholipids and essential amino acids) have been identified in garlic that could also contribute to the health benefits, but this still needs to be elucidated (Bozin *et al.,* 2008). Garlic contains water-soluble allyl amino acid derivatives, which account for most of its organosulfur content, lipid-soluble allylsulfides (allicin), flavonoids, saponins, and essential macro- and micronutrients that demonstrate antioxidant activity (Amagase *et al.,* 1996; Omurtag *et al.,* 2005; Chowdhury *et al.,* 2008). Other antioxidants in garlic include phenolic compounds, notably alexin, whose phenolic hydroxyl group confers antioxidant activity, *N*-fructosyl glutamate, *N*-fructosyl arginine, and selenium (Ide and Lau 1997). Furthermore, trace elements such as germanium (normalizer and immunostimulant) and selenium (for optimal function of the antioxidant enzyme glutathione peroxidase) were also shown to be present in garlic (Ross 2003). Adding to the complex chemical nature of garlic is the number of additional compounds produced in the plant by the aging process. The characteristic odor of garlic are caused by allicin (allyl-2-propene thiosulfinate or diallylthiosulfinate) and other oil-soluble sulphur components (Amagase *et al.,* 2001).

Based on 19 human studies, the relationship between the intake of garlic and/or garlic constituents and a reduced incidence of certain cancers i.e. stomach, colon, breast, rectal, lung and endometrial has been consistently supported (Fleischauer and Arab 2001; Kim and Kwon 2009). Several animal models have also reported on

the modulatory effects garlic extracts and/or individual garlic compounds have on liver, kidney, mammary, lung and skin tumourigenesis (Tsai *et al.,* 2012). Cancer can be seen as a disease of complex etiology with three distinct phases identified i.e; initiation, promotion and progression during the transformation of normal cells into cancerous cells and ultimately malignant tumours. According to the World Health Organisation (WHO), worldwide lung, stomach, liver, colon and breast cancer cause the most cancer deaths each year [http://www.who.int/mediacentre/factsheets/fs297/en/ (7 May 2012)]. Liver cancer is one of the top twenty causes of death [http://www.worldlifeexpectancy.com/south-africa-liver-cancer (7 May 2012)] and accounting for 6% of all cancers in South Africa [http://www.mrc.ac.za/bod/faqcancer.htm(7 May 2012)]. Thirty percent of these cancers are modifiable by our dietary behaviour and lifestyle, thus the development of alternative therapies to prevent/modify this disease seems reasonable. Plants have shown to possess a widespread variety of compounds, some of which are powerful modifiers of chemical carcinogenesis. As mentioned previously, garlic extracts and garlic-related organosulphur molecules have shown to modulate the development of certain cancers with possible mechanisms including inhibition of cell proliferation, inducing apoptosis and cell cycle arrest (Shukla and Kalra 2007; Tsai *et al.,* 2012). Certain organosulphur compounds also i.e. S-allyl cysteine sulfoxide, have also shown to exert antioxidant properties through their ability to inhibit lipid peroxidation, protect LDL cholesterol againts oxidative damage, protect DNA against free radical-mediated damage and mutations and reduce ischemic/reperfusion damage (Kim *et al.,* 2001; Shaarawy *et al.,* 2009). Stimulation of glutathione-peroxidase activity (Rahman 2003) by garlic oil also resulted in the modulation of the levels of lipid peroxidation. In addition, the formations of reactive oxygen species (ROS) such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) have been suppressed by garlic extracts by increasing the activity of important antioxidant enzymes i.e. superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Borek 2001). A common flavour compound found in garlic, diallylsulfide (DAS), has been found to play pivotal role in the inhibition of chemically induced carcinogenicity and cytotoxicity in various animal models (Hammons *et al.,* 1999). Diallylsulfide showed evidence of modulating phase II drug metabolizing enzymes i.e., epoxide hydrolase, quinine reductase, glutathione S-transferase, arylamine N-acetyltransferase and UDP-glucuronosyltransferase (Guyonnet *et al.,* 1999).

Traditionally, two indigenous South African wild garlic species, *Tulbaghia violacea* and *T. alliacea,* are utilised as remedies for a variety of infections and ailments (Van Wyk 1997; MacDonald *et al.,* 2004; Van Wyk *et al.,* 2009). *T. violacea* has been used for medicinal purposes, especially in the Eastern Cape and KwaZulu Natal regions of South Africa for the treatment of colds and fever, asthma, gastrointestinal ailments and tuberculosis, while *T. alliacea* as a remedy for fever, fits, rheumatism and paralysis (Van Wyk 1997). Very few studies are available on the biological activities of *T. violacea* (Bungu *et al.,* 2006; Raji *et al.,* 2012). Bioactivity of compounds isolated from the leaves of *T. violacea* includes antibacterial activity (Burton 1990), while more recently, *T. violacea* and *T. alliacea* were investigated for its antifungal activity against *Candida albicans* and important plant fungal pathogen (Motsei *et al.,* 2003; Thamburan *et al.,* 2006). Water extracts and specific compounds isolated from *T. violacea* have also been shown to induce apoptotic cell death in cancer cells (Lyantagaye and Rees 2003). Important components of *T. violacea* comprise several odour forming compounds (Kubec *et al.,* 2002) and bioflavonoids such as kaempferol and quercitin (Hutchings *et al.,* 1996). Allinase and several sulphur compounds have already been isolated from *T. violacea* (Burton 1990; Kubec *et al.,* 2002). Since *Tulbaghia* belong to the same family and exhibit similar characteristic sulphur smell, it has also been postulated that the plant have similar biological activities and secondary metabolites as commercial garlic (Van Wyk *et al.,* 1997; Van Wyk and Gericke 2000; Bungu *et al.,* 2006). These observations also indicate that there are some similarities between *T. violacea* and commercial garlic and therefore it might be possible that these plants have similar active compounds (Jacobsen *et al.,* 1968; Jansen *et al.,* 1989). The extensive consumption of *T. violacea*, however, has been linked to undesirable symptoms namely inflammation, abdominal pain and gastroenteritis (Kubec *et al.,* 2002). Based on these findings, *T. violacea* is shown to have immunostimulation and anti-inflammatory properties (Masika and Afolayan 2003; Bruck *et al.,* 2005). The *in vivo* effects of *T. violacea* on blood pressure in a salt-sensitive rat model showed a reduction in systemic arterial blood pressure in the Dahl rat by decreasing renal angiotensin II subtype 1 (AT1) receptor gene expression and hence modulating sodium and water homeostasis (Mackraj *et al.,* 2008).

Maize (*Zea mays*, L) is regarded as a dietary staple in sub-Saharan Africa (Van der Westhuizen *et al.,* 2011). Several fungi are associated with maize during pre-and postharvest periods, of which the genus *Fusarium* contains important toxigenic species. These species include *Fusarium verticillioides* (Sacc) Nirenberg (previously

known as *Fusarium moniliforme* Sheldon) and *F. proliferatum*, producers of fumonisin B (FB) mycotoxins in maize (Rheeder *et al.,* 2002). In South Africa, the former Transkei region of the Eastern Cape Province (EC), home-grown maize has been reported to be contaminated with high levels of fumonisin and therefore poses an important health risk (Shephard *et al.,* 2007). The contamination of maize with fumonisins has attracted much attention due to the associated human illnesses which include oesophageal and liver cancer in humans (Sun *et al.,* 2007). The co-occurrence of fumonisin with other natural occurring cancer promoting and initiating food contaminants such as aflatoxin B, could pose an increased risk for cancer (Gelderblom *et al.,* 2002). The toxicological and pathological effects of fumonisin B (FB), which are naturally produced by *F. verticillioides* on maize, have been extensively studied in laboratory animals. Fumonisin is shown to be a potent liver cancer promoters (Gelderblom *et al.,* 1988) which induced liver nodules in a short-term carcinogenesis model (Gelderblom *et al.,* 2002; Marnewick *et al.,* 2009) reported on the modulation of the oxidative stress during FB_1-induced cancer promotion by different herbal teas. Important events such as cell proliferation and apoptosis are critically influenced by reactive oxygen species and their role in carcinogenesis (Klaunig and Kamendulis 2004). It is important to note that the strength of chemoprevention can greatly be influenced by a number of natural compounds. Furthermore, pure compounds do not always behave in the same way as in natural plant products because of important synergistic relationships among these compounds (Meckes *et al.,* 1993; Liu 2004). For this reason we evaluated the modulatory activity of crude aqueous extracts (used traditionally) of three garlic species, *A. sativum, T. violacea* and *T. alliacea* species on DEN-initiated, FB-promoted liver carcinogenesis.

4.3 Matrerials and Methods

4.3.1 Chemicals and diets

Butylated hydroxytoluene/Ethanol; butanol; perchloric acid (PCA); triton X-100 and dimethyl sulfoxide (DMSO) was purchased from Merck (Pty) Ltd (Cape Town, South Africa). Sodium pentobarbital; KCl; thiobarbituric acid (TBA); NaCl; fluorescein; 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH); trichloro acetic acid (TCA); ethylenediaminetetraacetic acid (EDTA); 3 mM 1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate (M2VP); glutathione reductase; 5,5'-dithiobis-2-nitrobenzoic acid (DTNB); nicotinamide adenine dinucleotide phosphate (NADPH); 1-chloro-2,4-dinitrobenzene (CDNB); 3,4-dichloro-nitrobenzene (DCNB) *p*-

nitrophenol; uridine diphosphate-glucuronic acid; Tris-HCl; $MgCl_2$; NaOH; diethylnitrosamine (DEN) was purchased from Sigma (Sigma–Aldrich, Cape Town, South Africa). The fumonisin-containing diet for the rats was prepared by incorporating lyophilized culture material of *F. verticillioides* MRC 826 (CMF) batch p131 at a level of 5% (250 mg fumonisin/kg) into the rat mash prepared from rat cubes (Epol Ltd., Johannesburg, South Africa). The diet was stored under nitrogen at 4°C.

4.3.2 Plant material and preparation

T. violacea (wild garlic) used in the *in vitro* tests were obtained from Cape Peninsula University of Technology's Nursery garden, Cape Town while *T. alliacea* (social garlic) was obtained from herb traders, Cape Town, South Africa. *A. sativum* (commercial garlic) was bought from a retail outlet in Cape Town. The bulbs of the different garlic species were cut into smaller pieces. Freshly boiled tap water was added to the plant material at concentrations of 1 g/100 ml and at 2 g/100 ml for each plant, respectively and homogenised using a Waring blender. The aqueous extracts were allowed to cool to room temperature. After cooling, it was filtered (Whatman no. 4) and dispensed into water bottles of the experimental rats. Aqueous garlic extracts were freshly prepared every second day.

4.3.3 Treatment of animals

The study was approved by the Ethics Committee for Research on Animals (ECRA) of the Medical Research Council (MRC, South Africa). The experiments were conducted in agreement with the laws and regulations controlling experiments on live animals in South Africa. Seventy male Fischer rats (150–170 g), obtained from the Primate Unit, MRC (Tygerberg, South Africa), were housed separately in wired top and bottom cages fitted with Perspex™ houses and kept in a controlled environment of 23-24 °C. Humidity was kept at 50 % and a 12 h light/dark cycle was maintained. Rats were randomly divided into ten treatment groups of seven each. Initiation was effected by a single dose of diethylnitrosamine (DEN; 200 mg/kg body weight, i.p.). During this 1 week period of initiation, rats had free access to tap water and Epol rat mash. The various aqueous garlic extracts were given to the rats as their sole source of drinking fluid one week after initiation until the end of the experiment. The control groups received tap water. Promotion started three weeks after initiation by feeding the relevant rats with the study diet (250 mg FB/kg diet) for 21 days (Figure 1). The positive control rats received DEN initiation and CMF promotion treatments (DEN-CMF) in the absence of the plant extracts. Other control

groups received either DEN or DMSO (carrier solvent) treatments with the normal mash diet and tap water as drinking fluid. All the rats were averaged fed during the CMF promotion treatment period, according to the daily feed intake of the positive (DEN-CMF) control group. The body weights of the rats were monitored on a weekly basis. The average weekly feed and CMF intake was calculated using the mean daily rat feed intake as a function of the body weight and expressed as gram feed. At termination, rats were fasted (16 hr), euthanized by i.p. injection of sodium pentobarbital (0.15 ml /100g bw). Blood was collected from the abdominal aorta and serum samples prepared for clinical chemical analyses. Livers were excised, weighed and sections processed in buffered formalin for histological examination. The remaining liver tissue were frozen immediately in liquid nitrogen and stored at -80°C for biochemical analyses.

4.3.4 Total polyphenolic and soluble solid analyses

The soluble solid content of the different garlic extracts was determined by drying 1ml aliquots of the respective garlic extracts at 90 °C for 12 h. The phenolic content of the garlic extracts was determined using the Folin- Ciocalteu according to Waterhouse (2005) with gallic acid as standard. Gallic acid standard was added to a clear 96 well plate, while the garlic samples were added in triplicate. The absorbance was read at 765 nm using a microplate reader (Multiskan Spectrum Thermo Electron Corporation).

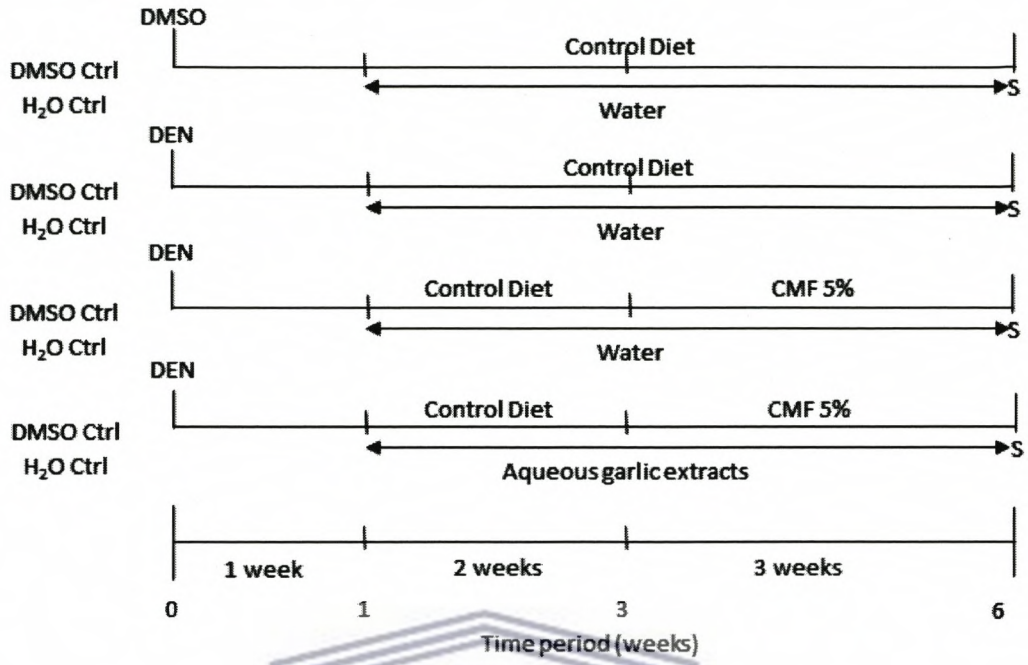

Figure 4.1. Schematic diagram illustrating the experimental regimens utilized to investigate the modulating properties of the different garlic treatments on cancer promotion by CMF in rat liver. DEN = diethylnitrosamine (200 mg/kg body weight; i.p., single dose); CMF (250 mg fumonisin/kg diet for 3 weeks) as promotion regimen; s = sacrifice animals; ctrl = control.

4.3.5 Clinical chemistry

Measurement of the clinical biochemical parameters including serum creatinine, aspartate transaminase (AST), alanine transaminase (ALT), total iron and alkaline phosphatase (ALP) were performed on a Technicon RA 1000 automated analyzer. The serum levels of AST, ALT and ALP were determined to assess liver function and tissue damage, while creatinine levels were determined as a marker for kidney function.

4.3.6 Oxidative stress parameters

4.3.6.1 *Lipid peroxidation*

When polyunsaturated fatty acids of membrane phospholipids go through peroxidation, malondialdehyde (MDA) is produced. Lipid peroxidation was assessed by determining MDA concentration [measured as thiobarbituric acid reacting substances (TBARS)] in liver homogenates. The modified method of Esterbauer

and Cheeseman (1990) were used to determine the level of TBARS. Sub samples of the excised livers (0.1 g) were homogenized on ice in 500 µl of KCl (0.15M) and 0.01 M phosphate buffer (pH 7.4). The supernatant (50 µl) was mixed with 6.25 µl (0.01%) of butylated hydroxytoluene/ethanol and 50 µl ortho-phosphoric acid. The resultant homogenate was combined with 6.25µl of 0.67% thiobarbituric acid (TBA) solution and incubated at 90 °C for 450 min. The mixture was allowed to cool to room temperature and mixed with 1000 µl butanol and 50 µl NaCl (saturated solution). The samples were centrifuged (12000 *g*) for 2 minute at 4 °C. The absorbance was measured at 532 nm using a Multiscan spectrophotometer. Results were expressed as nmole MDA per mg protein using a molar extinction coefficient of 1.56 $x10^5$ M^{-1} cm^{-1} for MDA (Esterbauer and Cheeseman, 1990). Lipid peroxidation was also assessed by measurement of conjugated dienes (CDs). The method of Recknagel and Glende (1984) was used for the estimation of CDs in liver homogenates. A 2:1 solution of chloroform and methanol were prepared of which 400 µl was added to 50 µl of tissue sample in an eppendorf tube. The mixture was vortexed for 60 seconds and centrifuged at 8000 *g* for 15 min at 4 °C. The top aqueous layer was removed and discarded. The lipid layer was removed by plunging a glass Pasteur pipette along the wall of the tube through the protein layer and transferred into a clean eppendorf tube. The sample was dried under liquid nitrogen for 30 min. One ml cyclohexane was then added and the solution vortexed for 60 seconds. The absorbance was read at 234 nm using a Multiscan spectrophotometer after transferring 300 µl into a clear 96-well plate. The samples were done in duplicate. The CDs were expressed as µmole/g liver using a molar extinction coefficient of 26550 M^{-1} cm^{-1}.

4.3.6.2 *Oxygen radical absorbance capacity (ORAC)*

Frozen liver samples (0.1 g) were homogenized in 500 µl phosphate buffer (75 mM, pH 7.0) using a Potter-Elvehjem Teflon pestle and glass tube tissue homogeniser (10 strokes) and centrifuged at 12000 *g* for 15 min at 4 °C. The supernatant was deproteinised with an equal volume of (0.25 M) perchloric acid (PCA) and centrifuged at 14000 *g* for 15 min. The ORAC assay was conducted according to the method of (Cao and Prior 1999) in a black 96 well microtiter plate using a Fluoroscan Ascent (Thermo Electron Corporation) fluorescence spectrophotometer. The reaction consisted of 12 µl of diluted sample (1:4) and 138 µl fluorescein (95 nM), which was used as a target for free radical attack. The addition of 50 µl (92 mM) 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) initiated the reaction. The fluorescence (emission 530 nm, excitation 485 nm) was recorded every 5 min

until the reading had declined to less than 5 % of the initial reading. The ORAC values were calculated using a regression equation ($Y = a + bx + cx^2$) between Trolox concentration (Y) (uM) and the nett area under the fluorescence decay curve (x) and expressed as µmole/l Trolox equivalents/g wet liver weight.

4.3.6.3 *Glutathione analysis*

The determination of total glutathione (GSH and GSSG) was based on the modified method of Tietze (1969). Liver samples were homogenized (1:10) on ice in 15% (w/v) trichloro acetic acid (TCA) containing 1 mM ethylenediaminetetraacetic acid (EDTA) for GSH determination and in 6% (v/v) perchloric acid (PCA) containing freshly prepared 3 mM 1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate (M2VP) and 1 mM EDTA for GSSG determination. After centrifugation at 10000 g for 10 min, 50 µl of supernatant was added to glutathione reductase (1U) and 0.3 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). The reaction was initiated by addition of 1.0 mM nicotinamide adenine dinucleotide phosphate (NADPH) to a final volume of 200 µl. The change in absorbance was monitored at 410 nm for 5 min and levels calculated using pure GSH and GSSG as standards. The GSH/GSSG ratio are calculated as Ratio = GSH ÷ GSSG.

4.3.7 Preparation of microsomal and cytosolic liver fractions

For the preparation of the subcellular fractions, 300 mg frozen liver samples was homogenised using a Potter Elvehjem tissue homogeniser in 900 µl of ice-cold KCl (0.15 M) solution for 10 seconds. Thereafter the homogenates were filtered using double layer cheesecloth and homogenized with a glass dounce (10 strokes) using a loose pestle. The homogenates were centrifuged at 9000 *g* for 10 min. The cytosolic and microsomal preparations were collected after centrifugation of the supernatant at 100 000 *g* for 1 hr at 4 °C. The microsome pellet was washed 10 times using a Pasteur pipette. The pellet was then resuspended in 1 ml ice cold 0.15 M KCl using a glass dounce and centrifuged at 100 000 *g* for 1 h, resuspended in 0.15 M KCl, and stored with the cytosolic fractions at -80 °C. All procedures were performed on ice. The protein determination for microsomal and cytosolic fractions were performed by the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

4.3.8 Phase II enzyme assays

4.3.8.1 *Glutathione S-Transferase (GST) assay*

The GST-α activity was measured according to the method of Habig *et al* (1974) with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. A reaction mixture of 0.1 M potassium phosphate buffer (pH 6.5), 200 mM GSH, and 100 mM CDNB was prepared. With the addition of 2 µl cytosol (10 mg protein/ml), the reaction was initiated. The absorbance was recorded at 340 nm for 3 min at 25 °C due to the formation of CDNB-GSH conjugates (Multiskan Spectrum, Thermo Electron Corporation). The specific activity (µmole/ml/min) was calculated using a millimolar extinction coefficient of 9.6 for CDNB-GSH conjugate. The activity of GST-µ was measured using 60 mM 3,4-dichloro-nitrobenzene (DCNB) as substrate (Habig *et al.,* 1974). The reaction mixture contained 0.1 M potassium phosphate buffer (pH7.5), 86.1 mM GSH, and 60 mM DCNB as substrate cytosolic protein (0.3 g/mL). Absorbance was measured at 344 nm for 3 min at 25 °C (Multiskan Spectrum, Thermo Electron Corporation). The specific activity was expressed as µmole/ml/min protein using a millimolar extinction coefficient of 8.5 for the DCNB-GSH conjugate.

4.3.8.2 *Uridine diphosphate-glucuronosyltransferase (UDP-GT) assay*

The activity of microsomal UDP-GT was determined spectrophotometrically using *p*-nitrophenol and UDP-glucuronic acid as substrates (Bock *et al.,* 1983). The incubation mixture consisted of liver microsomes (1 mg of protein/ml), activated with 0.25% (w/v) Triton X-100, and incubated with 50 µl (0.1 M) Tris-HCl (pH 7.4), 50 µl (50 mM) $MgCl_2$, and 50 µl (5 mM) *p*-nitrophenol for 2 min at 37 °C. The addition of 50 µl UDP-glucuronic acid (30 mM) initiated the reaction which was then terminated after 10 min by the addition of ice cold 500 µl (0.5 M) trichloro acetic acid (TCA) and kept on ice for 5 min. After centrifugation (3000 *g*) for 10 min, 100 µl (2 M) NaOH was added to 0.4 ml supernatant, and the absorbance was determined at 405 nm (Multiskan Spectrum, Thermo Electron Corporation). The specific activity was expressed as nmole/min/mg protein using the millimolar extinction coefficient of 18.1.

4.3.9 Activity of antioxidant enzymes

The superoxide dismutase (SOD) and catalase (CAT) activity was determined by the modified method of (Ellerby and Bredesen 2000), for a microplate reader (Multiskan Spectrum Thermo Electron Corporation). Catalase activity was determined spectrophotometrically at 240 nm by monitoring the decomposition of H_2O_2 and expressed as µmoles H_2O_2/min/µg protein. For both CAT and SOD determinations, tissue sample (200 mg) were homogenised with 2 ml phosphate buffer in a Potter-Elvehjam homogeniser for five strokes on ice. The homogenate

was sonicated for two 15 second bursts on ice and centrifuged for 10 min at 15 000 *g*, 4°C and stored at -80 °C. For the CAT assay, the stored samples were diluted with phosphate buffer to a concentration of 0.1 µg/µl protein. A 96-well plate was prepared by mixing 75 µl of H_2O_2 with 170 µl of phosphate buffer and 10 µl diluted homogenate (0.1 µg/µl) added and mixed. A linear absorbance at 240 nm decrease/min was read for at least 1 min in 15 second intervals. The activity was expressed as umole/min/mg protein using the millimolar extinction coefficient of 0.000394. For the SOD assay, the stored samples were diluted with SOD assay buffer to a protein concentration of 0.1 µg/µl protein. The samples (12 µl) were added in duplicate to a 96-well plate followed by 15 µl (1.6 mM) of 6-hydroxydopamine (6-HD). The diethylenetriaminepentaacetic acid (DETAPAC) (173 µl, 0.1 mM) solution was added last to ensure sufficient mixing with samples. The auto-oxidation of 6-HD was recorded at 490 nm for 4 min in 1 min intervals. SOD activity was expressed as the amount of protein (ng) required to produce a 50% inhibition of auto-oxidation of 6-hydroxydopamine.

4.3.10 GST-Pi immunohistochemical assay

Histochemical staining for the placental form of glutathione-S-transferase (GST-P^+) was conducted on dewaxed liver sections using a three-stage indirect strepavidin–biotin technique to identify GST-P^+ stained hepatocytes (Ogawa *et al.,* 1980). The enzyme altered foci were quantified microscopically (10X magnification), according to their number and size (internal diameter). The foci was grouped according to the following sizes, 0.04-0.5mm^2 (mini foci), 0.51-1.0mm^2, 1.1-1.5mm^2 and > 1.5mm^2. Foci were expressed as number of foci/mm^2 of the liver section, the area of which was determined by using NIS-Elements (Nikon) image analysis software. The relative amount of each focal size category was expressed as a % of the total GST-P^+ foci. The total GST-P^+ foci area were also determined.

4.3.11 Statistical analysis

All statistical analyses were carried out using the SPSS statistical package with the analysis of variance and Duncan's multiple range test. The statistical comparisons were carried out between DEN-CMF treated rats, DEN, DMSO as controls and various garlic treated groups. Differences were considered statistically significant at $P<0.05$.

4.4 Results

4.4.1 Garlic, phenolic intake and CMF intake profiles

During the second week of the experiment, a significant (P<0.05) reduction in feed intake was noticed in the positive control group (2.99 mg/100 g BW/day) as well as the garlic treated groups (between 2.03 and 3.81 mg/100 g BW/day) due to CMF-induced toxicity. No marked differences between garlic treated and control groups were recorded for garlic intake and tap water respectively, during the same period. During the fourth week, feed intake levels normalised and were similar to that recorded in the first week (6.21 mg/100 g BW/day). The average *T. violacea* and *T. alliacea* consumption for both concentrations (1%, 2% w/v) was significantly less (P<0.05) when compared to the commercially available *A. sativum* (Table 4.1). The lowest consumption of garlic was observed for *T. alliacea* (2% w/v) when all the garlic treatment groups were compared. The total phenolic intake per day was lowest for rats consuming *T. violacea* (1% and 2%) followed by *T. alliacea* (1% and 2% w/v) while rats treated with *A. sativum* (2% w/v) showed a significantly highest phenolic consumption per day compared to all garlic treated groups. There was no significant difference between the CMF intake profiles when comparing the different groups, except for rats consuming *A. sativum* (1% w/v). A significantly (P<0.05) lower intake was recorded when compared with the positive (DEN-CMF) control group (Table 4.1).

4.4.2 *Effect of body weight parameters*

The total body weight gain of the positive control group was significantly (P<0.05) reduced when compared to the DMSO control group, with a non-significant (P=0.217) reduction when compared to the DEN control group (Table 4.1). The various garlic treatments exhibited effects to the total body weight gains, with rats consuming *A. sativum* (2 % w/v) showing similar gains to that of the positive control group, while lower gains were observed for *T. violacea* (1% and 2% w/v), *T. alliacea* (1%, 2% w/v) and *A. sativum* (1% w/v) when compared to the positive (DEN-CMF) control group. Among the various garlic treatment groups, *A. sativum* (2% w/v) showed a significantly (P<0.05) higher total body weight gain when compared to *T. violacea* and *T. alliacea.* There were no significant differences in the relative liver weights of rats when considering all treatment and control groups.

4.4.3 *Clinical biochemical parameters*

The effect of the different aqueous garlic extracts on serum levels of AST, ALT, ALP creatinine and total iron are summarised in Table 4.2. The DEN treatment group exhibited no significant differences in AST, ALT and ALP serum levels compared to

the DMSO control group. The positive control group significantly (P<0.05) increased the serum levels of AST, ALT, ALP when compared to the DMSO and DEN control groups. None of the garlic extracts caused any further increase in the levels of AST, ALT and ALP. However, it was evident that *A. sativum* (2% w/v) resulted in a significantly reduction in the ALT and ALP serum levels when compared to the positive control group. Similarly, the serum ALP level was also significantly (P<0.05) reduced by *T. alliacea,* at both concentration levels, when compared to the positive control group. When comparing the garlic treatment groups, it was noticed that *T. violacea* and *A. sativum* significantly (P<0.05) increased the ALP levels compared to treatment with *T. alliacea*.

The DEN-CMF treatment resulted in a significant (P<0.05) increase in the serum creatinine level compared to the DMSO and DEN control groups. However, the DEN-CMF-induced increase of creatinine serum levels were significantly (P<0.05) reduced by all garlic treatments (Table 4.2). Similarly, the serum total iron levels were significantly (P<0.05) increased by the DEN-CMF treatment when compared to DMSO and DEN control treatment. None of the garlic treatments resulted in any significant further change when compared with the positive control group.

Table 4.1. Effect of DEN-CMF and various garlic treatments on the rat body weight parameters

Treatment	Ave garlic intake (ml/100 g BW/day)	total phenolic intake (mg gallic acid equivs/ day/100 g BW)	Ave CMF intake (mg/100 g BW/day)	Body weight gain (g)	Relative liver weight[A] (%)
DMSO	-	-	-	71.91 ± 15.12d	2.52 ± 0.22ab
DEN	-	-	-	48.92 ± 10.41c	2.52 ± 0.11ab
DEN-CMF	-	-	6.81 ± 0.81b	39.61 ± 12.81bc	2.71 ± 0.21ab
DEN-CMF-Tv 1%	16.91 ± 0.40b	2.10 ± 1.21a	6.04 ± 0.91ab	13.71 ± 9.21a	2.91 ± 0.22b
DEN- CMF -Tv 2%	17.82 ± 0.90bc	6.04 ± 0.12a	6.61 ± 0.61ab	27.21 ± 14.01ab	2.80 ± 0.12ab
DEN- CMF -Ta 1%	17.01 ± 1.51b	13.54 ± 0.35b	5.91 ± 0.92ab	20.02 ± 16.41a	2.91 ± 0.34ab
DEN- CMF -Ta 2%	13.82 ± 1.31a	20.37 ± 1.01bc	6.50 ± 1.32ab	12.71 ± 15.71a	2.34 ± 0.31a
DEN- CMF -As 1%	27.03 ± 1.32e	26.87 ± 1.21c	5.64 ± 0.73a	21.82 ± 14.42a	2.92 ± 0.34b
DEN- CMF -As 2%	29.11 ± 4.03f	38.41 ± 0.98d	5.84 ± 0.51ab	39.40 ± 10.81bc	2.74 ± 0.11ab

Values in columns represent the mean ± STD.
Abbreviations: Tv 1% = *Tulbaghia violacea* at 1% (w/v), Tv 2% = *T. violacea* at 2% (w/v), Ta 1% = *T. alliacea* at 1% (w/v), Ta 2% = *T. alliacea* at 2% (w/v), As 1% = *Allium sativum* at 1% (w/v) and As 2% = *Allium sativum* at 2% (w/v). DMSO = dimethyl sulfoxide, DEN = diethylnitrosamine, CMF = the fumonisin-containing diet. Values followed by the same letters do not differ significantly. When letters differ then $P < 0.05$.
[A] Relative liver weights equal liver weight/body weight x 100.
N = 7 rats per group.

Table 4.2. Effect of garlic treatments on selected serum biochemical parameters related to liver and kidney function

Treatment	AST (U/L)	ALT (U/L)	ALP (U/L)	Creatinine (µmole/L)	Total iron (µmole/L)
DMSO	111.31 ± 29a	75.01 ± 9a	68.91 ± 10b	65.31 ± 7ab	20.61 ± 13a
DEN	112.11 ± 22a	74.72 ± 10a	92.70 ± 14b	60.62 ± 7a	26.21 ± 18b
DEN-CMF	311.82 ± 49bc	311.82 ± 49cd	222.31 ± 17e	91.02 ± 7e	34.12 ± 17cd
DEN-CMF-Tv 1%	264.01 ± 36b	255.71 ± 40bc	200.51 ± 18cde	71.61 ± 10bc	34.32 ± 3cd
DEN-CMF-Tv 2%	286.61 ± 41bc	292.71 ± 69bcd	213.70 ± 22de	73.92 ± 3bc	32.10 ± 12cd
DEN-CMF-Ta 1%	283.12 ± 47b	274.60 ± 36bc	199.61 ± 14cd	72.42 ± 4bc	30.81 ± 9c
DEN-CMF-Ta 2%	283.03 ± 67bc	264.21 ± 45bc	185.71 ± 25c	77.34 ± 6cd	31.92 ± 23cd
DEN-CMF-As 1%	300.44 ± 40bc	267.60 ± 37bc	222.01 ± 30e	76.71 ± 6cd	35.21 ± 8d
DEN-CMF-As 2%	308.61 ± 52bc	250.31 ± 30b	195.62 ± 14cd	82.22 ± 3de	35.21 ± 14d

Values in columns represent the mean ± STD.
Abbreviations: Tv 1% = *Tulbaghia violacea* at 1% (w/v), Tv 2% = *T. violacea* at 2% (w/v), Ta 1% = *T. alliacea* at 1% (w/v), Ta 2% = *T. alliacea* at 2% (w/v), As 1% = *Allium sativum* at 1% (w/v) and As 2% = *Allium sativum* at 2% (w/v). DMSO = dimethyl sulfoxide, DEN = diethylnitrosamine, CMF = the fumonisin-containing diet.
Values followed by the same letters do not differ significantly. When letters differ then $P < 0.05$.
N = 7 rats per group.

Table 4.3.Effect of *Tulbaghia violacea*, *T. alliacea* and *Allium sativum* on total glutathione (GSH), oxidized glutathione (GSSG), the ratio GSH/GSSG, oxygen radical absorbance capacity (ORAC), oxidative enzyme parameters and lipid peroxidation in livers of rats

Treatment	ORAC (uM Trolox equivs/mg liver)	GSH (uM/mg protein)	GSSG (uM/mg protein)	GSH/GSSG ratio	CAT (umole/min/mg protein)	SOD U/ng protein	TBARS mmole/g liver	CD umole/g liver
DMSO	6.4 ± 1a	423.8 ± 154b	22.0 ± 4a	19.2 ± 6ab	15.0 ± 2a	51.2 ± 5bc	24.9 ± 8a	716.1 ± 50ab
DEN	6.5 ± 1a	442.9 ± 40b	22.6 ± 3a	19.6 ± 7ab	19.6 ± 3a	47.5 ± 4abc	22.5 ± 3a	947.7 ± 93d
DEN-CMF	7.0 ± 1a	577.1 ± 41c	21.1 ± 6a	27.3 ± 2bc	19.6 ± 2a	40.0 ± 7a	37.4 ± 5bc	862.3 ± 75bcd
DEN-CMF-Tv 1%	7.8 ± 1a	537.3 ± 46c	17.8 ± 5ab	29.8 ± 7c	18.9 ± 4a	58.7 ± 8c	56.3 ± 3d	690.2 ± 139a
DEN-CMF-Tv 2%	7.1 ± 4a	520.6 ± 71c	20.5 ± 0.5ab	25.4 ± 4bc	17.3 ± 2a	44.5 ± 9ab	45.0 ± 8cd	706.8 ± 121ab
DEN-CMF-Ta 1%	7.3 ± 1a	368.7 ± 33a	20.2 ± 5ab	18.2 ± 4a	18.1 ± 3a	52.1 ± 8bc	41.0 ± 7bcd	770.3 ± 98bc
DEN-CMF-Ta 2%	6.4 ± 1a	490.7 ± 34b	17.9 ± 2b	27.3 ± 4c	17.4 ± 1a	53.9 ± 5bc	39.4 ± 5bc	795.9 ± 69bcd
DEN-CMF-As 1%	7.4 ± 1a	465.2 ± 67b	19.2 ± 6ab	24.1 ± 10c	14.3 ± 2a	45.1 ± 6ab	43.6 ± 7bcd	792.3 ± 71bc
DEN-CMF-As 2%	6.4 ± 1a	463.5 ± 39b	21.5 ± 4ab	21.6 ± 4abc	16.5 ± 2a	35.8 ± 3a	42.2 ± 8bcd	836.3 ± 106bcd

Values in columns represent average of 6 values per group ± STD. Means followed by the same letter do not differ significantly, when letters differ then $P < 0.05$. CAT = catalase; SOD = superoxide dismutase.
Abbreviations: Tv 1% = *Tulbaghia violacea* at 1% (w/v), Tv 2% = *T. violacea* at 2% (w/v), Ta 1% = *T. alliacea* at 1% (w/v), Ta 2% = *T. alliacea* at 2% (w/v), As 1% = *Allium sativum* at 1% (w/v) and As 2% = *Allium sativum* at 2% (w/v). DMSO = dimethyl sulfoxide, DEN = diethylnitrosamine, CMF = the fumonisin-containing diet.

4.4.4 *Hepatic oxidative status*

4.4.4.1 *Oxygen radical absorbance capacity (ORAC)*

No significant differences were noticed in the hepatic ORAC status among the control groups. The various garlic treatments also did not results in any further change in the ORAC status when compared to the control groups (Table 4.3).

4.4.4.2 *Hepatic glutathione*

Total hepatic GSH levels were significantly (P<0.05) elevated in the positive control group when compared to the DMSO and DEN treated control groups (Table 4.3). When considering the different garlic treatments, *T. violacea* did not further enhance the GSH level when compared to the positive control group. However, a significant reduction in GSH levels were observed in rats consuming *T. alliacea* and *A. sativum* at both concentration levels, compared to the positive control group. Glutathione levels for rats consuming *T. alliacea* at 1% resulted in a significant (P<0.05) reduction when compared to the DEN-CMF treated control group and the other garlic treatment groups, respectively. Levels of GSSG were unaffected by the DEN-CMF treatment which resulted in a significant increase in GSH/GSSG ratio when compared to DMSO and DEN. The GSSG levels were marginally (P=0.524) decreased in liver of rats consuming *T. violacea* with a resultant increase in GSH/GSSG ratio, although not significant. A marginal (P=0.394) reduction was also noticed for rats consuming *A. sativum* (1%) when compared to DEN-CMF. Rats consuming *T. alliacea* (1% w/v) showed marginally (P=0.261) reduced GSSG hepatic levels, which contributed to a significantly (P<0.05) lower GSH/GSSG ratio compared to the positive control. None of the other garlic treatments resulted in a significant reduced GSH/GSSG ratio.

4.4.5 *Antioxidant enzyme activity*

DEN-CMF treatment caused no significant changes to CAT activity when compared to the DMSO and DEN control groups, while the various garlic treatments did not result in any further changes in CAT activity when compared to the positive control (Table 4.3). However, DEN-CMF treatment resulted in a significant (P<0.05) reduction in SOD activity when compared to the DMSO control group. Treatments with *T. violacea (1% w/v)* and *T. alliacea* (1,2% w/v) resulted in a significant (P<0.05) recovery in the DEN-CMF-induced decrease by increasing the SOD activity. No significant effect was noticed in SOD activity with the *T. violacea* (2% w/v) and *A. sativum* treatments when compared to the positive control.

4.4.6 *Lipid peroxidation*

The DEN-CMF treated rats showed a significant (P<0.05) increase in the hepatic TBARS levels (Table 4.3) when compared to the DMSO and DEN treated control groups. The rats consuming *T. violacea* (1% w/v) showed a further increase in the hepatic TBARS levels when compared to the DEN-CMF control group. Consumption of *T. violacea* (2%), *T. alliacea* (1%, 2%), as well as *A. sativum* (1%, 2%), showed no further significant (P>0.05) changes in the DEN-CMF-induced lipid peroxidation. When considering the levels of hepatic CDs, rats treated with DEN-CMF exhibited significantly (P<0.05) higher CD levels when compared to the DMSO treated rats. When considering the various garlic treatments, rats consuming *T. violacea* (1%, 2%) showed a significantly (P<0.05) lowered hepatic CD level when compared to the positive control group, while none of the other garlic treatments had any further effect on the CD levels .

4.4.7 *Cytosolic glutathione S-transferases (GST) and microsomal UDP-glucuronosyl transferases (UDP-GT)*

The DEN-CMF treated rats showed a significant (P<0.05) increase in the activity of GST-μ (mu), GST-α (alpha) and UDP-GT compared to the DMSO and DEN control groups (Table 4.4). All garlic extracts, except for *A. sativum* (2% w/v), resulted in a significant (P<0.05) recovery of the DEN-CMF-induced increase in GST-μ activity when compared to the positive control group, while GST-α activity was significantly (P<0.05) further enhanced by all the garlic extracts, except for *A. sativum* (2%) that was similar to the positive control group. No significant differences were noticed among the different garlic treated groups. When considering the UDP-GT activity, a significant (P<0.05) increase was evident following the DEN-CMF treatment when compared to the DEN and DMSO control treatments. However, no further significant differences were observed with the various garlic treatments, with *T. alliacea* (2% w/v) only marginally (P=0.056) increasing the UDP-GT activity when compared to the DEN-CMF treated group.

4.4.8 *Modulation of GST-P$^+$ foci*

No GST-P$^+$ foci were detected in the DMSO control group, while the number of hepatic GST-P$^+$ foci were significantly (P<0.05) increased by the DEN-CMF treatment when compared to the DEN control group. This was evident in the significant (P<0.05) increased number of foci in all the different size categories as well as the total area of foci, respectively (Table 4.5). In the smallest foci category (0.04-0.5 mm^2), *T. alliacea* (2%) and *A. sativum* (1%) significantly (P<0.05) reduced the number of foci compared to the DEN-CMF treated group, while the other garlic

treatments did not cause any further increase in these foci. No further increase or reduction in the number of the 0.51-1 mm^2 foci size category was observed after treatment with *T. violacea, T. alliacea* and *A. sativum* when compared with the DEN-CMF treated group. In the 1.1-1.5 mm^2 foci size category, no further increases were observed for the various garlic treatments when compared to the DEN-CMF treatment. In the largest GST-P^+ foci category (>1.5 mm^2), *T. violacea* (1%) significantly (P<0.05) increased the number of hepatic GST-P^+ foci, while all the other remaining garlic treated groups showed a marked, although not significant reduction in the number of GST-P^+ foci for this size category, when compared to the positive control group. When considering the total number of GST-P^+ foci, *T. alliacea* (2%) and *A. sativum* (1%) treatments resulted in a significant (P<0.05) decrease in the number when compared to the positive control group. For the recorded total GST-P^+ foci area, the treatment of the rats with *T. violacea* (2%), *T. alliacea* (2%) and *A. sativum* (1%, 2%) significantly (P<0.05) reduced the foci area compared to the DEN-CMF control group.

Table 4.4. Effect of *Tulbaghia violacea* (Tv), *T. alliacea* (Ta) and *Allium sativum* (As) treatments on the activities of the cytosolic Glutathione S-Transferases (GST) and the Microsomal UDP-Glucuronosyl Transferases (UDP-GT) in livers of rats

Groups	GST-μ(mu) (μmole/min/mgprotein)	GST-α (alpha) (μmole/min/mgprotein)	UDP-GT (nmole/min/mgprotein)
DMSO	0.3 ± 0.1a	5.8 ± 1a	18.9 ± 8a
DEN	0.3 ± 0.1a	5.3 ± 1a	16.2 ± 10a
DEN-CMF	0.5 ± 0.1b	6.8 ± 1b	29.1 ± 10bc
DEN-CMF-Tv 1%	0.3 ± 0.02a	7.9 ± 1c	25.8 ± 10b
DEN-CMF-Tv 2%	0.3 ± 0.04a	8.3 ± 1c	30.6 ± 5bc
DEN-CMF-Ta 1%	0.4 ± 0.03a	8.2 ± 1c	30.5 ± 1bc
DEN-CMF-Ta 2%	0.3 ± 0.04a	8.1 ± 1c	36.1 ± 1c
DEN-CMF-As 1%	0.3 ± 0.03a	7.9 ± 1c	28.7 ± 1bc
DEN-CMF-As 2%	0.5 ± 0.07b	6.4 ± 1b	24.8 ± 2b

Values in columns are means ± STD of 7 rats per group. Means followed by the same letter are not significantly different (P > 0.05). If letters differ, then P< 0.05. DCNB was used as substrate for GST-μ determination and CDNB for GST-α. Abbreviations: Tv 1% = *Tulbaghia violacea* at 1% (w/v), Tv 2% = *T. violacea* at 2% (w/v), Ta 1% = *T. alliacea* at 1% (w/v), Ta 2% = *T. alliacea* at 2% (w/v), As 1% = *Allium sativum* at 1% (w/v) and As 2% = *Allium sativum* at 2% (w/v). DMSO = dimethyl sulfoxide, DEN = diethylnitrosamine, CMF = the fumonisin-containing diet.

Table 4.5. Effect of *Tulbaghia violacea* (Tv), *T. alliacea* (Ta) and *Allium sativum* (As) on the induction of GST-P$^+$ foci by combined treatment of DEN and CMF.

Treatment	GST-P$^+$ Liver Foci										
	total area of liver (cm^2)	No foci (0.04-0.5mm^2)	% of the total no of foci	No foci (0.51-1.0mm^2)	% of the total no of foci	No foci (1.1-1.5mm^2)	% of the total no of foci	No foci (>1.5mm^2)	% of the total no of foci	total no of foci	total area of foci
DMSO	38 ± 70a	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
DEN	41 ± 55a	2 ± 1a	47 ± 8a	1 ± 1a	12 ± 3a	1 ± 2a	8 ± 2b	nd	nd	4 ± 3a	4 ± 6a
DEN-CMF	39 ± 51a	100 ± 27cd	83 ± 4b	12 ± 4bc	10 ± 2ab	5 ± 3b	4 ± 2a	4 ± 6a	3 ± 4ab	121 ± 36b	33 ± 9c
DEN-CMF-Tv 1%	38 ± 49a	112 ± 74d	91 ± 46b	16 ± 4c	14 ± 4b	6 ± 6b	5 ± 5a	19 ± 34b	15 ± 27b	121 ± 39b	56 ± 9c
DEN-CMF-Tv 2%	34 ± 42a	87 ± 53cd	83 ± 22b	8 ± 6b	12 ± 15ab	2 ± 2ab	3 ± 3a	1 ± 2a	2 ± 5ab	98 ± 50ab	25 ± 9b
DEN-CMF-Ta 1%	36 ± 42a	77 ± 35cd	78 ± 21b	11 ± 2bc	14 ± 9b	3 ± 1ab	5 ± 5a	2 ± 1a	4 ± 8ab	93 ± 33ab	28 ± 7bc
DEN-CMF-Ta 2%	33 ± 31a	47 ± 17b	82 ± 18b	7 ± 5b	12 ± 9ab	3 ± 4ab	5 ± 8a	1 ± 2a	1 ± 2a	57 ± 13a	22 ± 9b
DEN-CMF-As 1%	33 ± 127a	47 ± 43b	74 ± 26b	8 ± 7b	18 ± 17b	2 ± 3ab	5 ± 6a	1 ± 2a	3 ± 2ab	58 ± 39a	17 ± 4b
DEN-CMF-As 2%	34 ± 64a	71 ± 22cd	85 ± 6b	9 ± 5b	11 ± 4ab	2 ± 2ab	2 ± 2a	1 ± 2a	1 ± 3a	84 ± 26b	26 ± 9b

Values represent the average of ten rats per group ± STD.
Means (column) followed by the same letter do not differ significantly. When letters differ then $P < 0.05$.
nd = not detected. Abbreviations: Tv 1% = *Tulbaghia violacea* at 1% (w/v), Tv 2% = *T. violacea* at 2% (w/v), Ta 1% = *T. alliacea* at 1% (w/v), Ta 2% = *T. alliacea* at 2% (w/v), As 1% = *Allium sativum* at 1% (w/v) and As 2% = *Allium sativum* at 2% (w/v). DMSO = dimethyl sulfoxide, DEN = diethylnitrosamine, CMF = the fumonisin-containing diet.

4.5 Discussion

When normal cells are transformed into the malignant state, it has to proceed through several stages including initiation and later events defined as tumor promotion (Borek 2001; 2002). Previous studies have shown FB_1 to be a non-genotoxic liver cancer promoter as well as hepato- and nephrocarcinogenic in rats (Gelderblom *et al.,* 1991; Howard *et al.,* 2001). The disruption of sphingolipid, phospholipid and fatty acid metabolism by FB_1 at a cellular level are suggested underlying mediators responsible for cancer promotion in the liver (Gelderblom *et al.,* 2001; Riley *et al.,* 2001; Burger *et al.,* 2007; Marnewick *et al.,* 2009). Phytochemicals appear to be an increasingly feasible approach for cancer prevention and/or modulation. Several studies on fruits, vegetables, spices, teas, herbs, and medicinal plants have been proven to be successful in modulating experimental carcinogenesis in various organs (Borek 2001; Sengupta *et al.,* 2004). A large number of these phytochemical compounds can prevent the occurrence of cancer, which contribute to the strength of chemoprevention. For this reason we evaluated the activity of crude aqueous garlic extracts which are the nearest form to preparation used traditionally. Furthermore, pure compounds do not always behave in the same way as in natural plant products because of important synergistic relationships among these compounds (Meckes *et al.,* 1993; Liu 2004). Since garlic contains various biochemically active substances including thioallyl compounds, and since its extracts have been known to protect organs from various injuries (Ip and Lisk 1995; Münchberg *et al.,* 2007) the present study was conducted to examine the modulatory effect of crude aqueous extracts of commercial and wild garlic species, on preneoplastic foci formation in DEN-initiated, CMF-promoted rat hepatocarcinogenesis. Previously garlic homogenates were shown to induce morphological changes in rat liver and kidney (Banerjee *et al.,* 2001). A 1000 mg/kg/day dose of *A. sativum* resulted in marked histopathological and ultrastructural changes in both liver and kidneys. The results therefore, propose that low doses of garlic have the potential to increase the endogenous antioxidant status. However, at higher doses of garlic, a reversal of these effects was observed. In our study the garlic dose ranged between 340 to 1300 mg/kg/day.

In the current study, the fumonisin-induced hepatotoxic and nephrotoxic effects were confirmed by the significant ($P<0.05$) increase in the clinical chemical parameters related to liver and kidney function and decreased body weight gain suggesting that

severe, acute liver injury had been induced in the positive control (DEN-CMF) rats. This increase in ALT, ALP and AST could be due to the production of free radicals during the metabolism of DEN and fumonisin, which subsequently lead to damage to the hepatocellular membranes. The increased ALT enzyme level was significantly reduced after treatment with *A. sativum* (2% w/v), while treatment with *T. alliacea* (1%, 2% w/v) and *A. sativum* (2% w/v) also resulted in a decrease in ALP enzyme levels when compared to the positive control. ALP is a marker enzyme for plasma membrane and a significant amount of this enzyme would suggest partial solubilisation of the plasma membrane (Holdsworth and Coleman 1975) indicating damage to the hepatocyte. Furthermore, serum creatinine levels were significantly reduced by all garlic treatment groups except for *A. sativum* (2%), implying a reduced nephrotoxic effect. The observed reduction in these enzymes suggests that certain of the garlic extracts may preserve the structural integrity of the tissues, protecting it against the toxic effect induced by fumonisin contained in the culture material that was fed to the rats. Previously it was reported that in general, culture material from *F. verticillioides* which were fed to rats, produced micro- and macronodular cirrhosis, cholangiofibrosis and primary hepatocellular carcinomas (Gelderblom *et al.*, 1988; Gelderblom *et al.,* 1991). These findings in rats, as a result of feeding infected culture material, led the way for studies into the cancer-initiating capability of purified FB_1, FB_2, and FB_3 (Gelderblom *et al.,* 1992; Gelderblom *et al.,* 1993; Gelderblom *et al.,* 1994). Previously it was shown that organosulfur components (i.e diallyl sulfides), present in garlic, exhibit protective effects against several toxicants such as cadmium, lead nitrate, arsenic, tobacco and zearalenone toxicity (Kwak *et al.,* 1994; Singh and Singh 1997; Chowdhury *et al.,* 2008; Abid-Essefi *et al.,* 2011). DEN-CMF treatment significantly increased the total serum iron levels, while the garlic treatments did not further enhance these increased serum iron levels. A previous study showed garlic oil pre-treatment to decrease the level of serum iron in rats by increasing the iron binding capacity and thereby preventing hemolysis. This effect subsequently led to preventing iron catalyzed lipid peroxidation (Saravanan and Prakash 2004).

It has been documented that free radicals and lipid peroxidation are known to cause initiation and promotion of carcinogenesis (Baur and Wendel 1980; Zhang *et al.,* 2006). Lipid peroxidation is also generally increased during the carcinogenic processes. A fair assumption could therefore be that agents that can reduce the generation of free radicals *in vivo* may be considered to have the potential for chemoprevention. Oxidative

stress therefore, is a result of damage brought about by attack on cellular macromolecules such as lipids and DNA by free radicals (van Rossen *et al.,* 2000; Giftson *et al.,* 2010). In this study, two different lipid peroxidation products were measured in the liver (TBARS and CDs). The level of malondialdehyde (measured as TBARS) was significantly increased in the DEN-CMF treated group. This level was further enhanced by *T. violacea* (1%), while the other garlic treatments had no further significant impact on the TBARS levels. It should be noted that the TBARS assay is non-specific for MDA and other components could react with TBA (Janero 1990), thus the level of another marker, conjugated dienes was also measured. The level for conjugated dienes was significantly reduced by *T. violacea* (1%) when compared to the positive control group, with none of the other garlic treatments further affected the CD levels. This reduced level of CDs however was not associated with a suppression of GST-P$^+$ foci. Previous studies have reported on the reduction of hepatic TBARS levels by certain garlic compounds/extracts from *Allium sativum* (Takada *et al.,* 1994; Obioha *et al.,* 2009). This discrepancy could be ascribed to factors such as the concentration of the garlic compounds/extract, variations in processing methods of extracts and plant species differences.

Reduced glutathione is seen as a powerful intracellular antioxidant playing a very important role in stabilizing several enzymes (Wang *et al.,* 2000; Marnewick *et al.,* 2003). The increase in GSH content could therefore be seen as a defence mechanism against the increase in oxidative stress. In the current study, the GSH levels were significantly increased by the fumonisins intake and associated with the increased development of GST-P+ foci. Previous studies have shown, gamma glutamyl transpeptidase positive (GGT$^+$) foci to be associated with an increased level of GSH, providing these foci with a growth advantage over the surrounding tissue (Gelderblom *et al.,* 1991; Abel and Gelderblom 1998). The GSH levels were significantly decreased by extracts of both *T. alliacea* and *A. sativum* also resulting in a significant reduction in the GST-P$^+$ foci (*T. alliacea* 2 % and *A. sativum* 1 %), confirming this phenomenon. A previous study has also shown GSH levels to be significantly increased in hepatic pre-neoplastic lesions (Marnewick *et al.*, 2009). When considering the GSSG levels, only treatment with *T. alliacea* (2 %) resulted in a significantly reduced level when compared to the positive control, but had no further effect on the GSH/GSSG ratio compared to DEN-CMF treated rats. Our results therefore support the notion that depletion of tissue

GSH is one of the major factors leading to subsequent tissue damage (Sener *et al.,* 2003).

Under our experimental conditions, DEN-CMF treatment resulted in an increased level of lipid peroxidation (TBARS) with a decrease in SOD activity compared to DMSO control rats. The decreased activities of SOD and an increased level of lipid peroxidation in DEN-CMF treated animals substantiate the occurrence of induced oxidative stress in the liver tissue. In excess of 90 % of the oxygen utilized by the mitochondria will be transformed into water and the rest to superoxide radicals (O_{2}-) (Das and Saha 2009). The resultant ROS are normal by-products of cellular metabolism and are counteracted by cellular defences provided by antioxidant enzymes such as CAT and SOD (Singh *et al.,* 1996; Valentine and Hart 2003). The extent of oxidative damage to important cellular fractions is therefore determined by the balance between the production of oxidants and the scavenging of those oxidants by the antioxidant defence system. In the present study, the activities of SOD were reduced by DEN-CMF, thus exposing the tissues to the peroxidative damage. In this study the various garlic treatments and control treatments had no significant effect on the CAT activities. However, the consumption of *T. violacea* (1% w/v) and *T. alliacea* (1%, 2% w/v) resulted in an increase in the activity of SOD. The increased activity of SOD in the liver tissue of rats treated with aqueous garlic extracts suggests a certain role to combat oxygen free radical in two-stage carcinogenesis (Werts and Gould 1986). Phytochemicals in aqueous garlic extracts may therefore act in a synergistic or maybe additive way. Their antioxidant action may be exerted by scavenging ROS (Borek 2001), enhancing the cellular antioxidant enzymes (CAT, SOD) and/or by increasing glutathione.

Generally, the induction of phase II drug-metabolizing enzymes, the suppression of phase I enzymes or the combination of these actions are seen as mechanisms liable for the defence against toxic and neoplastic effects of carcinogens (Talalay 1989). Furthermore, a variety of mechanisms exist to produce such protection. Numerous studies support the fact that phase II enzymes play important roles in the detoxification of xenobiotics. The up-regulation of these enzymes provides protection against potentially harmful insults from the environment (Kong *et al.,* 2001). The modulation of carcinogen metabolism is believed to be associated with the cancer-chemopreventive effect of garlic and its organosulfur compounds. This include effects on both phase I

and II detoxification enzymes (Tsai *et al.,* 2012). Phase I enzymes, mainly cytochrome P450 (CYP), is involved in the detoxification of several endogenous and exogenous chemicals and the activation of many carcinogens. Phase II enzymes are involved in catalyzing the conjugation of phase I metabolites into different water-soluble molecules, which include, glucuronic acid, or sulfate, to accelerate the metabolite excretion rate (Tsai *et al.,* 2012). It was shown previously that the efficacy of DAS, DADS, and DATS in the transcriptional regulation of phase I and II detoxification enzyme expression correlates positively with the inhibition of aflatoxin B_1- and benzo[*a*]pyrene-induced fore-stomach and liver neoplastic development in mice and rats (Hu *et al.,* 1996; Guyonnet *et al.,* 2002). The induction of several phase II detoxification enzymes and other antioxidants and enzymes are associated with the reduction of tumor and LPOs (Das and Saha 2009). Previously it was shown that GST, a phase II enzyme, played a physiological role in initiating the detoxification of numerous alkylating agents and environmental chemicals as well as mutagens and carcinogens (Testa *et al.,* 2007). In our study, fumonisins significantly increased the GST-α and GST-μ activity when compared to the negative and DEN controls. The activity of GST-α was found to be significantly further enhanced by all garlic treatments, with the exception of *A. sativum* (2%), indicative of a beneficial effect of these garlic extracts (Table 4.4). When considering GST-μ activity, all the garlic treatments, with exception of *A. sativum* (2%), resulted in a significant reduction of the fumonisin-induced increase. This is in agreement with several studies that garlic or its organosulfur compounds increased GST activity in some tissues, such as liver and small intestine (Hatono *et al.,* 1996; Ip and Lisk 1997). The GST activity is therefore known for being induced by exposure to carcinogens (Manson *et al.,* 1997). GST activity was also showed to be higher in tumor bearing tissue when compared to normal tissues (Stout and Becker 1986; Kweon *et al.,* 2003). Our findings regarding the induced activity of GST-α in the garlic treated groups are in association with suppressing the GST-P^+ foci formation in the same garlic treated groups. While fumonisin showed an induction of another phase II enzyme, UDP-GT, some of the garlic treatments also marginally (P=0.059) increased the activity of this enzyme in the rat hepatic tissue.

The total area of GST-P^+ foci, a biomarker of preneoplastic lesions, was significantly reduced by aqueous extracts of *T. violacea* (2%), *T. alliacea* (2%) as well as *A. sativum* (1% and 2%) compared to the positive control. When considering total number of GST-

P^+ altered cells, the consumption of *T. alliacea* (2%) and *A. sativum* (1%) significantly reduced the quantity in the presence of fumonisins. Previously it was shown that garlic powder-containing diets inhibited the number and size of GST-P^+ foci in rat a hepatocarcinogenesis model (Kweon *et al.,* 2003). It has been reported that garlic and its compounds have been potent chemopreventive agents in breast (Ip and Lisk 1995; Schaffer *et al.,* 1997), colon (Hatono *et al.,* 1996) and stomach cancers (Hadjiolov *et al.,* 1993). Some studies also reported that the organosulfur compounds from garlic promoted hepatocarcinogenesis (Takahashi *et al.,* 1992; Takada *et al.,* 1994). However, Haber Mignard *et al.* (1996) demonstrated that diallylsulfide and diallyl disulphide significantly suppressed preneoplastic foci formation which was induced by DEN or aflatoxin B_1 injections with partial hepatectomy.

It is thus proposed that the induction potency of the garlic aqueous extracts on phase II enzymes could be associated with their chemoprevention efficacy (Jana and Mandlekar 2009; Tan and Spivack 2009). The present study indicates that *T. alliaceae* (2 % w/v) and *A. sativum* (1% w/v) treatment suppressed GST-P^+ foci formation with the modulation of certain phase II detoxification enzymes, as well as the antioxidant enzyme, SOD (*T. alliaceae)* and decreased glutathione levels as being possible mechanisms of protection. It is important to acknowledge that other possible mechanisms of protection due to the organosulfur compounds in garlic may also be involved. In the current study, *T. alliaceae* (2% w/v) and *A. sativum* (1% w/v) resulted in a significant protection against fumonisin-induced cancer promotion. The possible chemopreventive properties of *T. alliaceae* should be further evaluated, as it could make a significant contribution to traditional healers within the South African context, where wild garlic is commonly used for treating their patients for various ailments.

4.5 References

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CHAPTER 5

Summary, Conclusions and Recommendations

5.1 Introduction

Maize (*Zea mays*, L) is a dietary staple in sub-Saharan Africa (Van der Westhuizen *et al.,* 2011) and can be associated with several fungi during pre - and postharvest periods. *Fusarium verticillioides* (Sacc) Nirenberg (formerly known as *Fusarium moniliforme* Sheldon) is regarded as one of the most economically important phytopathogens of maize worldwide (Nelson *et al.,* 1981; Munkvold and Desjardins 1997). During the development of the maize plant, *F. verticillioides* is associated with disease at all stages, infecting the roots, stalk, and kernels. It is also amongst the most common fungi found colonizing symptomless maize plants (Munkvold and Desjardins 1997). The endophytic relationship between this fungus and its plant host is of major concern as it may lead to significant losses of grain and seed quality and the potential occurrence of mycotoxins such as fumonisins (FB) (Kpodo *et al.,* 2000); (Marasas 2001).

Available commercial fungicides are not adequate in controlling *F. verticillioides* (Munkvold and Desjardins, 1997). *In vitro* experiments also showed that current preservatives used in the food industry are unsuccessful in controlling the production of fumonisins by *Fusarium* species and that they are not necessarily effective in restricting the number of species either (Marin *et al.,* 1999). *Fusarium* infections of humans have also emerged as a major fungal disease with increasing frequency, irrespective of antifungal therapies (Gaur and Flynn 2001). Fusariosis was shown to be more prominent in the immunocompromised patients and those with haematological malignancies (Campo *et al.,* 2009 ; De Pauw *et al.,* 2008). The treatment of this disease with a standard broad spectrum antifungal agent such as amphotericin B, has been challenged with resistance (Gómez-López *et al.,* 2003). Worldwide surveillance studies have revealed that multi-drug resistance (MDR) in pathogenic fungi are also becoming an increased phenomenon (Karchmer 2000). (De Waard 1996; 1997) reported on the mechanisms of fungicide MDR and development of subsequent strategies for effective fungicide resistance management.

In 1988, Gelderblom *et al,* reported on the identification of fumonisins from cultures of *F. moniliforme* and that these toxins exhibit cancer promoting activity (Gelderblom *et al.,* 1988) in rat liver. The co-occurrence of other natural occurring cancer initiating and promoting food contaminants such as aflatoxin B with fumonisin, could pose an increased risk for cancer (Gelderblom *et al.,* 2002). Fumonisins were shown to cause severe animal diseases such as equine leukoencephalomalacia (ELEM) in horses (Marasas *et al.,* 1988) and porcine pulmonary edema (PPE) in swine (Colvin and Harrison 1992; Halloy 2005). Aside from their hepatotoxicity (Gelderblom *et al.,* 2001) and nephrotoxicity (Edrington *et al.,* 1995), fumonisins were also shown to affect the immune system (Bhandari *et al.,* 2002; Dombrink-Kurtzman 2003). Fumonisins have attracted much attention due to the association with human illnesses including oesophageal and liver cancer in humans (Sun *et al.,* 2007). Studies of fumonisin toxicity in foods and feeds are therefore regarded as being critical (Miller 2008; Shephard 2008).

Therefore, the current study focussed on South African wild garlic plant extracts in the development of antifungal control options against *F. verticillioides* and how these plant extracts could potentially alleviate health-related problems associated with fumonisin toxicity. The contributions against fumonisin toxicity led to investigations into the antioxidant status modulation and consequently the possible cancer modulating and/or chemoprevention properties of the two wild garlic species compared to one commercially available garlic species.

5.2 *In Vitro* Synergistic Studies

Natural plant extracts as alternatives to synthetic chemicals have received much attention in recent years in the scientific community to control several pathogens in food sources (Fu *et al.,* 2007; Rota *et al.,* 2008). In order for plants to protect themselves against microbial pathogens, they produce an enormous variety of secondary metabolites (Dixon 2001). However, it is evident that a different paradigm - "synergy" - exist in plants to combat infections (Hemaiswarya *et al.,* 2008). This synergy can be demonstrated by combination treatments, whereby the dosages of one (synthetic) component can be reduced and combined with safer and effective agents primarily from plant sources, without the loss of effective control of the pathogen targeted. These

combinations will result in a reduced risk for resistance of the target pathogen to either component (Gisi 1996; Lorbeer 1996).

In this study it was hypothesised that natural compounds found in three South African wild garlic species exhibit synergistic activity with synthetic antifungal compounds. Mixtures of four fungicides, Sporekill™, Thiram, Itraconazole and Fluconazole (6.25, 12.5, 25 and 50 mg/ml) with aqueous plant extracts of *Tulbaghia violacea*, *T. alliacea* and *T. simmleri* at dosages of 6.25, 12.5, 25 and 50 mg/ml were tested on the radial growth inhibition of *F. verticillioides* (MRC 826) on potato dextrose agar. The dosages used for Sporekill™ and Thiram, according to the supplier's specification, was 0.000125, 0.00025, 0.0005 and 0.001 mg/ml; 0.001, 0.002, 0.004 and 0.008 mg/ml respectively. The percentage (%) inhibition (treatment/control X 100) was calculated from the radial growth while synergistic ratio (SR) for percentage inhibition was based on the Abbott formula (Abbott 1925) as described by (Gisi 1996) (SR>1=Synergistic, SR=1=Additive; SR<1=antagonistic). A dose response for percentage growth inhibition was observed when the respective fungicides were tested alone while no linear response was noticed for single garlic treatments with an increase in dose. When combinations of plant extracts with fungicides were assessed, a significant increase in inhibitory response was observed with several synergistic interactions. When considering Sporekill, from 16 combinations with *Tulbaghia violacea, T. alliacea* and *T. simmleri*, respectively, 63 %, 69 % and 25 % of the mixtures exhibited synergistic interactions. However, significant growth inhibition was only obtained for 2 (13 %), 10 (63 %) and 4 (25 %) combinations for *T. violacea*, *T. alliacea* and *T. simmleri*, respectively when compared to Sporekill™ and garlic extracts alone. *F. verticillioides* showed more sensitivity towards combinations of Sporekill™ with *T. alliacea*. *Tulbaghia alliacea* also exhibited better growth inhibition against *F. verticillioides* with combinations with Thiram. High synergistic interactions were also measured for the combinations with *T. alliacea*. When considering the azole mixtures, combinations with *T. simmleri* resulted in 94 % synergistic interactions and 11 mixtures showing significant higher growth inhibition against *F. verticillioides*. From the results, it could be concluded that the combination of these fungicides with the various garlic extracts showed excellent antifungal properties, and strong synergy was also observed against *F. verticillioides* at low and sublethal dosages of fungicides. Our investigation indicates an important option in developing plant-based pesticides which are ecofriendly for the

management of the fungus. Furthermore, these interactions with plant extracts is a potential option to overcome the resistance mechanisms of fungi.

5.3 Modulation of hepatic drug metabolizing enzymes and oxidative status in rats

Abel and Gelderblom (1998) provided important data showing a close association between oxidative damage and FB_1-induced liver cancer initiation *in vivo*. Oxidative cellular damage is possible when cellular defenses are not functioning optimally or when free radicals are over produced. According to Reddy *et al* (2003), many natural products have been employed as a means of chemoprotection against commonly occurring cancers. We evaluated the modulation properties of a commercially available garlic specie, *Allium sativum* and two South African wild garlic species *Tulbaghia violacea* and *T. alliacea* in male Fischer rats. Due to the complex nature of their phytochemical composition, a battery of assays were assessed to evaluate their antioxidant potential. No adverse effects were noticed in the liver and kidneys of the rats treated with various garlic species. The observed maintenance and/or decrease in the liver function enzymes levels suggests that these aqueous garlic extracts may preserve the structural integrity of the tissues. Also, total plasma iron was similarly affected showing no evidence for iron catalyzed lipid peroxidation (Saravanan and Prakash 2004; Prakash *et al.,* 2007). Aqueous extracts of the wild garlic (*T. violacea* and *T. alliacea*) resulted in increased hepatic ORAC values. However, we cannot conclude that a relationship exists between the phenolic intake by the rats and the increased hepatic ORAC levels. An increase in GSH level was observed for the respective garlic (*T. alliacea* and *A. sativum*) treatments and could also be a result of the modulation of several enzyme systems. In addition, the observed significant ($P<0.05$) increase in GSH/GSSG ratio in the liver may be indicative of an increased antioxidant capacity in the cell or possible reduced oxidative stress. Therefore, oxidative stress-induced tissue damage can be prevented by assisting the balance towards a lower oxidative status. When considering the phase II enzymes, the activity of GST-μ was significantly ($P<0.05$) enhanced while a non-significant increase in the activity of GST-α (alpha) in rat liver was also noted indicating an increased ability for detoxification of carcinogens in rats consuming garlic (*T. violacea* and *T. alliacea*). In summary, we have shown in our study that cells pre-treated with aqueous garlic extracts resulted in a significant elevation in GSH levels, induction of GST-μ and UDP-

GT and modulation of CAT and SOD. Therefore, *T. violacea, T. alliacea* and *A. sativum* administration to male Fischer rats modulated the oxidative status as well as phase II drug metabolizing enzymes in the liver and may be crucial to protect the liver against the adverse effects related to oxidative damage and mutagenesis.

5.4 Chemoprotective properties of *Allium sativum*, *Tulbaghia violacea* and *Tulbaghia alliacea*

Subsequent to our findings in 5.3 above, we evaluated the activity of crude aqueous garlic extracts to examine the chemoprotective properties of commercially available garlic species, *Allium sativum* and the two South African wild garlic species, *Tulbaghia violacea* and *T. alliacea* at different levels on preneoplastic foci formation promoted by culture material of *F. verticillioides* MRC 826 (CMF) in rat liver using diethylnitrosamine (DEN) as cancer initiator. Clinical chemical parameters related to liver and kidney function and decreased body weight gain suggesting that severe, acute liver injury had been induced in the positive control (DEN-CMF) rats, while the levels were mostly reduced by the various garlic treatments. Activities of antioxidant enzymes like SOD, CAT and GSH status were differently affected by the various garlic treatments. The GSH levels were significantly decreased by extracts of both *T. alliacea* and *A. sativum* also resulting in a significant reduction in the GST-P$^+$ foci. In our study, fumonisins present in the culture material, significantly increased the GST-α and GST-μ activity which were found to be significantly further enhanced by all garlic treatments, with the exception of *A. sativum* (2% w/v). Our findings regarding the induced activity of GST-α in the garlic treated groups are in association with suppressing the GST-P$^+$ foci formation in the same garlic treated groups. Therefore, the induction potency of the garlic aqueous extracts on phase II enzymes could be associated with their chemoprevention efficacy. The present study further indicates that *T. alliaceae* (2 % w/v) and *A. sativum* (1% w/v) treatment suppressed GST-P$^+$ foci formation with the modulation of certain phase II detoxification enzymes, as well as the antioxidant enzyme, SOD (*T. alliaceae)* and decreased GSH levels as being possible mechanisms of protection. It is important to acknowledge that other possible mechanisms of protection due to the organosulfur compounds in garlic may also be involved. It can therefore be concluded that *T. alliaceae* and *A. sativum* treatments inhibited/decreased cancer promotion effected by the CMF (250 mg fumonisin/kg) diet.

5.6 Conclusion

In conclusion, we present preliminary evidence for the antifungal activity of garlic extract against *F. verticillioides*. The South African indigenous plant species were able to produce modulators that potentiate the activity of fungicides. Furthermore, this approach allow for reduced fungicide concentrations while maintaining adequate fungal control. This is also the first report on the modulation and chemopreventive properties properties of *T. alliaceae* and *T. violacea.* These results provide new evidence showing the modulation of phase II drug metabolizing enzymes and the oxidative status in the liver of rats by the South African wild garlic species as well as *A. sativum.* Furthermore, our study forms a foundation that could lead to future studies into the successful control of *F. verticillioides* on maize and maize-based foods during storage conditions while residual extract could potentially have similar effects observed in rats when consumed. However, one has to be causious when extrapolating findings obtained in animal studies to humans. The possible chemopreventive properties shown in the study should be further evaluated, as it could make a significant contribution to traditional healers within the South African context, where wild garlic is commonly used for treating their patients for various ailments.

5.7 Recommendations

From the survey carried out, it has emerged that pathogen development and mycotoxin production are not strictly related. Further studies should be performed to evaluate combination therapy on secondary metabolite production of *F. verticillioides*.

In order to assess the potential of these combinations for therapeutic application, further work is necessary to explore the mode of action of aqueous extracts and to understand the molecular mechanisms of their synergy with various fungicides.

Further studies are required for the selection of appropriate dose, duration of treatment and their possible effects on chemoprevention against other cancer.

Phytochemical analyses, of the garlic extracts used in this study, did not fall within the scope of this study, therefore future studies will have to focus on this important aspect, to assist in the elucidation of possible mechanisms involved

5.7 References

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