THE POTENTIAL OF SPICE OILS IN THE CONTROL OF MYCOTOXIN PRODUCING FUNGI

SARLA JUGLAL

Submitted in fulfilment of the requirements for the degree of

MASTER OF TECHNOLOGY : BIOTECHNOLOGY

in the
Department of Biological Sciences
M. L. Sultan Technikon
Durban

2000

DECLARATION

This study represents original work by the author and has not been submitted in any form to any institution. Where use was made of the work of authors it has been duly acknowledged in the text.

S. JUGLAL

TABLE OF CONTENTS

Acknowledgements i

Conference Presentations iii

List of Figures iv

List of Tables vi

List of Abbreviations viii

Abstract ix

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW 1

1.1 Current status of the mycotoxin problem 5

1.2 Aflatoxins 9

1.2.1 Structure 10

1.2.2 Biosynthesis of aflatoxin B_1 12

1.2.3 Substances affecting aflatoxin production 14

1.3 Fumonisins 18

1.3.1 Physical and chemical properties of fumonisins 21

1.3.2 Mode of action of fumonisins 22

1.3.3 Control of fumonisins 24

1.4 Spices as bio-control agents against fungi 26

1.4.1 Antifungal properties of chemical components of spices 29

1.4.2 Antibacterial and antifungal properties of pungent spices 31

1.4.2.1 Mustard and wasabi 31

1.4.2.2 Garlic 33

1.4.3 Mode of action of spice oils 33

CHAPTER TWO: MATERIALS AND METHODS 35

2.1. Extraction and purification of spice oils 35

2.2. Fungal strains 35

2.3. Effect of clove, oregano, eucalyptus and tumeric oils on mycotoxin-producing fungi 36

2.4. Effect of spice oils on aflatoxin- and fumonisin-producing fungi 37

2.4.1 Isolation of *Aspergillus* species from natural substrates 37

2.4.2 Identification of *Aspergillus* species 37

2.4.2.1 Macroscopic characteristics 38

2.4.2.2 Microscopic characteristics 38

2.4.3 Toxin producing aspergilli 39

2.5. Inhibition of *A. parasiticus* and *F. moniliforme* by spice oils 40

2.6. Effect of spice oils on aflatoxin and fumonisin production 40

2.6.1. Inhibition of aflatoxin production by spice oils 41

2.6.2. Effect of clove oil on aflatoxin and fumonisin B_1 production 41

2.6.3. Aflatoxin and fumonisin B_1 extraction 42

2.6.4. Thin layer chromatography of aflatoxins and fumonisin B_1 43

2.6.5. High pressure liquid chromatography (HPLC) of aflatoxins and fumonisin B_1 44

2.6.5.1. Preparation of standards 45

2.6.5.2. Preparation of samples 46

2.6.5.3. High pressure liquid chromatography optimization 46

2.6.5.4. Quantification 46

2.7. Analysis of the clove oil components inhibitory to *A. parasiticus* and *F. moniliforme* 48

2.8 Preliminary investigations of the use of cloves for the storage of grain 49

CHAPTER THREE: RESULTS 50

3.1 The effect of spice oils on mycotoxin-producing fungi 50

3.2 Isolation of aflatoxin producing fungi 51

3.2.1. Isolation and identification of mycotoxin-producing fungi 51

3.2.2. Production of aflatoxins by *Aspergillus parasiticus* 53

3.3 Inhibition of *Aspergillus parasiticus* and *Fusarium moniliforme* by spice oils . . 54

3.4 Inhibition of aflatoxin production by spice oils 57

3.5 Inhibition of *Fusarium moniliforme* by clove oil 59

3.6. Inhibition of aflatoxin and fumonisin B_1 production by clove oil 60

3.6.1 Thin layer chromatography 60

3.6.1.1. *Aspergillus parasiticus* 60

3.6.1.2. *Fusarium moniliforme* 60

3.6.2 High pressure liquid chromatography 62

3.6.2.1. *Aspergillus parasiticus* 62

3.6.2.2. *Fusarium moniliforme* 67

3.7 Components of clove oil inhibitory to *Aspergillus parasiticus* and *Fusarium moniliforme* 68

3.8 The effect of clove on fumonisin production in grain 70

3.8.1. Cost evaluation 71

CHAPTER FOUR: DISCUSSION 72

CHAPTER FIVE: REFERENCES 82

APPENDIX ONE 105

APPENDIX TWO 106

APPENDIX THREE 108

APPENDIX FOUR 109

For

my father, who taught me that knowledge is strength;

my mother, for her courage and inspiration;

my sister, Jaya, and brother-in-law, Reggie, for always

being there for me;

my daughter, Myuri 'for just being you';

my husband, Ashwin, for his unfailing support in all my endeavours

and for always being happy for my success.

Acknowledgements

The author expresses her sincere gratitude to:

Dr. B. Odhav, supervisor, acting H.O.D. of the Department of Biological Sciences, M. L. Sultan Technikon for her supervision, constructive criticism, patience, time and all her help during the research and preparation of this dissertation.

Prof. M. F. Dutton, co-supervisor and H.O.D. of the Department of Physiology, University of Natal - Durban for his expert guidance.

National Research Foundation (NRF) for funding this research project.

Robertsons Spices for the supply of spice oils.

Ms. S. Naidoo for her invaluable time and help; her assistance in HLPC analysis of aflatoxins and word processing is sincerely appreciated.

Ms. S. Naiker for her contribution in HPLC analysis of fumonisins.

Dr. R. Govinden for her much appreciated time and assistance in preparing this dissertation.

Mr. K. Ramsunder for his assistance with optimisation of the HPLC.

Mr. V. Mohanlall and Ms. R. Naidoo for their assistance with the use of computer technology.

Dr. R. Gengan for this advice in the field of organic chemistry.

Dr. K. Permaul for proof-reading.

Ms. L. Reddy for her continued support and encouragement.

My friends Jenni, Priscilla and Samantha for their moral support and encouragement during this study.

Reggie, for his time and help; and prompt assistance in literature search and retrieval

My sister Jaya, for her uplifting advice when the end seemed so far away and for her time and assistance.

For my daughter Myuri, for adapting so easily and 'giving' me the time to complete this dissertation.

My husband Ashwin, for his patience and for making the time for me to complete this study.

Conference Presentations

1 Aflatoxin and fumonisin levels in maize and maize related products in Kwa-Zulu Natal and the potential of plant extracts in preservation.
S. Juglal, M. F. Dutton, B. Odhav
Poster presented at **14th SAAFoST International Congress**
Pretoria, 1-4 September 1997

2 Control of mycotoxins using spice oils.
S. Juglal, M. F. Dutton, B. Odhav
Oral presentation at **Joint Kwa-Zulu Natal: Biochemistry and Microbiology Symposium**
University of Natal: Durban, October 1997

3 Control of mycotoxins using extracts of spice oils.
S. Juglal, M. F. Dutton, B. Odhav
Poster presentation at **International Conference on Frontiers in Biotechnology**
Trivandrum, India, November 1997

4 Control of mycotoxins using extracts of spice oils
S. Juglal, M. F. Dutton, B. Odhav
Oral presentation at **Regional Centre for Indigenous Plant Usage Research Symposium**
University of Natal: Durban, October 1997

5 Potential of spice oils in the control of mycotoxin producing fungi.
S. Juglal, S. Naiker, B. Odhav
Poster presentation at **15th Biennial SAAFoST International Congress**
Cape Town, September 1999

List of Figures

Figure 1.1: Structure of the major aflatoxins (Takahashi and Beebe, 1979) 11

Figure 1.2: Pathway for biosynthesis of aflatoxin B_1 (Zaika and Buchanan, 1987). 13

Figure 1.3: Life cycle of *Fusarium moniliforme* on maize (Bacon *et al.*, 1996). 20

Figure 1.4: Typical *Fusarium* ear rot showing white discolouration of kernels (Bacon *et al.*, 1992). 20

Figure 1.5: Structure of fumonisins B_1, B_2, B_3, B_4, A_1 and A_2 (Cawood *et al.*, 1991) . . . 22

Figure 1.6: Disruption of sphingolipid pathway by fumonisins. 23

Figure 1.7: Structural representation of the chemical constituents of clove oil (Mahmoud, 1994). 29

Figure 3.1: Effect of clove, oregano, eucalyptus and tumeric oils on mycotoxigenic fungi using the agar overlay technique. 50

Figure 3.2: A: Pure cultures of *Aspergillus parasiticus* isolated from pet food. B: Pet food ('dog bites') contaminated with *Aspergillus* fungi. 52

Figure 3.3: TLC plate showing aflatoxins produced by the *Aspergillus parasiticus* strain isolated from pet food. 53

Figure 3.4: Agar overlay showing inhibition of *Aspergillus parasiticus* by 10, 20 and 30 µl clove oil. 55

Figure 3.5: Agar overlay showing inhibition of *Fusarium moniliforme* by clove oil. *F. moniliforme* unchallenged by clove oil (control) and *F. moniliforme* challenged with 10 µl clove oil. 55

Figure 3.6: Comparison of the inhibition by spice oils of *Aspergillus parasiticus* and *Fusarium moniliforme*. 57

Figure 3.7: Inhibition of the growth of *Fusarium moniliforme* in broth cultures by clove oil showing a decrease in growth with increasing concentration of clove oil. Pink colour of broth indicates toxin presence and white indicates mycelial mass of *F. moniliforme*. 59

Figure 3.8: Diagrammatic representation of a TLC plate showing fumonisins produced by *Fusarium moniliforme* grown in the presence of varying concentrations of clove oil. 61

Figure 3.9: HPLC chromatogram of aflatoxin standards showing retention times for AFG_2, AFG_1, AFB_2 and AFB_1 respectively. 62

Figure 3.10: HPLC chromatogram of aflatoxins AFG_2, AFG_1, AFB_2 and AFB_1 produced by *Aspergillus parasiticus* grown in SMKY broth containing no spice oil (control). 63

Figure 3.11: HPLC chromatogram of aflatoxins AFG_2, AFG_1, AFB_2 and AFB_1 produced by *Aspergillus parasiticus* grown in SMKY broth containing 0.03 ppm clove oil 64

Figure 3.12: HPLC chromatogram of aflatoxins AFG_1, AFB_2 and AFB_1 produced by *Aspergillus parasiticus* grown in SMKY broth containing 0.05 ppm clove oil. 64

Figure 3.13: HPLC chromatogram of aflatoxin AFG_1 produced by *Aspergillus parasiticus* grown in SMKY broth containing 0.15 ppm clove oil. 65

Figure 3.14: A: TLC plate of clove oil and two of its active components, viz., cinnamic aldehyde and eugenol. B: Bioautography showing inhibition of *Aspergillus parasiticus* by clove oil and its active componennts. Clearings indicates inhibition 68

Figure 3.15: A: TLC plate of clove oil and two of its active components, viz., cinnamic aldehyde and eugenol. B: Bioautography showing inhibition of *Fusarium moniliforme* by clove oil and its active components. Yellow colour indicates growth of *F. moniliforme.* 69

Figure 3.16: Inhibition of the growth of *Fusarium moniliforme* in samp by whole cloves. Flask with 1 g of clove shows no visible growth of *F. moniliforme*. Growth is indicated by a white fuzzy mass. The cloves are black. 70

List of Tables

Table 1.1: Mycotoxin regulations for foods in Southern African countries (Sibanda *et al.*, 1997) 8

Table 1.2: The effect of various compounds on growth of and/or aflatoxin formation by *Aspergillus flavus* and *Aspergillus parasiticus* 15

Table 1.3: Antimicrobial, antibiotics and pesticides that inhibit aflatoxin formation through inhibition of growth of *Aspergillus flavus* and *Aspergillus parasiticus* 16

Table 1.4: Botanical substances and their effects on *Aspergillus flavus* and *Aspergillus parasiticus* growth 17

Table 1.5: Levels of fumonisins associated with high incidence of human oesophageal cancer (EC) 21

Table 3.1: Macroscopic and microscopic characteristics of fungi isolated from food products 52

Table 3.2: Inhibition of *Aspergillus parasiticus* and *Fusarium moniliforme* using varying volumes of spice oils 56

Table 3.3: Inhibition of aflatoxins in broth culture at varying spice oil concentrations 58

Table 3.4: Minimum inhibitory concentration of clove oil for aflatoxin production by *Aspergillus parasiticus* in SMKY broth culture determined by thin layer chromatography 60

Table 3.5: Concentration and reduction (%) of aflatoxin production by *Aspergillus parasiticus* grown in the presence of clove oil 66

Table 3.6: Minimum inhibitory concentration of clove oil for aflatoxin production by *Aspergillus parasiticus* in SMKY broth 66

Table 3.7: Inhibition of fumonisin B_1 production by *Fusarium moniliforme* by clove oil 67

Table 3.8: Relationship between fungal growth and toxin production of *Aspergillus parasiticus* and *Fusarium moniliforme* 67

Table 3.9: The effect of whole and ground cloves on the reduction of FB_1

levels in samp 71

Table 4.1: Summary of mycotoxin control measures and their advantages and disadvantages. 77

List of Abbreviations

CH_3CN	-	acetonitrile
AFB_1	-	aflatoxin B_1
AFB_2	-	aflatoxin B_2
AFG_1	-	aflatoxin G_1
AFG_2	-	aflatoxin G_2
$Ca(OH)_2$	-	calcium hydroxide
CYA	-	Czapek yeast extract agar
CYA20S	-	Czapek yeast extract agar 20% sucrose
DON	-	deoxynivalenol
CH_2Cl_2	-	dichloromethane
ELEM	-	equine leucoencephalomalacia
FB_1	-	fumonisin B_1
FB_2	-	fumonisin B_2
FB_3	-	fumonisin B_3
C_6H_{14}	-	hexane
HPLC	-	high performance liquid chromatography
LEM	-	leucoencephalomalacia
MEA	-	malt extract agar
MeOH	-	methanol
MIC	-	minimum inhibitory concentration
MON	-	moniliformin
OPA	-	*o*-phthaldialdehyde
R_f	-	response factor
SMKY	-	sucrose, magnesium sulphate, potassium nitrate yeast extract broth
TLC	-	thin layer chromatography
TFA	-	trifluoroacetic acid
a_w	-	water activity
ZEA	-	zearalenone

Abstract

Spice oils are known to exhibit antifungal activity and therefore have the potential to control mycotoxin production. There is a need in the food industry to find measures to control mycotoxins that are frequently associated with grains that form the staple diet of the majority of the population in South Africa. Clove, cinnamon, oregano, tumeric, eucalyptus, neem, aniseed, mace and nutmeg oils were tested to determine their inhibitory potential against growth of *Aspergillus parasiticus* and *Fusarium moniliforme* using the agar overlay technique. Varying concentrations of the spice oils, ranging from 0.1 ppm to 2.0 ppm, were incorporated into broth cultures of *A. parasiticus* and maize patty cultures of *F. moniliforme*. Levels of production of aflatoxins and fumonisin were determined using standard thin layer chromatography and high-pressure liquid chromatography methods. In addition, the active component of the spice oils were isolated, characterised and tested. The inhibitory potential of these compounds for field use was tested by incorporating clove oil, whole cloves and ground cloves in samp. The levels of aflatoxins and fumonisins were subsequently determined. Clove oil at 0.1 ppm prevented growth of *A. parasiticus* and production of aflatoxins B_1, B_2, G_1 and G_2 completely. At 2.0 ppm clove oil reduced the production of fumonisin B_1 by 78%. Eugenol and cinnamic aldehyde were identified as the active components in clove oil. 100 mg/g of whole cloves and ground cloves reduced the fumonisin levels by 92% and 87%, respectively. The findings of this study show that clove oil is a natural preservative. Eugenol and cinnamic aldehyde have potential for use in more established agricultural concerns. The use of whole cloves is a safe, economical and effective method of control for aflatoxins and fumonisins which are commonly co-occurring mycotoxins in local grain.

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

Mycotoxins are toxic metabolites produced by different genera of fungi (*Aspergillus, Fusarium, Penicillium, Claviceps,* etc.) that can contaminate a wide range of foods and feeds. They are natural contaminants and yet are some of the most poisonous toxins known to man. Mycotoxin producing fungi are ubiquitous and widespread at all levels of the food chain. Their presence is considered unavoidable and it is not possible to predict or entirely prevent their occurrence during cultivation, harvest, storage and processing operations by good agronomic and manufacturing practices. Under favourable conditions of temperature and humidity, these fungi grow on certain foods (grains, cereals, oil seeds, edible nuts, dried fruits), resulting in the production of toxins. Mycotoxins can also be metabolised by animals fed contaminated grains and pass into milk, eggs and other organs thus re-entering the food chain once again (Thiel, 1998).

Over three hundred mycotoxins, produced by some 350 fungal species, have been identified. The most agriculturally important toxins are aflatoxins, deoxynivalenol, nivalenol, T-2 toxin, zearalenone, ochratoxin, fumonisins and patulin.

There is a continuous need to protect the health of humans and susceptible animals by limiting their exposure to mycotoxins because of their toxicological manifestations which include acute and chronic ones such as cancer, immunosuppression, mutagenic and oestrogenic gastrointestinal, urogenital, vascular, kidney and nervous disorders (Peraica *et al.*, 1999).

Many countries legislate for or suggest permitted levels of mycotoxins in foods and feeds because of the public health implications and commercial consequences of mycotoxin contamination. In developing countries, mycotoxins also have profound economic implications. Losses in grain and animal production are also increased by losses of markets by non-tariff barriers due to mycotoxin level restrictions. In South Africa, the only mycotoxins that are regulated are the aflatoxins, despite fumonisins being frequently detected in many of the local grains (Thiel, 1998).

Aflatoxin B_1 has been shown to be one of the most potent chemical carcinogens tested in rats and is much more potent than certain man-made substances such as pesticides, insecticides and industrial chemicals (Gold *et al.*, 1984). The high carcinogenic potency of aflatoxins and their occurrence in major human staple foods justify their inclusion in food surveillance programmes. Aflatoxins have been detected at toxicologically significant levels in agricultural commodities which serve as the staple food for many populations (Thiel, 1998). Two important examples are maize and peanuts, which are consumed world-wide. The presence of aflatoxins in maize is, however, of much more significance to rural populations in developing countries, where maize consumption can be as high as 460 grams per person per day, than to industrialised populations. In Switzerland for instance, the maize content of the daily diet has been estimated to be in the order of only 5 grams per person per day (Shephard *et al.*, 1996).

Fumonisin B_1 is another mycotoxin for serious concern, as it occurs in the highest concentration under natural conditions (Thiel, 1998). Little information regarding fumonisins is available compared with aflatoxins to incriminate them as potentially dangerous to human health. The evidence, which has accumulated in the relatively short period since their

isolation, justifies careful monitoring of their presence in human foods. Factors emphasising the potential danger of fumonisin intake to human health include the following:

- Cancer promotion and cancer initiation activity in a rat liver bioassay procedure (Gelderblom *et al.*, 1992). Fumonisin B_1 has also been shown to induce cancer in the liver of rats in controlled experiments and are also the causative agents in the neurotoxic disease of horses, leukoencephalomalacia (Kellerman *et al.*, 1990).
- Pulmonary oedema in pigs (Colvin and Harrison, 1992).
- Hepatotoxic and nephrotoxic in species in which they have been tested (Thiel, 1998).

Fumonisins are especially important to populations dependent on maize as their major staple food. The potential danger of fumonisins to human health has been emphasised by the detection of extremely high levels of fumonisins in the maize produced by subsistence farmers in the Transkei area in South Africa. The quantities in some maize batches were similar to those in feeds which caused leukoencephalomalacia in horses and in rations which induced tumours in the livers of rats (Thiel *et al.*, 1992).

The most important factors indicating the potential danger of fumonisins to human health is, however, that they occur ubiquitously in maize (Shephard *et al.*, 1996). They are virtually always present in the crop, mostly at concentrations below acutely toxic levels but nevertheless in amounts, which could still be significant with respect to cancer development. Unlike many other mycotoxins, which are formed on agricultural commodities under poor storage conditions when the product becomes mouldy, fumonisins are formed in the field with often no mouldiness visible to the naked eye.

Current control measures are aimed at controlling fungal growth and the subsequent mycotoxin formation in stored grains by physical methods, viz., aeration, cooling and modified atmospheres (Pasteur *et al.*, 1988) or by chemical methods, viz., ammoniation, acids, bases and food preservatives (Moreck *et al.*, 1980). Herbs, spices and essential oils have been found to have antifungal activity and some of them also inhibit mycotoxin formation (Chatterjee, 1990; Daw *et al.*, 1995; El-Baroty, 1997). Recently, there has been increasing interest in using naturally occurring compounds, especially essential oils, to limit fungal growth and toxin production. These naturally occurring compounds are known to be quite safe for humans as they have been used not only for flavouring food but also for their antioxidant preservative and medicinal properties.

Thus, the purpose of this study was to evaluate the potential of common spice oils to control/prevent the production of aflatoxin and fumonisin. Experiments were designed to:

- Assess the inhibitory potential of various spice oils on the *Aspergillus parasiticus* and *Fusarium moniliforme* using the agar overlay method. To evaluate the potential of the most effective spice oil to reduce/inhibit aflatoxin and fumonisin production by using analytical techniques such as Thin layer chromatography (TLC) and High pressure liquid chromatography (HPLC).
- To demonstrate the active component of the most effective spice oil that inhibits fungal growth by bioautography.
- Simulation on a laboratory scale, the effectiveness of the most effective spice oil.

1.1. Current status of the mycotoxin problem

Mycotoxins are naturally occurring toxic chemical compounds produced by fungi (moulds) infecting agricultural crops, particularly cereals and oil seeds, both during growth and in storage as well as in processed foods and feeds. The presence of high levels of mycotoxins in grains poses a significant economic threat because animals that consume contaminated grains may perform poorly or even become sick and die. The threat of mycotoxins entering the human food supply through contaminated grain products and exposed animals is of serious concern to regulatory bodies. Currently, the only mycotoxins that are regulated are the aflatoxins. South Africa and most parts of Africa are plagued by the AIDS epidemic, and the majority of these people are at high risk to diseases due to their socio-economic status and their immuno-compromised immunity.

Mycotoxins have been implicated in the aetiology of diseases like kwashiorkor, marasmic kwashiorkor, hepatocellular carcinoma in humans and hepatocarcinomas, encephalopathy and other acute diseases in animals (Miller, 1998). In developing countries, the impact on human health is significant. Up to 36% of disease burdens measured Disability-Adjusted Life Years lost are due to diseases known to be modulated by mycotoxins (Miller, 1998). In both the developing and fully developed market economies, mycotoxins also have a major impact on animal productivity (CAST 1989; Charmley *et al.*, 1994). In the fully developed market economies, these are primarily felt by the grain and animal production industries; while in developing countries, by the subsistence farmer (Bhat and Miller, 1991). In years with high mycotoxin contamination, food producers must spend millions of dollars to perform chemical analyses and source raw material from mycotoxin-free areas (Charmley *et al.*, 1994). In developing countries mycotoxin-free commodities are exported to urban populations (Bhat and Miller, 1991).

The limited supply of food in Africa has forced people not to reject any material that can be used as food even if moulds have changed its organoleptic quality (FAO/UN, 1979). The precise dimensions of the mycotoxin problem are not well understood in countries where climates and agricultural food handling systems are conducive to contamination by moulds and their subsequent production of mycotoxins in food. With increasing populations, especially in Africa, there is growing pressure on the limited food resources. Most African countries have embarked upon increasing food production to meet the needs of their expanding populations. Increased demand for food has also called for large scale storage and transport facilities that have magnified the problems resulting from waste and spoilage, and in addition could result in mycotoxin contamination, presenting, a major health and environmental hazard (FAO, 1987; WHO/ UNEP, 1987).

In Africa, reports on mycotoxicoses have not been as widespread as those for the rest of the world, and this has been due to limited knowledge in disease diagnosis, occurrence and the acute and chronic effect of mycotoxins (Sibanda *et al.*, 1997). To date, only a few mycotoxins have been studied to any extent and studies have looked at their occurrence, detection, and diagnosis of causative mycotoxin for reported cases, prevention and control. Many countries are still struggling to set up prevention, control and surveillance strategies to curb the incidence of mycotoxins in foods. Technological advances in agricultural systems in Africa are still lagging behind. The situation is aggravated by the fact that, in most African countries, communal farmers contribute a significant amount towards the national yield. In most cases, their pre- and post-harvest treatment of crops is not stringent enough to discourage fungal infection, growth and the subsequent production of mycotoxins (Sibanda *et al.*, 1997).

The current status of mycotoxin research in South Africa is outlined by Sibanda *et al.* (1997). Aflatoxins, zearalenone (ZEA), deoxynivalenol (DON), moniliformin (MON), fumonisins and patulin are the major mycotoxins, which have received attention in South Africa. Thiel *et al.* (1982) carried out a study on ZEA in Transkei in an attempt to establish the cause for the aetiology of oesophageal cancer. This study sought to establish a statistical relationship between oesophageal cancer rates in the Butterworth district of Transkei in South Africa and the ZEA levels in maize. Thiel *et al.* (1982) reported the occurrence of DON in maize from this region. Results from this study showed that ZEA and DON caused toxicity and feed refusal in experimental animals due to *F. graminearum* contamination. In an effort to establish the causal mycotoxin in the aetiology of human oesophageal cancer in Transkei, other *Fusarium* mycotoxins were isolated, including MON. This was the first time MON was found to be naturally occurring in maize (Sibanda *et al.*, 1997). This study was carried out in a high oesophageal cancer rate area with an attempt to link MON to this ailment (Marasas *et al.*, 1988). In the same study, T-2 toxin, nivalenol and tricarbollyic acid were found to occur with MON, fumonisin, DON and ZEA (Sydenham *et al.*, 1990).

Maximum permissible levels of mycotoxins in most countries have only been considered for aflatoxins. The regulatory programmes for mycotoxins in Southern African countries are outlined in Table 1.1. Aflatoxins and patulin are the only mycotoxins that are currently being monitored.

Table 1.1: Mycotoxin regulations for foods in Southern African countries (Sibanda *et al.*, 1997)

Country	**Authority institution**	**Food item**	**Maximum permissible level (ppb)**	**Mycotoxin**
Zimbabwe	Ministry of Agriculture	Peanut All foods Maize	20 20 4	AFB_1 AFB_1
Malawi	Government	All foods Peanut for export	45 5	Aflatoxins Aflatoxins
Senegal	Ministry of Commerce and Public Health	Peanut and peanut products Straight foods Foodstuffs ingredients	50 300	Aflatoxin
Nigeria	Food and Drug Administration	Foodstuffs Fluid milk All foods Infant foods	50 1 20 0	Aflatoxins Aflatoxins Aflatoxins Aflatoxins
Kenya	Ministry of Health	Peanut and peanut products Vegetable oil	20 20	Aflatoxins Aflatoxins
South Africa	Government Gazette No. 4959-16.01	All foods Fruit juice All foods	5 10 50	AFB_1 $B_1+B_2+G_1+G_2$ Patulin
Ivory Coast	Ministry of Public Health; Production of Commerce	Straight Animal foods Feedstuffs Swine and poultry Dairy cattle	100 75 38 50	$B_1+B_2+G_1+G_2$

Unlike the cereals grown in North America, the co-occurrence of two or more mycotoxins in crops is apparently common in Asia and Africa (Yamashita *et al.*, 1995; Wang *et al.*, 1995; Doko *et al.*, 1996). In domestic and laboratory animals, there is increasing evidence of synergism between these potent toxins (Miller, 1991; 1993). The combination of aflatoxin and fumonisin is of special concern in that fumonisin B_1 is a powerful cancer promoter and aflatoxin B_1 is a potent human carcinogen (IARC, 1993; Riley *et al.*, 1996). In many regions

of Africa and Asia, a significant percentage of the population is seropositive for hepatitis B and/or C. The potency of these carcinogenic viruses is affected by aflatoxin exposure (IARC, 1993; Quain *et al.*, 1994). The common co-occurrence of the potent human carcinogen, aflatoxin and the cancer-promoting fumonisin B_1 in Africa, and high incidence of fumonisins in rural South Africa encourages the need to investigate control measures that may be used in these conditions.

1.2. Aflatoxins

Aflatoxins are a group of secondary metabolites produced by the molds *Aspergillus flavus* and *Aspergillus parasiticus.* Little was known about these highly toxic and highly carcinogenic compounds before the outbreak of the 'Turkey X disease' in 1960 in England. The cause of poultry deaths was shown to be dietary in nature and associated with the Brazilian peanut meal used as a common ingredient in the feed (Blount, 1961). A toxic component isolated from chloroform extracts of the meal induced the disease in ducklings (Alcroft *et al.*, 1961). In 1961, Sargeant *et al.* described a paper chromatographic technique for detection of the toxin based on a blue-fluorescing spot under UV light. Nesbitt *et al.* (1962) and Hartley *et al.* (1963) showed that the single fluorescent "aflatoxin" spot could be resolved into four main components by thin layer chromatography on silica gel using chloroform-methanol as the mobile phase. Two spots fluoresced blue and two fluoresced green under UV light resulting in the assignment of the trivial names, B_1, B_2, G_1 and G_2. The molecular formulae for aflatoxin B_1 and aflatoxin G_1 were determined as $C_{17}H_{12}O_6$ and $C_{17}H_{12}O_7$, respectively, from elemental analysis and mass-spectral data (Zaika and Buchanan, 1987).

1.2.1. Structure

The structures of aflatoxins B_1 and G_1 were determined by Asao *et al.* (1965) and aflatoxins B_2 and G_2 where shown to be the dihydro derivatives of aflatoxin B_1 and G_1, respectively (Fig 1.1). The basic skeleton of the aflatoxin molecule is a condensed bisfuran/coumarin ring system. Not all strains of *A. flavus* and *A. parasiticus* produce aflatoxins. Toxigenic *A. parasiticus* strains generally produce aflatoxins B_1, B_2, G_1 and G_2, while toxigenic *A. flavus* strains produce B_1 and B_2 toxins. Aflatoxins M_1 and M_2 are typically mammalian metabolites of aflatoxin B_1 and B_2, respectively, although they may also occur in the fungal cultures. Other aflatoxins isolated from *A. flavus* cultures include aflatoxin B_{2a} and aflatoxin G_{2a} (Dutton and Heathcote, 1966). Aflatoxin B_1 is the most commonly produced aflatoxin and is also the most toxic and carcinogenic (Zaika and Buchanan, 1987).

Since the discovery of aflatoxins, numerous substances and environmental conditions have been examined in an effort to find an agent that would effectively control growth and aflatoxin production by *A. flavus* and *A. parasiticus.* Accordingly, considerable information is available on production of aflatoxins (Zaika and Buchanan, 1987), both under laboratory conditions and in different food and feed commodities. Comparatively less is, however, known about the regulatory mechanisms controlling aflatoxin biosynthesis.

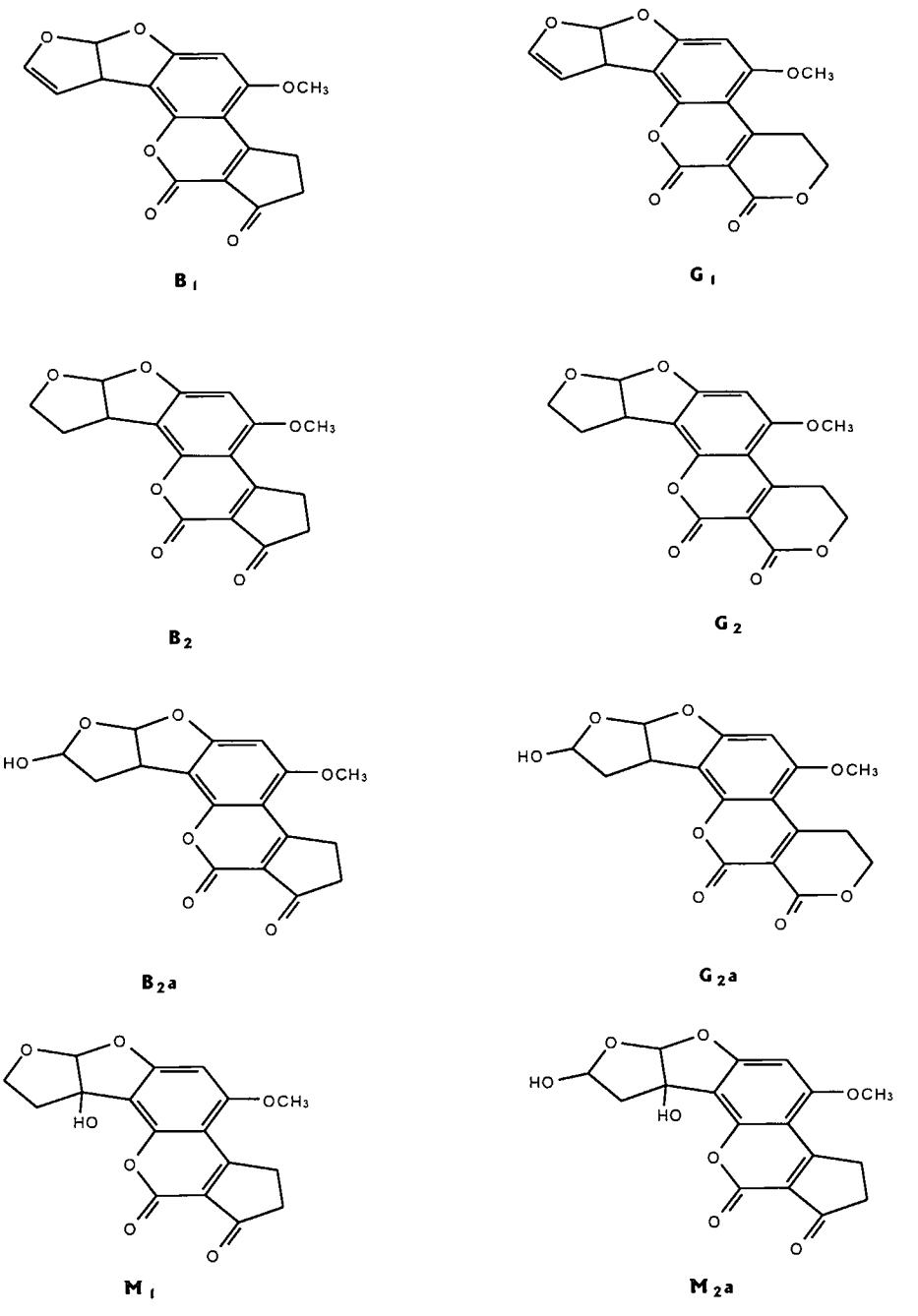


Figure 1.1: Structures of the major aflatoxins (Takahashi and Beebe, 1979).

1.2.2. Biosynthesis of aflatoxin B_1

According to Biollaz *et al.* (1968, 1970), aflatoxin B_1 is an acetate-derived compound. The skeleton of aflatoxin B_1 is derived entirely from acetate while the methoxy group carbon is derived from methionine. Experimental evidence currently available indicates that aflatoxin B_1 is synthesized from acetate by way of the C_{20} polyketide intermediate compound involving the sequential formation of norsolorinic acid, averantin, averufin, versiconal hemiacetal acetate, versicolorin A and sterigmatocystin (Fig. 1.2) (Steyn *et al.*, 1980; Applebaum and Marth, 1981; Bennet and Christensen, 1983; Prasad, 1983).

A number of these intermediate compounds have been detected in cultures of aspergilli other than *A. flavus* and *A. parasiticus*, particularly *A. versicolor*. It is of interest to note that many *Aspergillus* species can produce sterigmatocystin, an intermediate compound thought to occur late in the metabolic pathway.

While many strains that produce only the B-aflatoxins have been isolated from nature, strains that produce only the G-aflatoxins are unknown (Bennet and Christensen, 1983). Therefore most workers concur with Biollaz *et al.* (1970) that aflatoxin G_1 is derived oxidatively from aflatoxin B_1. Strains of *A. flavus* accumulating aflatoxin B_2 but not B_1 have, however, been reported (Gunasekaran, 1981). According to Shroeder and Carlton (1973), aflatoxin B_1 is not a precursor of aflatoxin B_2. Dutton (1985) also showed that aflatoxin B_1 and B_2 originate independently from versicolorin A and versicolorin C, respectively. These workers have also shown that aflatoxins M_1 and M_2 arise from aflatoxins $B_1$and B_2, respectively.

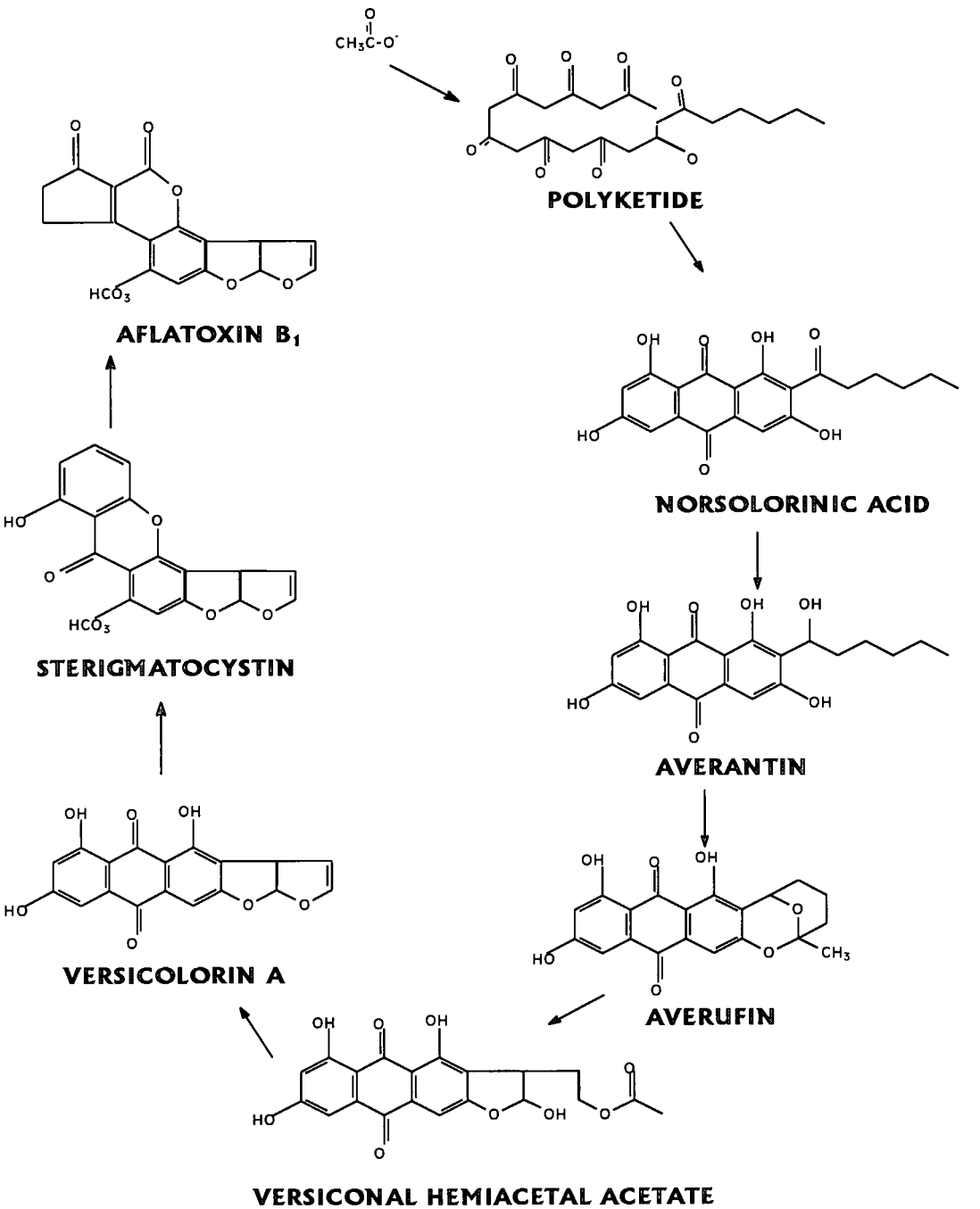


Figure 1.2: Pathway for biosynthesis of aflatoxin B_1 (Zaika and Buchanan, 1987).

1.2.3. Substances affecting aflatoxin production

A number of substances have been identified that inhibit toxin production, which is often due to their effect on fungal growth (Table 1.2). Of the compounds tested, some had little or no effect on aflatoxin production and others induced increased growth and/or aflatoxin production by *A. flavus* and *A. parasiticus*. Compounds that stimulated aflatoxin production included acetone, ethanol, isoprothiolane, nisin and phytate. Compounds that had no effect included butylated hydroxytoluene, propyl gallate, pyridazinone herbicides and steriodal carbamates (Table 1.2).

A range of other compounds including antimicrobial agents, antibiotics and pesticides also influence aflatoxin production (Table 1.3). Some of these compounds inhibit aflatoxin formation by inhibiting the growth of *A. flavus* and *A. parasiticus*.

The effects of plant derived substances and their components on *A. flavus* and *A. parasiticus* have also been studied (Table 1.4). All of these compounds exhibited inhibitory effects on fungal growth, viz., clove oil (Bullerman *et al.*, 1977), cinnamon (Azzouz and Bullerman, 1982), phytoalexins (Wotton and Strange, 1985) and plant extracts (Bahk and Marth, 1983b). It would appear that naturally occurring compounds of botanical origin show more inhibitory potential than chemical compounds, pesticides and antimicrobial agents.

Table 1.2: The effect of various compounds on growth of and/or aflatoxin formation by *Aspergillus flavus* and *Aspergillus parasiticus*

SUBSTANCE	REFERENCE	REMARKS
Acetone	Bennett *et al.*(1976)	Resting cells; aflatoxin stimulated at < 1M*
	Floyd *et al.* (1982)	Stimulatory at 0.1%
BHT (butylated hydroxytoluene)	Foudin *et al.* (1978)	No effect
	Fung *et al.* (1977)	No effect
	Lin and Fung (1983)	No effect
Ethanol	Bennett *et al.*(1976)	Resting cells; aflatoxin Stimulated at <1M*
DL-ethionine	Reiss (1982)	No effect on growth at 1000μg/ml; enhanced growth at 10 μg/ml.
Isoprothiolane	Yousef and Marth (1984)	Stimulates aflatoxin B_1 accumulation*
Methoxychlor	Draughon and Ayres (1981)	100 mg/ml inhibits aflatoxin by 6%*
Nisin	Yousef *et al.* (1980)	Initial delay of growth followed by stimulation of toxin*
Phytate	Ehrlich and Ciegler (1984)	No effect at pH 4.5; aflatoxin production stimulated at pH 6.0*
	Ehrlich and Ciegler (1985)	No effect; degradation to phosphate
Propyl gallate	Lin and Fung (1983)	No effect
Pyridazinone herbicides	Bean and Southall (1983)	No effect on growth (20-60 μl/ml).
Steroidal carbamates	Jung and Suh (1985)	No effect on growth; Aflatoxin inhibited*

* - effect on aflatoxin production.

Table 1.3: Antimicrobial, antibiotics and pesticides that inhibit aflatoxin formation through inhibition of growth of *Aspergillus flavus* and *Aspergillus parasiticus*

SUBSTANCE	REFERENCE	REMARKS
p-aminobenzoic	Davies and Diener (1967)	
Antifungal agents	Holmquist *et al.* (1983)	Aflatoxins not analysed
Benzoic acid derivatives	Chipley and Uraih (1980)	
t-butrylhydroquinone	Lin and Fung (1983)	
Butyric acid	Ghosh and Häggblom (1985)	
Coconut shell smoke	Arseculeratne *et al.* (1976)	
Crystal violet	Stewart *et al.* (1977)	
Diacetyl	Rama Devi and Polasa (1985)	total inhibition at 250 ppm
2,6-dichloro-4-nitroaniline	King *et al.* (1979)	MIC 12.5 ppm
Dimethyl sulfoxide	Bean and Rambo (1975)	
Fatty acids	Mayura *et al.* (1985)	
	Tiwari *et al.* (1986)	
Ferulic acid	Sinha and Singh (1981)	
	Bilgrami *et al.* (1981)	cereals and seeds
Fluoroacetic acid	Reiss (1982)	conidia inhibited; no growth at >600 ppm
Gentian violet	King *et al.* (1979)	MIC 10 ppm
p-hydroxylbenzoic acid	Sinha and Singh (1981)	
Lauric acid derivatives	Rama Devi and Polasa (1984)	
	Chipley *et al.* (1981)	
Malachite green	King *et al.* (1979)	MIC 25 ppm
Natamycin (pimaricin)	Azzouz and Bullerman (1982)	
Nystatin	Yousef and Marth (1984)	
Obtusastyrene (4-cinnamylphenol)	Jurd *et al.* (1971)	MIC 100 ppm
Pentachlorophenol	King *et al.* (1979)	MIC 5 ppm
Phenylboric acid	Reiss (1982)	total inhibition at 1000 ppm
o-phenylphenol	Jurd *et al.* (1971)	MIC 100 ppm
Phloroglucinol	Sinha and Singh (1981)	
Propionic acid	Ghosh and Häggblom (1985)	
	Tsai *et al.* (1984)	
	Stewart *et al.* (1977)	Fungicidal at >3.0 ppm
	Buchanan and Ayres (1976)	
	Mallozi *et al.* (1985)	
Proyl paraben	Jurd *et al.* (1971)	
Pyrocatechol	Sinha and Singh (1981)	
Sevin (carbaryl)	Draughon and Ayres (1981)	
Sodium nitrite	Obioha *et al.* (1983)	Temp. inhibition.; converts NO_2^- to NO_3^-
Sodium chloride	Uraih and Chipley (1976)	
	Buchanan and Ayres (1976)	
	El-Gazzar *et al.* (1986)	
Sucrose esters	Marshall and Bullerman (1986)	
6-thioguanine	Reiss (1982)	total inhibition at 1000ppm; increased aflatoxin B_1 at 10 ppm
Thiram 80	King *et al.* (1979)	MIC 5 ppm
Tolnaftate	Khan *et al.* (1978)	yeastlike form at 100 ppm; total inhibition at 500 ppm
o-vanillin	Sinha and Singh (1981)	
	Bilgrami *et al.* (1982)	cereals and seeds
Wood smoke	Alverez-Barrea *et al.* (1982)	
	Uraih and Ogbadu (1982)	

Table 1.4: Botanical substances and their effects on *Aspergillus flavus* and *Aspergillus parasiticus* growth

SUBSTANCE	REFERENCE	REMARKS
Anethole	Hitokoto *et al.* (1980)	
Caffeic acid	Swaminathan and Koehler (1976)	
Carrot root extract	Batt *et al.* (1980)	raw carrots
Carrot seed oil	Batt *et al.* (1983)	
Cinnamaldehyde	Bullerman *et al.* (1977)	
Cinnamon	Bullerman (1974)	
	Hitokoto *et al.* (1978)	Complete inhibition
	Azzouz and Bullerman (1982)	complete inhibition
Cinnamon oil	Bullerman *et al.* (1977)	
Citral	Batt *et al.* (1983)	
Citrus oils	Karapinar (1985)	
	Alderman and Marth (1976)	
Clove oil	Bullerman *et al.* 1977)	
Corn ear volatile compounds	Gueldner *et al.* (1985)	
5,7-dimethoxyisoflavone	Turner *et al.* (1975)	
Eugenol	Bullerman (1983)	
	Hitokoto *et al.* (1980)	
Geraniol	Batt *et al.* (1983)	
β-ionone	Gueldner *et al.* (1985)	
Limonene	Batt *et al.* (1983)	
	Alderman and Marth (1976)	
o-Methoxycinnamaldehyde	Morozumi (1978)	Complete inhibition at 100 ppm
Onion extracts	Sharma *et al.* (1979)	
	Madhyastha and Bhat (1984)	as substrate
	Seenappa and Kempton (1980)	as substrate
Pepper oil	Madhyastha and Bhat (1984)	
Phytoalexins	Wotton and Strange (1985)	inhibit spore germination
Piperine	Madhyastha and Bhat (1984)	
Plant extracts	Bahk and Marth (1983b)	Honeysuckle flower inhibitory
	Salomao and Purchio (1982)	sisal inhibitory
	Hitokoto *et al.* (1980)	
	Bilgrami *et al.* (1979)	
	Bilgrami *et al.* (1980)	*Ricinus communis* complete inhibition
Saponins	Bahk *et al.* (1985)	
Saponin (ginseng)	Bahk and Marth (1983a)	
Terpinene	Batt *et al.* (1983)	
Terpineol	Batt *et al.* (1983)	
Thiopropanal-*S*-oxide	Sharma *et al.* (1979)	Lachrymator from onion
Spices	Azzouz and Bullerman (1982)	
	Hitokoto *et al.* (1978)	
	Hitokoto *et al.* (1980)	
	Flanigan and Hui (1976)	as substrates
	Karapinar (1985)	
	Llewellyn *et al* (1981a)	as substrates
	Llewellyn *et al* (1981b)	as substrates
	Mabrouk *et al* (1985)	
Thymol	Hitokoto *et al.* (1980)	
	Buchanan and Shepherd (1981)	
White potato	Swaminathan and Koehler (1976)	a phenolic acid

1.3. Fumonisins

Fumonisins are amino-polyalcohols produced by *Fusarium moniliforme* and *F. proliferatum* that contaminate maize and maize-based products world-wide. Fumonisin B_1 has been found as a common contaminant of maize-based food and feed in China, Africa and South America (Ueno *et al.*, 1993; Beardall and Miller, 1994; Wang *et al.*, 1995; Yamashita *et al.*, 1995; Doko *et al.*, 1996; Visconti, 1996). More than ten fumonisins have been isolated and characterized. Of these, fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) are the major fumonisins produced in nature. The most prevalent of these homologs in naturally contaminated maize is FB_1, which causes leukoencephalomalacia (LEM) in horses, pulmonary odema in swines, and cancer in rats. There is also evidence linking fumonisin with cancer in humans (Sydenham *et al.*, 1990).

Mycotoxins from *F. moniliforme* were characterised in 1988 by South African investigators and given the trivial name, fumonisins (Gelderblom *et al.*, 1988). This compound is part of a family of toxins discovered in 1988 by two groups working independently. One group was investigating the cause of human oesophageal cancer in parts of Southern Africa, while the other was attempting to find the cause of a well known disease of horses, equine leukoencephalomalacia (ELEM), a liquefaction of the cerebral hemispheres (Marasas, 1996).

Most of the fumonisin research in Africa has been restricted to South Africa where elucidation of the various fumonisin structures (FB_1, FB_2, FB_3, and FB_4) has been carried out by Marasas *et al.* (1988). Use of maize culture material of *F. moniliforme* isolated from mouldy maize used as feed, in Transkei, reproduced ELEM and hepatocarcinogenesis in rats (Marasas *et al.*, 1988). Studies by Sydenham *et al.* (1991) revealed that some commercially available cereal food products from five countries including Egypt and South Africa were

contaminated with fumonisins. Egypt had the highest levels of fumonisins and South Africa the lowest. Fumonisins were found to occur naturally in home-grown maize from households in the Kentani district (high oesophageal cancer incidence area) of Transkei where levels were statistically correlated with the incidence of human oesophageal cancer (Sydenham *et al.*, 1990). Epidemiological evidence strongly suggests a link between dietary fumonisin and oesophageal cancer in Transkei (Marasas, 1996). It has been established that environmental conditions in the high oesophageal cancer areas are conducive to fusarium kernel rot and hence the accumulation of fumonisins. Exposure to other mycotoxins is low. Thus, the populations in the high and low oesophageal cancer areas have similar lifestyles and similar high consumption of maize with one being chronically exposed to fumonisins and the other much less so. There are also areas in Italy and USA where consumption of maize containing fumonisin has been associated with elevated rates of oesophageal cancer (Marasas, 1996).

F. moniliforme is a non-obligate non-host specific pathogen. Thus, the fungus has been found in sorghum, wheat, cotton, beans, tomatoes, peanuts, bananas, soybean, green peppers and several forages. The fungus is, however, predominantly found in maize (90% incidence). The life cycle of the fungus entails a saprophytic and a parasitic stage as shown in Fig. 1.3. During the saprophytic stage, *F. moniliforme* can obtain nutrients from non-living tissues such as dried tissues, thereby producing the infective structure for disease establishment. Usually, most non-obligate pathogens kill their host cells in advance of infection and then obtain nutrients from the non-living tissues. During the parasitic stage, the fungus mostly gets nutrients from living host cells after intracellular colonisation. Disease symptoms and death of maize plants which may not be common cases of the parasitic stage, are responsible for most of the economic losses of maize. Several major diseases causing economic losses are

attributed to *F. moniliforme* including seedling blight, stalk rot, root rot, seed rot and ear rot (Fig.1.4).

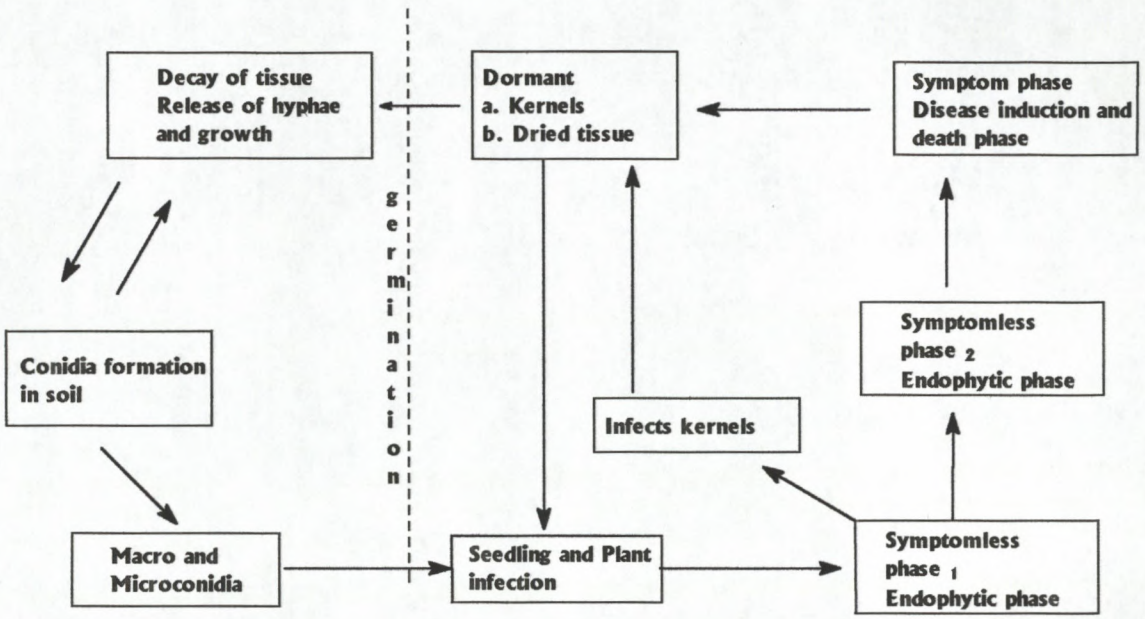


Figure 1.3: Life cycle of *Fusarium moniliforme* on maize (Bacon *et al.*, 1996).

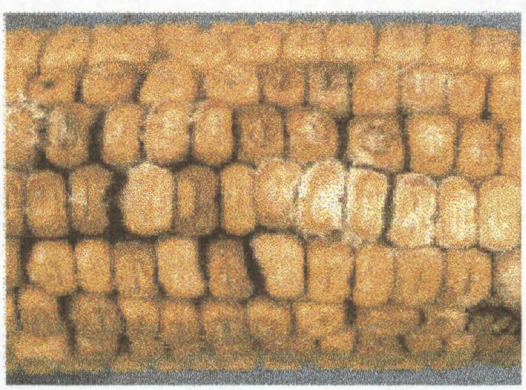


Figure 1.4: Typical *Fusarium* ear rot showing white discolouration of kernels (Bacon *et al.*, 1992).

Most maize and maize-based foods and feeds are likely to be contaminated with fumonisins at detectable levels (Marasas, 1996). The degree of contamination is highly dependant on environmental conditions as shown in Table 1.5. Environmental stresses such as heat and drought may be factors that increase colonisation by *Fusarium* spp. and stimulate the production of fumonisins.

Table 1.5: Levels of fumonisins associated with high incidence of human oesophageal cancer (EC)

The high human EC areas	**Fumonisin levels (μg/g)**	**References**
Transkei, South Africa (1985)	10.2 (good maize) 63.2 (mouldy maize)	Rheeder *et al.*, 1992
Transkei (1989)	6.7 (good maize) 140.5 (mouldy maize)	Rheeder *et al.*, 1992
Charleston, South Carolina, USA	0.17 – 2.4 FB_1 (maize meal samples)	Sydenham *et al.*, 1991
Linxian County, People's Republic of China	18 – 155 FB_1 (heavy mouldy maize, 16 of 31 household samples and 20 – 60 FB_1 (the other 15 samples) with low levels of aflatoxins	Chu and Li, 1994
Linxian County	0.87 FB_1 and 0.45 FB_2	Yoshizawa *et al.*, 1994

1.3.1. Physical and chemical properties of the fumonisins

At present, six fumonisins are known to be metabolites of *F. moniliforme* (Cawood *et al.*, 1991). Their chemical structures are shown in (Fig 1.5). FB_1 is the most abundant. Both FB_1 and FB_2 are described as amorphous solids (Gelderblom *et al.*, 1988); with a melting point of 103 -105°C being reported for FB_1 by Vesonder *et al.* (1992). Fumonisins are strongly polar compounds. They are soluble in water, more soluble in acetonitrile-water and also very soluble in methanol, but are not soluble in non-polar solvents (Laurent *et al.*, 1990).

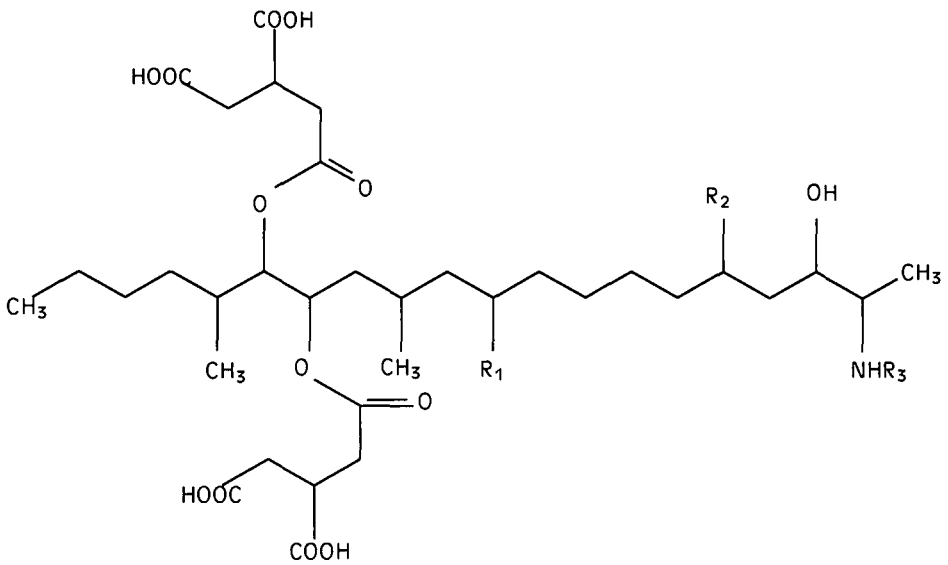


Figure 1.5: Structure of fumonisin B_1 ($R_1 = R_2 = OH$, $R_3 = H$), fumonisin B_2,($R_1 = H$, $R_2 = OH$, $R_3 = H$), fumonisin B_3 ($R_1 = OH$, $R_2 = R_3 = H$), fumonisin B_4 ($R_1 = R_2 = R_3 = H$), fumonisin A_1 ($R_1 = R_2 = OH$, $R_3 = CH_3CO$), and fumonisin A_2 ($R_1 = H$, $R_2 = OH$, $R_3 = CH_3CO$) (Cawood *et al.*, 1991).

1.3.2. Mode of action of fumonisins

There is considerable interest in the mechanism of action of the fumonisins FB_1 and FB_2. The structural similarity of fumonisins with sphingolipids has led to the hypothesis that fumonisins may inhibit both sphingosine and sphinganine *N*-acyltransferase, as shown in Fig. 1.6. Sphingolipids have important roles in membrane and lipoprotein structure, cell-cell communication, interaction between cells and the extracellular matrix, regulation of growth factor receptors and as second messengers for a wide range of factors, including the tumour necrosis factor and interleukin-1 (Marasas, 1996). The resulting accumulation of free sphinganine inhibits growth and is toxic to the cells. The loss of complex sphingolipid synthesis would be expected to alter cell behaviour and could also lead to cell death, as is suggested by the findings with serine palymityltransferase the initial enzyme of sphingolipid biosynthesis (Wang *et al.*, 1991; Norred *et al.*, 1992; Yoo *et al.*, 1992).

The fumonisins (B_1, B_2, and hydrolysed B_1) were shown to be the first naturally occurring specific inhibitors of *de novo* sphingolipid biosynthesis (Wang *et al.*, 1991). In addition, to inhibition of *de novo* biosynthesis, fumonisins also appear to inhibit the reacylation of sphingosine within the sphingolipid turnover pathway and may inhibit acylation of dietary sphingosine. This raises the interesting possibility that the animal disease associated with the consumption of fumonisins may result from altered sphingolipid biosynthesis. While other compounds and conditions can alter the kinetics and/or direction of carbon flux in the biosynthetic and turnover pathways, only fumonisins have been shown to specifically inhibit ceramide synthases (sphingosine and sphinganine *N*-acyltransferase) (Riley *et al.*, 1993).

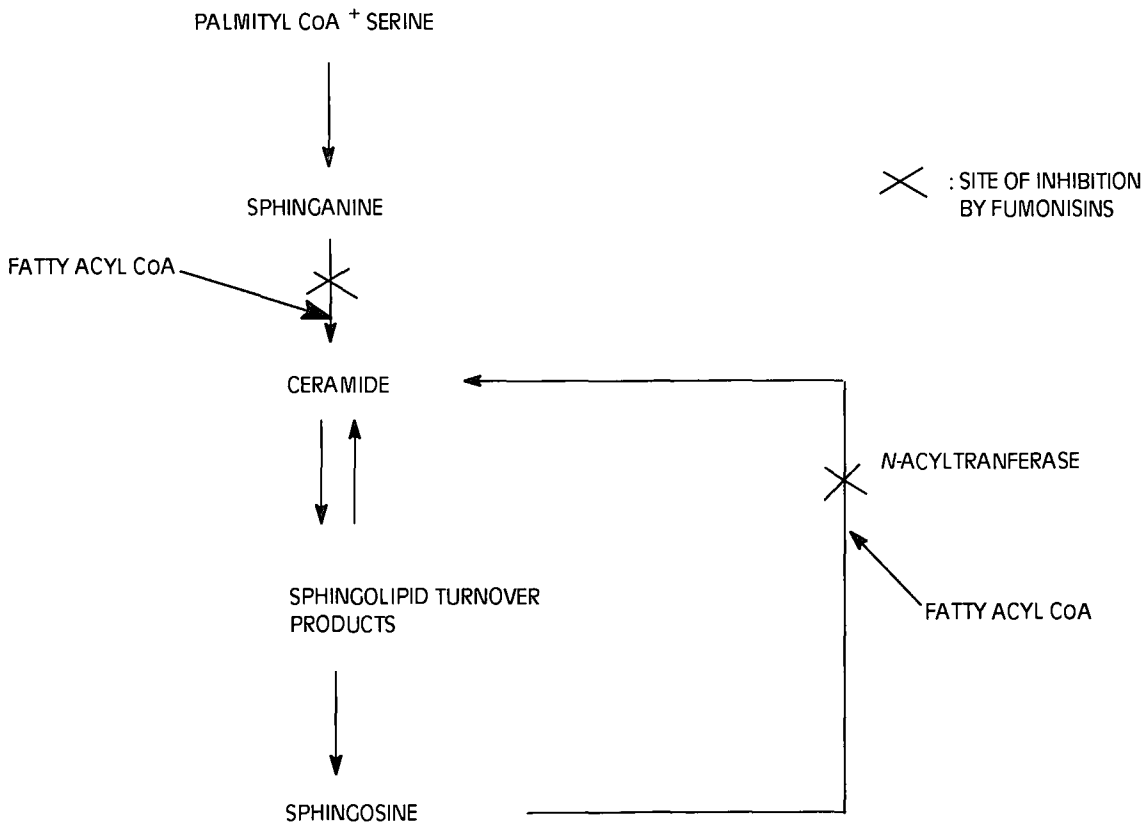


Figure 1.6: Disruption of the sphingolipid pathway by fumonisins.

1.3.3. Control of fumonisins

Fusarium mycotoxins found in food are generated primarily in the field, although some toxin synthesis may occur during storage. Temperature and moisture conditions during the growing season and insect infestations are critical factors affecting fungal infection and toxin synthesis. Wet and cool weather during flowering of wheat is conducive to infection with *F. graminearum* which produces DON, while late season rainfall increases infection of maize silk with *F. moniliforme*, the main producer of fumonisins (Munkvold and Desjardins, 1997).

Although climate cannot be controlled, investigators are exploring various approaches to influence rates of infection and toxin production in the field. Breeding for resistance to *Fusarium* infection has been carried out by traditional methods, with several varieties of wheat developed in China showing some degree of resistance to these fungi (Mesterhazy, 1997). These programs in Canada have produced varieties of maize with resistance to infection by fungal growth through the maize silk. More recently, genetic engineering techniques have been utilised to transfer antifungal genes from plants or microbes into wheat and potatoes (Mesterhazy, 1997). Some preliminary experiments demonstrated that these plants have an enhanced resistance to *Fusarium* spp.

Other field conditions affecting the infection process and fungal growth may also be amenable to change. Evidence is accumulating that infection rates are higher in crops planted in fields previously planted with maize, particularly when crop residues are left in the field. Fungicide treatments were found to reduce the incidence of *Fusarium* infections in wheat but as yet, there are no fungicides approved for use late in the season (at flowering). Research into the potential for using microbes antagonistic to *Fusarium* spp. is being conducted with rice plants

susceptible to *F. moniliforme* (Rosales and Mew, 1997). Preliminary results indicate that some species may suppress growth of toxigenic fungi.

Since these toxins are heat-stable, ordinary cooking and procedures for heat processing do not substantially reduce toxin levels. Other processing steps may, however, decrease toxin levels. Recent data from Kansas (Rumbeiha and Oehme, 1997) and the U.K. (Patel *et al.*, 1997) demonstrated that certain maize products contain relatively high levels of fumonisins: maize meal (up to 39 ng/g), polenta (up to 2124 ng/g) and maize flour (up to 167.7 ng/g). Other maize-based foods generally had low mycotoxin levels but some individual samples contained significantly higher levels (Patel *et al.*, 1997). Still other products including maize oils, maize syrups, tortilla shells and canned maize had little or no detectable fumonisins. These differences in toxin levels in different products may result from physical or mechanical steps during processing. Since fumonisins are present at higher concentrations in rice husks and in maize screenings from infected plants, polishing of rice and removal of small particles from maize processing can significantly reduce toxin levels. Aqueous extraction of fumonisin-contaminated material also removes significant amounts of this water-soluble toxin and thereby reduces toxicity of the material (Voss *et al.*, 1996). Wet milling of contaminated maize produces a starch fraction with very little or no fumonisin while the steep water, gluten, germ and fibre fractions contain most of the toxin (Munkvold and Desjardins, 1997).

Removal or destruction of mycotoxins by means of chemical reactions has also been investigated. The low incidence of fumonisins in tortillas suggested that nixtamalization (treatment of maize flour with calcium hydroxide) prior to making of tortillas may detoxify fumonisins (Voss *et al.*, 1996). Although nixtamalisation does hydrolyse fumonisins, experiments with rats demonstrated that the fumonisin compounds are not completely

detoxified and still exert hepatotoxic and nephrotoxic effects (Voss *et al.*, 1996). Ammoniation (which can detoxify aflatoxins) and fermentation are not effective in detoxifying fumonisins. A recently described procedure for reacting the amine group of FB_1 with fructose in a non-enzymatic browning reaction has been shown to eliminate the hepatotoxicity and cancer-promoting ability of this toxin (Lu *et al.*, 1997). Further experiments are under way to determine the stability of this conjugate and the possible practical applications of this technique. Although the effect of spice oils/ naturally occurring compounds on aflatoxin production has been widely reported in literature no reports on the effects of spice oils on fumonisin has been documented.

1.4. Spices as biocontrol agents against fungi

In modern food processing, spices are added to foods primarily as flavouring agents. The commonly used spices and their botanical names are listed in Appendix 3. The functional properties viz. flavour, aroma and antimicrobial factors of a spice reside in its essential oil. The antimicrobial properties of these spices have been known and utilised for centuries. For example, cinnamon, cumin and thyme were used in mummification in ancient Egypt. Spices were used in ancient India and China to preserve foods as well as for medicinal purposes. In ancient Greece and Rome, coriander was used to extend the preservation period for meat, and mint was used to prevent milk from spoiling (Hirasa and Takemasa, 1998). Infectious diseases, such as cholera and typhus, prevalent in the medieval period, were treated using spices, presumably for bactericidal as well as for medical reasons.

Research on the antimicrobial properties of spices began in the 1880s and mustard, clove and cinnamon were proven to have antimicrobial effects. Since the early twentieth century, research on spice extracts and the essential oils of spices has been conducted in this regard.

Zaika (1988) reviewed the literature reporting the antimicrobial activity of many spices and classified their inhibitory activities as strong, medium or weak. According to this ranking, cinnamon and clove were listed as exhibiting a strong inhibitory effect. Conner and Beuchat (1984) screened 32 essential oils from plant sources for inhibitory effects on 13 food spoilage and industrial yeasts and identified cinnamon and clove among the most active oils. Oregano and thyme have been classified as exhibiting medium inhibitory activity Martindale (1910) found both clove oil and cinnamon oil to be effective in retarding microbial growth. Other researchers discovered that oregano oil and peppermint oil had relatively strong antimicrobial effects. Most volatile oils and terpeneless oils have exhibited antibacterial activity for at least one of the microbes tested and the volatile oils of cinnamon, cumin, dill, weed and thyme were found to exhibit relatively strong antibacterial activities (Hirasa and Takemasa, 1998).

Studies on the inhibition of toxin formation and growth of toxin-producing fungi utilising spices have been attracting attention recently. Azzouz and Bullerman (1982) reported that a 2% level of oregano in potato dextrose agar completely inhibited the growth of seven mycotoxigenic molds. Maruzzella and Liguori (1958) reported that the volatile (essential) oils from oregano, savoury and thyme possessed substantial antifungal activities against 18 pathogenic and non-pathogenic fungi when tested *in vitro* using the standard zone of inhibition method. Thymol has been shown to be inhibitory to several micro-organisms, including toxigenic fungi (Hitokoto *et al.*, 1980; Buchanan and Shepherd, 1981). Thymol concentrations of ≥ 500 ppm were completely inhibitory to the growth of *A. parasiticus*; while lower thymol concentrations caused partial or transitory growth and toxin production patterns (Buchanan and Shepherd, 1981). Hitokoto *et al.* (1980) reported that a level of ≥ 0.4 mg/ml thymol caused complete inhibition of the growth of both *A. flavus* and *A. versicolor.*

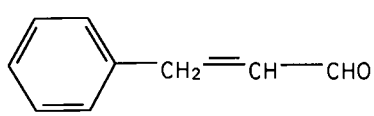
Farag *et al.* (1989b) investigated the effectiveness of the essential oil of thyme against the growth of *A. parasiticus* and its aflatoxin production and compared its activity with that of the oils of sage, clove, caraway, cumin and rosemary. In that study, the essential oil of thyme was found to inhibit mould growth and aflatoxin production at a concentration of 0.2 mg/ml. Oils of cumin, caraway and clove were found to inhibit total aflatoxin production at relatively low concentrations, although their inhibitory actions were less effective than that of thyme. Bullerman (1974), influenced by the fact that cinnamon bread tended not to become mouldy, conducted a study to see whether cinnamon would suppress mould growth as well as prevent aflatoxin production. Mould growth was suppressed with the addition of cinnamon. The addition of 0.02% cinnamon powder suppressed aflatoxin production by 21-25%. Aflatoxin was almost completely inhibited at cinnamon concentration of 2%. Cinnamon bread usually contains 0.5-1.0% cinnamon indicating that mould growth and aflatoxin production should be almost completely inhibited. Ethanol extracts of cinnamon was confirmed to possess strong inhibitory activity to both mould and aflatoxin production. It is interesting to note that the addition of 0.02% ethanol extract of cinnamon inhibited production of aflatoxin B_1, B_2, G_1 and G_2 by 74-89%, although mould growth was not inhibited at this concentration. This would indicate that higher spice concentrations may be necessary to inhibit mould growth than those required to suppress aflatoxin production.

In a later study, Bullerman *et al.* (1977) demonstrated that 200 ppm essential oil of cinnamon was inhibitory to growth and (subsequent) toxin production by *A. parasiticus* and that cinnamic aldehyde, the major component of cinnamon oil, was inhibitory at a level of 150 ppm. Basilico and Basilico (1999) studied the antifungal activity of essential oils of oregano, mint, basil, sage and coriander on mycelial growth on ochratoxin A production by *A. ochraceus* NRRL 3174. The authors suggested the use of the above mentioned spice essential

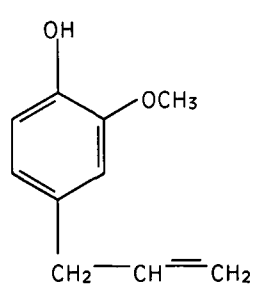
oils in the prevention of mycotoxin contamination in many foods instead of synthetic anti-fungal products.

1.4.1. Antifungal properties of chemical components of spices

In addition to the above research on spices and their essential oils, the antimicrobial activity of various constituents found in these spices have also been studied. The antimicrobial activity of cinnamon, cloves and allspice is attributed to eugenol (2-methoxy-4-allyl phenol) and cinnamic aldheyde (Fig. 1.7), which are major constitutes of the volatile oils of these spices. The anti-aflatoxigenic actions of eugenol may be related to inhibition of the ternary steps of aflatoxin biosynthesis involving lipid peroxidation and oxygenation (Jayashree and Subramanyan, 1999). Cinnamon contains 0.5-1.0% volatile oil, which is made up of 65-75% cinnamic aldheyde and 8% eugenol. Clove buds have an average essential oil content of 17% comprising 93-95% eugenol (Bullerman *et al.*, 1977; Farrell, 1985).



CINNAMALDEHYDE



EUGENOL

Figure 1.7: Structural representation of chemical constituents of clove oil (Mahmoud, 1994).

Eugenol exerted complete inhibition of *Acinetobacter* sp. and yeast at a concentration of 200 ppm and of *Bacillus megaterium* and *Pseudomonas* sp. at 800 ppm. It has been also reported that both *A. flavus* and *A. versicolor* were completely inhibited by eugenol at a concentration of 250 ppm (Hitokoto *et al.*, 1980). Bullerman *et al.* (1977) also reported that clove oil at 250 ppm inhibited growth and aflatoxin production by *A. parasiticus* and that eugenol, the principle component of clove oil, was inhibitory at 125 ppm. The effect of 29 spices (not including cinnamon) on three toxigenic *Aspergillus* sp. (*A. flavus, A. ochraceus* and *A. versicolor*) was reported by Hitokoto *et al.* (1980). Their study showed that clove, star anise and allspice caused complete inhibition of all three *Aspergillus* strains.

The terpenes, carvacrol, *p*-cymene and thymol, are the major volatile components of oregano and thyme and are most likely responsible for the antimicrobial activity (Beuchat, 1976; Zaika and Kissinger, 1981; Farag *et al.*, 1989a). Thymol is a major constituent of seed oils, with carvacrol and *p*-cymene. These compounds are shown to be safe for humans (Dwivedi and Singh, 1999). The essential oil of oregano contains up to 50% thymol, white thyme has 43% thymol and 36% *p*-cymene (Farrell, 1985; Farag *et al.*, 1989a).

Anetol, the major volatile compound of anise seed, and thymol, also showed inhibitory activities against these *Aspergillus* species and against aflatoxin production. Their activities were, however, lower than eugenol. Anetol and thymol inhibited the growth of molds completely at 0.2% and 200 ppm, respectively.

Kurita *et al.* (1979) examined the antifungal activities of perillaldehyde, cinnamaldehyde and other chemical compounds. The antifungal activities of each compound were evaluated for the duration of growth inhibition of 18 fungi. Cinnamaldehyde, at a concentration of 0.66

mM exhibited inhibitory effects on most fungi tested. Perillaldehyde also showed antifungal activity against some fungi, although its antifungal activity was lower than that of cinnamaldehye. Cinnamaldehyde also showed strong inhibitory effects on *Saccharomyces cerevisiae* as well as on *Penicillium* and *Aspergillus* spp. (Bullerman, 1974). Chemical compounds with a hydroxyl group (-OH) or and aldehyde group (-CHO) tend to exhibit strong antimicrobial activity. It is well known that a hydroxyl group can form hydrogen bonds with the active enzyme, resulting in its deactivation. The growth inhibition by the aldehyde group is considered to be due partially to their reactions with sulphydryl groups involved in microbial growth Farag *et al.* (1989a).

1.4.2. Antibacterial and antifungal properties of pungent spices

Most pungent spices are said to have relatively strong antimicrobial properties and this brief has been supported by numerous studies (Hirasa and Takemasa, 1998). Most antimicrobial components of pungent spices are found in volatile components of their essential oils, except in the case of red and black/white pepper. The inhibitory activities of red and black/white pepper, which are not contained in their essential oils, are also thought to be due to their pungent compounds. In this section we consider the antimicrobial effects of individual pungent spices.

1.4.2.1. Mustard and wasabi

Mustard belongs to the Cruciferae family and its seeds are utilised as a spice. Pungent mustard oil is obtained by mixing milled seeds with water. Mustard oil is known to be effective in preventing the growth of a wide variety of micro-organisms. The major antimicrobial principle of black mustard (*Brassica nigra*) is allyl-isothiocyanate, which is produced by the action of glucosinolase myrosinase on glycoside sinigrine, and that of white

mustard (*Sinapis alba*) is *p*-hydroxy-benzyl-isothiocyanate, produced by the action of the same glucosinolase on the glycoside, sinalpine (Hirasa and Takemasa, 1998). Wasabi (*Wasabia japonica*) and horseradish, which also belong to the Cruciferea family, produce allyl-isothiocyanate by the action of myrosinase when their plant tissues are disrupted and they also show strong antimicrobial activities. Many different kinds of isothiocyanate compounds exist in both types of mustard, wasabi and horseradish as forms of glucosides. These compounds are also said to have antimicrobial activities, although their effects are relatively small compared with the major pungent compounds. Most of these compounds are volatile and lose their effectiveness quickly, although their antimicrobial activities are in general, stronger than those of ginger and red pepper.

The effects of allyl-isothiocyanate was compared to those of other spice components including carvacrol (thyme) and salicylaldehyde (cinnamon). Allyl-isothiocyanate was found to retard the growth of most kinds of micro-organism tested, but its effect on gram-positive bacteria was relatively low. Salicylaldehyde and carvacrol were confirmed to be effective for both gram-negative and gram-positive bacteria. The effectiveness of allyl-isothiocyanate against some kinds of molds is enhanced synergistically when it is combined with other compounds. Recently, some products making use of the antimicrobial properties of allyl-isothiocyanate have been commercialised. These are now being used to preserve, prevent mould, decrease the number of microbes and prevent insect infestation in a number of food products, including breads, pasta, lettuce, grains, dried mushroom, and fruits (Hirasa and Takemasa, 1998).

Many studies have reported on how spice oils or pungent compounds inhibit the growth of many types of micro-organisms. White mustard soaked in water was confirmed to be effective against various fungi existing on human skin (Hirasa and Takemasa, 1998).

1.4.2.2. Garlic

Garlic has long been used in foods as a preservative as well as to give its typical flavour. Cavallito and Bailey (1944) and Chain (1941) tested the antimicrobial activities of garlic and its constituents using the cylinder plate method. Natural diallyl sulphides, which are major flavour compounds of garlic, did not show antimicrobial activity against either gram-positive or gram-negative bacteria. Noda *et al.* (1985) investigated the antifungal activity of garlic against typical fungi (*S. cerevisiae* and *A. oryzae*) and found that it was drastically reduced when heated (heating deactivates the enzyme leading to the production of allicin) before it was used.

1.4.3. Mode of action of spice oils

Some essential oils, plant extracts, and oleoresins influence different biochemical and/or metabolic functions, such as respiration and production of toxins or acids. This indicates that the active components in various oils and oleoresins may have different specificities with regard to target sites on or in microbial cells (Conner, 1993). It is reasonable to assume that since many of the components of essential oils, such as eugenol and thymol, are similar in structure to active phenolic antimicrobials, they have a similar mode of action to that of the phenolic antimicrobials (Conner, 1993).

Conner *et al.* (1984) investigated the influence of essential oils and oleoresins on specific metabolic activities, including ethanol production, respiration, and sporulation of yeasts.

They indicated that the effects observed may have been due in part to depletion of cellular energy. Baranowski *et al.* (1980) and Baranowski and Nagel (1983) suggested that allyl hydroxycinnamates, which are similar to the phenolic derivatives found as major constituents in the essential oils tested by Conner and co-workers (1984) and included allspice (eugenol), clove (eugenol), cinnamon (cinnamic aldehyde and eugenol), oregano (carvacrol), thyme (thymol) and savoury (thymol), inhibited the growth of *S. cerevisiae* and *Pseudomanas fluorescens* by a specific mode of action related to cellular energy depletion. In summarising his work, Conner (1983) concluded that essential oils and oleoresins have the ability to affect a variety of fungal enzyme systems, including those involved in cellular energy and structural synthesis. Furthermore, environmental conditions play a role in the expression of antimicrobial activity. Thompson (1990) showed that pH greatly affects that ability of thymol and carvacrol to inhibit the mycelial growth of eight toxigenic aspergilli.

CHAPTER TWO: MATERIALS AND METHODS

In this study, the effect of spice oils were tested for their inhibitory potential on mycotoxin-producing fungi. The spice oils were then investigated for their effect on the growth of *A. parasiticus* and *F. moniliforme* and their toxin production. The active ingredient of the most effective spice oil was subsequently identified. The effect of spice oils were tested by agar overlay technique and by growing the fungi in broth incorporated with the oils. The toxin production was investigated by TLC and HPLC. Bioautography was used to determine the active component of the most effective spice oil and pilot experiments that would simulate the application of these oils in natural environments were studied.

2.1. Extraction and purification of spice oils

All spice oils used in this study were supplied by Robertsons (Durban). The spice oils were distilled from the natural spice using the steam volatile oil extraction method (Somera[1] *per. comm.*). Distilled water was added to 50 g of sample (natural spice) and boiled for approximately 6 hours. The distillate was transferred to a separator and the oil layer was removed. This produced 100% pure oil. The oils were collected, dispensed into vials and stored at room temperature.

2.2. Fungal strains

Aspergillus oryzae (ATCC 22788), *A. flavus* (ATCC 15546), *A. versicolor* (ATCC 44605) and *A. parasiticus* (ATCC 11970) were obtained from the American Type Culture Collection and *Fusarium moniliforme* (MRC 826) from the Medical Research Council, Tygerberg. The

[1] K. Somera. Robertsons Spices. Durban.

fungal cultures were inoculated on Sabouraud agar (Merck) and incubated at 28°C for 4 –7 days until sporulation occurred. The reference and working stock cultures were maintained at 4°C.

2.3. Effect of clove, oregano, eucalyptus and tumeric oils on mycotoxin-producing fungi

A survey to determine the effect of clove, oregano, eucalyptus and tumeric oils on the growth of known mycotoxin-producing fungi, viz. *A. oryzae* (aflatoxins), *A. flavus* (aflatoxin), *A. versicolor* (sterigmatocystin), *F. moniliforme* (fumonisins) and *A. parasiticus* (aflatoxins) was carried out. The fungal cultures were grown on Sabouraud agar (Merck) until sporulation occurred. The plates were washed with distilled water and 2 ml of spores were collected and dispensed into glass Bijou bottles containing 5 ml of sterile distilled water.

The effect of spice oils on fungal growth was determined using the agar overlay method (Leven *et al.*, 1979). A 0.5 ml spore suspension (10^6/ml) (Appendix 1) of each of the above-mentioned fungi were suspended in molten agar (45°C) and overlayed on pre-set Sabouraud agar plates. One well was made in each agar plate and 20 µl of clove, oregano, eucalyptus, and tumeric oils were dispensed into each well. No oils were added to the control plates. Plates were incubated at 28°C for 48 hours and the growth of each fungus on the test plate was compared to the control plate. Results were expressed as zones of inhibition (mm).

2.4. Effect of spice oils on aflatoxin- and fumonisin-producing fungi

The objective was to find a spice oil that could have potential use in the food industry. Several spice oils were tested against *F. moniliforme* and aflatoxin-producing fungi, which were first isolated from, pet food, identified and tested for toxin production.

2.4.1. Isolation of *Aspergillus* species from natural substrates

Aflatoxin-producing fungi were isolated from peanuts, pet food, maize and beans using the single spore technique. Single spore isolation was carried out according to the method recommended by Rong[1] (*per. comm.*). A spore suspension of the *Aspergillus* species was made in 10 ml sterile distilled water (1–10 conidia per drop: under 10 × magnification). An aliquot of 0.1 ml of the spore suspension was distributed evenly onto water agar (15 g agar in 1 liter water) plates. The plates were incubated for 24 hours at room temperature and examined using a dissecting microscope (Kyowa Optical, Model SD-2PL, Japan). Single germinated spores were removed and placed on Sabouraud agar plates and incubated at 28°C for 3 days.

2.4.2. Identification of *Aspergillus* species

Aspergillus species were identified using the key devised by Klich and Pitt (1992). This identification system is based on 3 different media at 2 temperatures. The media and incubation conditions used were: Czapek yeast extract agar (CYA) (Merck), incubated at 25°C and 37°C, malt extract agar (MEA) (Merck), incubated at 25°C and Czapek yeast extract agar with 20% sucrose (CY20S) (Merck) (Appendix 2). Four Petri dishes (90 mm) were used for each culture, 2 of CYA, 1 of MEA and 1 of CY20S. Each plate was inoculated with a spore

[1] I. H. Rong. Mycology Unit. ARC-Plant Protection Research Institute. Pretoria.

suspension at 3 points equi-distant from the edge of the plate and each other. One CYA plate was incubated at 37°C and all of the others were incubated at 25°C in the dark. All plates were observed after 7 days of incubation. All tests were carried out in triplicate. After 7 days isolates were examined both macroscopically and microscopically. Isolates with immature ascocarps at 7 days were re-examined at 14 days or later if necessary.

2.4.2.1 Macroscopic characteristics

The characteristics used in the identification were:

- Colony diameters measured on the agar side (reverse) of the plate. A mean of 3 measurements was used for the key.
- Conidial colours
- Mycelial colours
- Ascorcarp colours
- Exudate (droplets on the surface of the colony) presence and colour
- Colony reverse colour (agar immediately under the colony and the agar adjacent to the colony)
- Colony depth, texture, and sulcation [wrinkling] (the conidia may be borne on the aspergillum in a columnar manner or radiately).

2.4.2.2 Microscopic characteristics

Microscopic characteristics were observed by making wet mount preparations as follows:

Two drops of a general mounting fluid were placed 1 cm apart on a single 25 × 75 mm microscope slide. A small amount of material was removed from the edge of the sporulating portion of a colony on CYA with a needle and placed on one of the droplets. A second sample

of material was removed from close to the centre of the same colony and placed in the same droplet. The same procedure was used to mount material from a colony on MEA into the second droplet on the slide. If CYA or MEA colonies were not sporulating well, material from another growth medium was used. A 22 × 22 mm cover slip was placed over each droplet on the slide. This method was used to observe media-influenced differences in microscopic characteristics. The slide was examined and colours noted. Lacto-fuschin (0.1% acid fuschin in 85% lactic acid) was used to aid observation.

The microscopic characteristics used in the identification were:

- The material on both of the mounts on the slide was examined using low (10-16 ×) and high (40-60 ×) objectives.
- Stipe lengths and general microscopic characteristics were determined under these magnifications.
- Measurements of other structures and determination of surface textures was done with a 100 × oil immersion objective. The shape and size of the vesicles, metulae, phialides, conidia, ascocarps and ascospores were used in the identification.
- The surface texture of the conidia and ascospores were described as smooth, coarsely or finely roughened, striated (i.e. have raised bars) or spinose. The nature of the ascocarp wall was important in determining teleomorph genera.

2.4.3. Isolation of toxin-producing aspergilli

Aspergillus strains isolated were tested for toxin production using a TLC system (Ramjee, 1990). This was done by cultivating the isolated fungi in SMKY broth (200 g sucrose, 0.5 g magnesium sulphate, 3.0 g potassium nitrate, 7 g yeast extract) (Diener and Davis, 1966) and

analysing for AFB_1, AFB_2, AFG_1 and AFG_2 against known standards (Sigma). An aliquot of 0.5 ml of a spore suspension of *A. parasiticus* (10^6 spores/ml) was inoculated into flasks containing 100 ml of SMKY broth and incubated at 28°C for 14 days in an orbital shaker (Certomat, Switzerland). The test was carried out in duplicate. The broth cultures were filtered (Whatman No.4) and concentrated to 10 ml using a solvent evaporating unit (Pierce 18780, Illinois). Concentrated broth was analysed by TLC as described in section 2.6.4.

2.5. Inhibition of *A. parasiticus* and *F. moniliforme* by spice oils

The agar overlay well diffusion technique was used to investigate the effect of clove, cinnamon, oregano, nutmeg, mace, aniseed, tumeric, neem and eucalyptus oils on *A. parasiticus* and *F. moniliforme*. An aliquot of 0.5 ml molten Sabouraud agar (45°C) was inoculated with 10^6 spores/ml of *A. parasiticus* and *F. moniliforme* respectively and overlayed on Sabouraud dextrose agar plates. One well was prepared on each plate, after the overlay had set. Each spice oil was then dispensed into the wells in aliquots of 10, 20, 30, 40 and 50 µl. The control plates did not contain oils. The plates were incubated for 48 hours at 28°C. Results were expressed as zones of inhibition around the wells (mm). All tests were carried out in duplicate. The growth of *A. parasiticus* and *F. moniliforme* on the test plates were compared to the controls.

2.6. Effect of spice oils on aflatoxin and fumonisin production

Spice oils were first tested for their effect on fungal growth. The most effective spice oil was then tested to determine its minimum inhibitory concentration (MIC) and its active ingredient was identified.

2.6.1. Inhibition of aflatoxin production by spice oils

Initial experiments involved testing clove, nutmeg, mace, eucalyptus and tumeric oils on aflatoxin production. An aliquot of 0.5 ml (10^6 spores/ml) of a spore suspension of *A. parasiticus* was inoculated into 100 ml of SMKY broth. Varying concentrations (0.1, 0.25, 0.5, 1, 1.5 and 2 ppm) of the above mentioned oils were added to the broth cultures. No oils were added to the control broth. The flasks were incubated at 28°C for 14 days on an orbital shaker and the toxin produced was identified by TLC. All tests were carried out in duplicate.

2.6.2 Effect of clove oil on aflatoxin and fumonisin B_1 production.

To test the effect of clove oil on aflatoxin production, 100 ml of SMKY broth was inoculated with a spore suspension of *A. parasiticus* (10^6/ml) with increasing concentrations of clove oil (0.03, 0.05, 0.1, 0.15, 0.2 and 0.25 ppm) and flasks were incubated at 28°C for 14 days. Clove oil was not added to the control broth. Each experiment was carried out in duplicate. Aflatoxins were detected qualitatively by TLC (Ramjee, 1990) and quantitatively using HPLC (Takahashi and Beebe, 1979).

To test the effect of clove oil on fumonisin B_1 production, 100 ml of A broth (Appendix 2) (Alberts *et al.*, 1994) was inoculated with a 0.5 ml (10^6 spores/ml) spore suspension of *F. moniliforme* and increasing concentrations of clove oil (0.1 to 5 ppm). Flasks were inoculated at 28°C for 21 days in an orbital shaker. Clove oil was not added to the control broth. All tests were carried out in duplicate. Extraction of FB_1 was carried out with sax columns (Varian) and fumonitest (Vicam). Both the extraction methods proved to be unsuccessful on broth cultures. Therefore the effect of clove oil on FB_1 production was tested in patty cultures.

Maize patty cultures were used to test the effect of clove oil on FB_1 production. A mixture of maize flour (Nyala) and water (1 ml/g) was prepared to make 20 g maize patties, in conical flasks/glass petri dishes according to the procedure of Alberts *et al.* (1993). The flasks were autoclaved for 1 hour at 121°C and 15 psi and autoclaving was repeated the following day. The water activity (a_w) was measured and adjusted to 0.85, using a water activity meter (Novasina, Switzerland). The maize patties were inoculated with 0.5 ml *F. moniliforme* spore suspension (10^6/ml) using varying concentrations (0.2, 0.5, 1.0 and 2.0 ppm) of clove oil. The control was set up without clove oil. Control and test samples were incubated for 25 days at 28°C. Each experiment was conducted in duplicate and the test results were expressed as an average.

2.6.3 Aflatoxin and fumonisin B_1 extraction

The liquid-liquid extraction procedure described by Takahashi and Beebe (1979) was used to extract the aflatoxin from SMKY broth. Three hundred ml of methanol (MeOH) and 200 ml of dichloromethane (CH_2Cl_2) were added to 200 ml broth in a 1000 ml separator. After shaking vigorously for 1 min, the layers were allowed to separate. The CH_2Cl_2 layer was then transferred to a second 1000 ml separator containing 200 ml of water. Extraction was repeated in the first separator with 100 ml of CH_2Cl_2 and added to the second separator. The second separator was then gently shaken for 5-10 seconds and the layers were allowed to separate. The CH_2Cl_2 layer was extracted and evaporated to dryness under a gentle stream of N_2 and concentrated to approximately 10 ml using a solvent-evaporating unit.

Fumonisins were extracted from maize patty cultures using the method recommended by Microsep (Vicam instructional manual : Fumonitest, HPLC). Ten g of patty culture, 1 g of

NaCl and 20 ml methanol:water (8:2) were homogenised using a Culatti mill (Janke and Kunkel 7813, Staufen) at high speed for 1 min and filtered (Whatman No. 4). Eight ml of diluting solution (2.5% sodium chloride, 0.5% sodium bicarbonate, 0.01% Tween-20 in water) were added to 10 ml filtrate. The diluted extract was filtered and the pH adjusted to 5.8. The extract was cleaned using Fumonitest - HPLC affinity columns that were attached to the outlet of Vac -Elut system and connected to a vacuum pump and pressure gauge (Varian: California).

One ml of each filtered dilute extract was pipetted into the columns and a steady flow rate (1 drop/min) was maintained through the column. After the extract had completely passed through the column, 2 ml of diluting solution was passed through the column at a faster flow-rate. The column was then washed with 1 ml distilled water and a clean vial was placed under the outlet column. Toxin was eluted with 1 ml of HPLC grade methanol. The eluate was analysed for FB_1 using TLC and HPLC (Thiel *et al.*, 1993).

2.6.4. Thin layer chromatography of aflatoxins and fumonisin B_1

Aflatoxin standards (0.1 μg/ml of B_1, 0.5 μg/ml of B_2, 0.1 μg/ml of G_1, and 0.5 μg/ml of G_2) were prepared in chloroform. The aflatoxin standard mixture (5 μl) and samples (5 μl) were spotted 1 cm apart on the fluorescing TLC plate (Merck) using capillary tubes. The plate was developed in toluene:ethylacetate:formic acid (6 :3 :1). Once the solvent front had reached 0.5 cm from the top edge of the plate, the plate was removed from the tank and allowed to dry. The aflatoxin spots were viewed under UV light (365 nm). Aflatoxins B_1, B_2, G_1 and G_2 were identified by comparison of their R_f values with those of aflatoxin standards on the same plate.

Fumonisins were analysed by the TLC method outlined by Dutton[1] (*per. comm.*). The mobile phase was made up of butanol:acetic acid:water (6:1.5:2.5) and was allowed to equilibrate in the TLC tank. One mg of FB_1 was dissolved in acetonitrile and water (1:1) to make a stock solution of 50 µg/ml. Aliquots of 10 µl of standard, control and sample extracts were spotted 1 cm apart on normal phase, non-fluorescent silica plates (Merck). Plates were developed and sprayed by the method of Vesonder *et al.* (1990). Plates were sprayed with [*p*-anisaldehyde (5 ml sulphuric acid in 70 ml methanol + 10 ml acetic acid and 500 µl *p*-methoxybenaldehyde)] and heated at 110°C for 5 min. and R_f values calculated.

The R_f values were calculated as follows:

$$R_f = \frac{\text{distance spot travelled from origin}}{\text{distance solvent travelled from origin}}$$

2.6.5. High pressure liquid chromatography (HPLC) of aflatoxins and fumonisin B_1

The method used for aflatoxin detection was that devised by Takahashi and Beebe (1979). Quantitative analysis was carried out by HPLC using a La Chrom D-7000 HPLC system manager (Merck, NT) with a L-7480 fluorescent detector and a column: stainless steel, in-line reverse phase C-18 guard column, an autosampler (L-7280 Robat), 150W Xenon lamp and pump (7100) was used. The mobile phase was made up of water:acetonitrile:methanol (75:15:10). This was vacuum filtered (0.45 µm water HV membrane) and degassed in an ultrasonic water bath for 10 min.

[1] M. F. Dutton. Department of Physiology. University of Natal-Durban.

The HPLC method employing reaction with *o*-phthaldialdehyde (OPA) was adapted for accurate and sensitive quantification of FB_1 in agricultural commodities (Thiel *et al.*, 1993). Quantitative analysis of the fumonisins was carried out using the HPLC system as described above, using an in-line reversed phase C-8 guard column. The mobile phase, methanol:0.1 M sodium dihydrogenphosphate (4:1), was adjusted to pH 3.35 with *o*-phosphoric acid, vacuum filtered (0.45 μm Waters HV membrane) and degassed in ultrasonic water bath for 10 min.

2.6.5.1. Preparation of standards

An aliquot of 20 μl of each aflatoxin standard (Sigma) was dispensed into a 10 ml volumetric flask and was allowed to evaporate to dryness under a gentle stream of N_2. One hundred μl of trifluoroacetic acid (TFA) (Merck) was added to the flask to wet the residues completely. This was then dissolved and diluted to volume in a water [H_2O]:acetonitrile [CH_3CN] (9:1) mixture.

A stock solution of 50 μg/ml FB_1 standard (Sigma) was made with acetonitrile:water (1:1) and stored at -20°C. Forty mg OPA (Merck) was dissolved in 1 ml methanol and diluted with 5 ml 0.1 M di-sodium tetraborate. Fifty μl of 2-mercaptoethanol was added, mixed and stored in an amber bottle in the dark. This reagent is shelf-stable for a period of 1 week. Fifty μl of FB_1 standard was added to 450 μl OPA reagent and mixed. Twenty μl were injected within 1 min. (time between addition of reagent and injection is critical due to fluorescence derivative decay).

2.6.5.2. Preparation of samples

A 1 ml aliquot of aflatoxin sample was evaporated to dryness under a gentle stream of N_2. Two hundred µl of hexane (C_6H_{14}) was added to the sample to dissolve waxy residues. Fifty µl of TFA was then added to completely wet the residue, followed by 5 ml of water: acetonitrile (9:1). Fumonisin samples were prepared by derivatising 50 µl of sample extract with 450 µl OPA. Twenty µl was immediately injected.

2.6.5.3. High pressure liquid chromatography optimisation

The HPLC was initialised via the computer and the method was set up in the D-7000 HMS programme. For the detection of aflatoxin, the detector was set at a wavelength of 365 nm (excitation) and 400 nm (emission). The system was purged and flushed with ultra-pure water, until a steady baseline was obtained. The system was equilibrated at a flow rate of 1.5 ml/min. A sample table was set up with an injection volume of 100 µl.

The detector was set at a wavelength of 335 nm (excitation) and 440 nm (emission) for the detection of FB_1. The system was purged with ultra-pure water until the solvent lines were free of air bubbles. Thereafter, the HPLC was flushed with water until a steady baseline was obtained. The pump was stopped and the solvent delivery line A was added to the degassed mobile phase and the system was equilibrated at a flow rate of 1.2 ml/min. A sample table was set up with an injection volume of 20 µl.

2.6.5.4. Quantification

The aflatoxin content of the samples was calculated from chromatograph peak areas as follows:

$$B_1\ (\mu g/kg) = \frac{P \times C \times V' \times D}{P' \times V \times W}$$

P = area or peak height of sample

C = concentration of B_1 in standard (μg/ml)

V' = μl of standard injected

D = dilution of sample extract (μl)

P' = area or peak height of standard

V = μl of sample injected

W = sample weight (g)

The FB_1 content of the samples were calculated from the chromatographic peak areas as shown below :

$$A\ (ng) = \frac{G \times S}{H}$$

Where:

A = ng of fumonisin present in the test injected into HPLC

G = fumonisin peak area of the test sample

S = amount of fumonisin injected into HPLC

H = fumonisin peak area of standard (50 μg/ml)

The concentration [C] of FB_1 present in the test sample in ng/g was calculated as follows:

$$C\ (ng/g) = \frac{A \times T \times D}{I \times W}$$

Where:

A = as calculated above

T = total volume of derivatised test solution (500 μl)

D = dilution factor (0.11)

I = injection volume used (20 μl)

W = test portion equivalent weight derivatised (2.5 g)

2.7. Analysis of clove oil components inhibitory to *A. parasiticus* and *F. moniliforme*

The main components of clove oil are eugenol and cinnamic aldehyde (Conner, 1993). Authentic samples of eugenol, cinnamic aldehyde and clove oil were separated on TLC. The active components that affect fungal growth were determined using a bioautographic method outlined by Homans and Fuchs (1970).

Five µl of eugenol (Merck):water (1:50), cinnamic aldehyde (Merck):water (1:50) and clove oil:water (1:50), were spotted 1 cm apart on fluorescent TLC plates. The plates were developed in toluene:ethylacetate:formic acid (6:3:1) in a TLC tank. Once the solvent front had reached 0.5 cm from the top edge of the plates, the plates were removed from the tank, allowed to dry and R_f values calculated.

One plate was used as a template and the other plates (test plates) were sprayed with spores of *A. parasiticus* and *F. moniliforme* (10 ml of 10^6/ml) in a fungal culture medium comprising of solution A and solution B. [solution A:7 g KH_2PO_4, 3 g NaH_2PO_4-H_2O, 4 g KNO_3, 1 g NaCl in water (70 ml) and solution B:30 % glucose solution (10 ml). Solution A and solution B were filter-sterilised separately before the spores were added]. The template was not sprayed with spores and was used as a reference plate.

The test plates for *A. parasiticus* and *F. moniliforme* were placed in a moist chamber and incubated for 2 and 4 days, respectively at 28°C. The plates were examined for zones of inhibition at the corresponding spots that the components of clove oil and the standards of eugenol and cinnamic aldehyde separated on the plate.

2.8. Preliminary investigation of the use of cloves for the storage of grain.

The effect of whole cloves and ground cloves on fumonisin production was tested in samp (Nyala). Whole cloves and ground cloves (Robertsons Spices) in varying amounts (0.1- 1.0 g) were each incorporated into 10 g of samp inoculated with 0.5 ml *F. moniliforme* spores (10^6 /ml). The water activity (a_w) was measured and adjusted to 0.85. Cloves were not added to the control. The flasks were incubated at 28°C for 25 days. FB_1 was extracted as described in section 2.5.4. and analysed qualitatively (TLC) and quantitatively (HPLC) for FB_1. The effects of whole clove and ground clove on FB_1 production were compared by determining the percentage reduction of FB_1 in each test relative to the control.

CHAPTER THREE: RESULTS

3.1. The effect of spice oils on mycotoxin-producing fungi

This was a preliminary study to ascertain whether spices have an inhibitory effect on the growth of mycotoxin-producing fungi. The agar overlay technique was used to evaluate the inhibitory effect of four spice oils, viz. clove, oregano, eucalyptus and tumeric against several mycotoxin-producing fungi (Fig. 3.1.). The results indicated that some spice oils had an inhibitory effect. Clove and oregano oils inhibited the growth of *A. oryzae, A. flavus, A. versicolor, F. moniliforme* and *A. parasiticus,* showing zones of inhibition of 14–16 mm and of 12–15 mm, respectively. Eucalyptus and tumeric oils had no effect on the growth of the fungi.

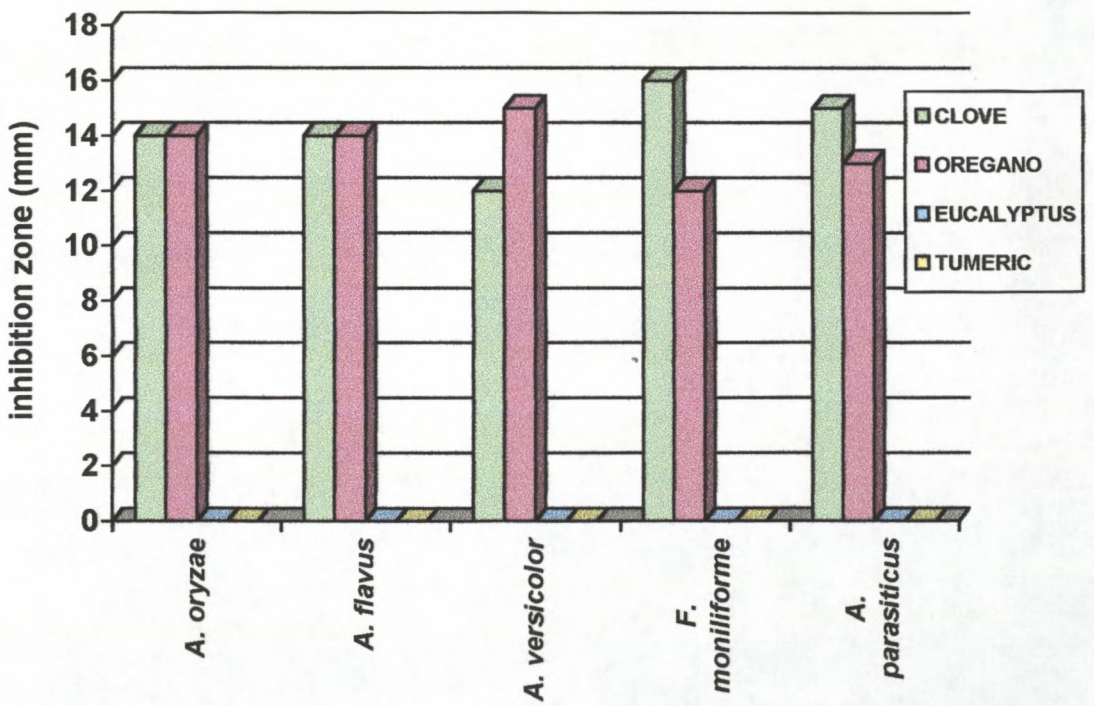


Figure 3.1: Effect of clove, oregano, eucalyptus and tumeric oils on mycotoxigenic fungi using the agar overlay technique.

Once it was established that spice oils were cabable of inhibiting mycotoxin-producing fungi, the study was focused on the effect of the two most prevalent toxins, viz. aflatoxins and fumonisins and the two most commonly co-occuring fungi, viz. *Aspergillus* and *Fusarium* that produce these toxins. *F. moniliforme* produced fumonisin B_1. *A. parasiticus* produces all four aflatoxins. Since an *A. parasiticus* strain was not available, a strain had to be isolated from food products and tested for toxin production as detailed in section 3.2.

3.2. Isolation of aflatoxin producing fungi

3.2.1. Isolation and identification of mycotoxin-producing fungi

Food products, viz. peanuts, pet food (Fig 3.2), maize and beans were source material for the isolation of fungi. The fungi were identified from the key devised by Klich and Pitt (1992). The macroscopic and microscopic characteristics of the fungi isolated are listed in Table 3.1. The distinguishing characteristic between *A. parasiticus and A. flavus* is the texture of the conidia. *A. parasiticus* (produces AFB_1, AFB_2, AFG_1 and AFG_2) was isolated from pet food and peanuts and *A. flavus* (produces AFB_1 and AFB_2) from beans and maize. *A. tamarii* and *A. niger* were also isolated from food products but were not used in this study as they did not produce the required toxins.

Table 3.1: Macroscopic and microscopic characteristics of fungi isolated from food products

	Peanuts	Pet food	Maize	Beans
Macroscopic:				
Colony diameter	CYA – 50 mm	CYA – 50 mm	CYA – 60 mm	CYA – 63 mm
Conidial colours	Dark green	Dark green	Olive green	Olive green
Mycelial colours	White	White	White	White
Exudate	Hyaline	Hyaline	Uncoloured	Uncoloured
Colony reverse	Dark yellow	Dark yellow	Dark yellow	Dark yellow
Microscopic:				
Stipe	Rough-walled	Rough-walled	Rough-walled	Rough-walled
Vesicles	Spherical	Spherical	Spherical	Spherical
Conidia	5 µm, coarsely, rough-walled, globose	4 µm , rough-walled, globose	6 µm, smooth-walled, globose	6 µm, finely rough-walled, globose
Foot cell	Present	Present	Present	Present
Conidiogenous cells	Phialidic conidia	Phialidic conidia	Phialidic conidia	Phialidic conidia
Conidiogenous opening	Basipetal succession	Basipetal succession	Basipetal succession	Basipetal succession
Diagnosis	*A. parasiticus*	*A. parasiticus*	*A. flavus*	*A. flavus*

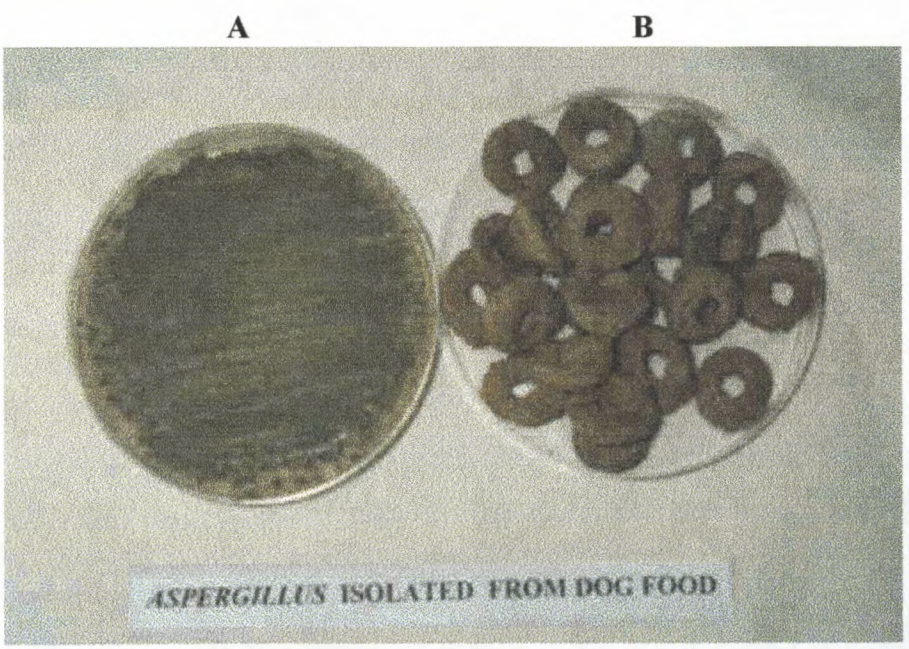
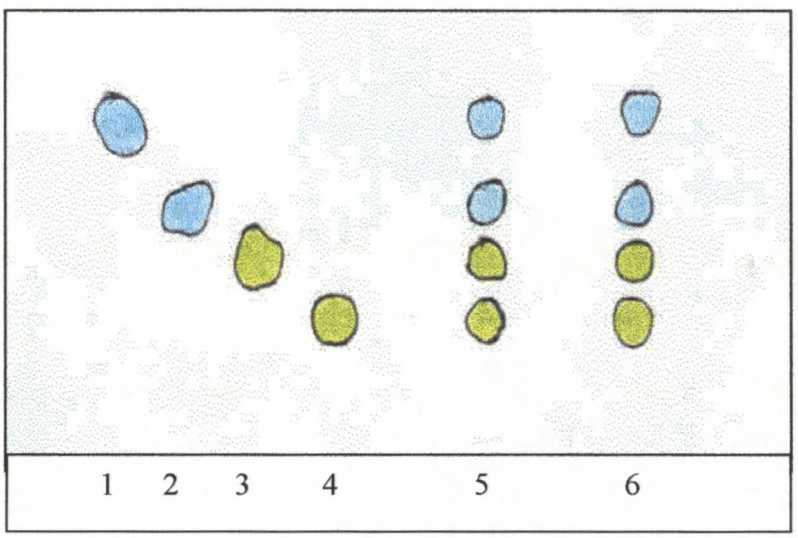


Figure 3.2: A: Pure culture of *Aspergillus parasiticus* isolated from pet food.
B: Pet food 'dog bites' contaminated with fungi.

3.2.2. Production of aflatoxins by *Aspergillus parasiticus*

TLC was carried out to verify that the *A. parasiticus* isolated from pet food produced all four aflatoxins. Four distinct spots (Fig. 3.3) with the same R_f values as the aflatoxin standards AFB_1, AFB_2, AFG_1 and AFG_2 were resolved, thus indicating that this isolate of *A. parasiticus* was capable of producing all four aflatoxins in laboratory culture. This isolate was therefore used in the experiments to determine the effect of spice oils on aflatoxin production.



1	= AFB_1	R_f	=	0.33
2	= AFB_2	R_f	=	0.23
3	= AFG_1	R_f	=	0.19
4	= AFG_2	R_f	=	0.13
5	= BROTH CULTURE	R_f	=	0.32, 0.22, 0.19, 0.12
6	= BROTH CULTURE	R_f	=	0.32, 0.23, 0.19, 0.12

Figure 3.3: TLC plate showing aflatoxins produced by the *Aspergillus parasiticus* strain isolated from pet food.

3.3. Inhibition of *Aspergillus parasiticus* and *Fusarium moniliforme* by spice oils

The agar overlay technique was used to evaluate the effect of spice oils on the growth of *A. parasiticus* (Fig. 3.4) and *F. moniliforme* (Fig. 3.5). *A. parasiticus* was inhibited by clove, cinnamon, oregano, mace, nutmeg, aniseed and tumeric oils. Of all the oils tested, clove oil was the most effective oil, as the lowest concentration 10 µl produced a 10 mm zone of inhibition (Table 3.2). The second most effective oil was cinnamon (8 mm), followed by oregano (5 mm), mace (5 mm), nutmeg (2 mm), tumeric (1 mm) and aniseed (0.5 mm). Neem and eucalyptus oils had no effect on growth. A comparison of the different oils showed that 10 µl clove oil was twice as effective as mace and oregano and was 10-fold more effective than tumeric oil. Complete inhibition of *A. parasiticus* was obtained with 40 µl and more of clove and cinnamon oils. Mace and nutmeg produced three and four fold increased inhibition respectively, when the volumes were increased five fold.

F. moniliforme was inhibited by clove, cinnamon, oregano, mace, nutmeg, aniseed and tumeric oils. Clove oil was the most effective oil, with the lowest concentration (10 µl) producing a zone of inhibition of 14 mm (Table 3.2). This was followed by cinnamon (8 mm), oregano (5 mm), mace (5 mm), nutmeg (4 mm), aniseed (2 mm) and tumeric (1 mm) (Table 3.10). A comparison of the inhibitory potential on fungal growth of the different spice oils shows cinnamon to be only 57% inhibitory as clove oil, followed by oregano (36%), mace (36%) nutmeg (29%), aniseed (14%) and tumeric (7%). Neem and eucalyptus oils had no inhibitory effects.

F. moniliforme is more sensitive than *A. parasiticus* to clove, nutmeg and aniseed (Table 3.2 and Fig. 3.6). Clove oil completely inhibited *F. moniliforme* at 30 µl. Both *F. moniliforme* and *A.*

paraiticus were completely inhibited by clove and cinnamon at 40 µl. Cinnamon produced similar zones of inhibition in both fungi while oregano and mace was more effective against *A. parasiticus* than *F. moniliforme*. Oregano produced complete inhibition at 50 µl, however, mace did not completely inhibit either fungi at this concentration.

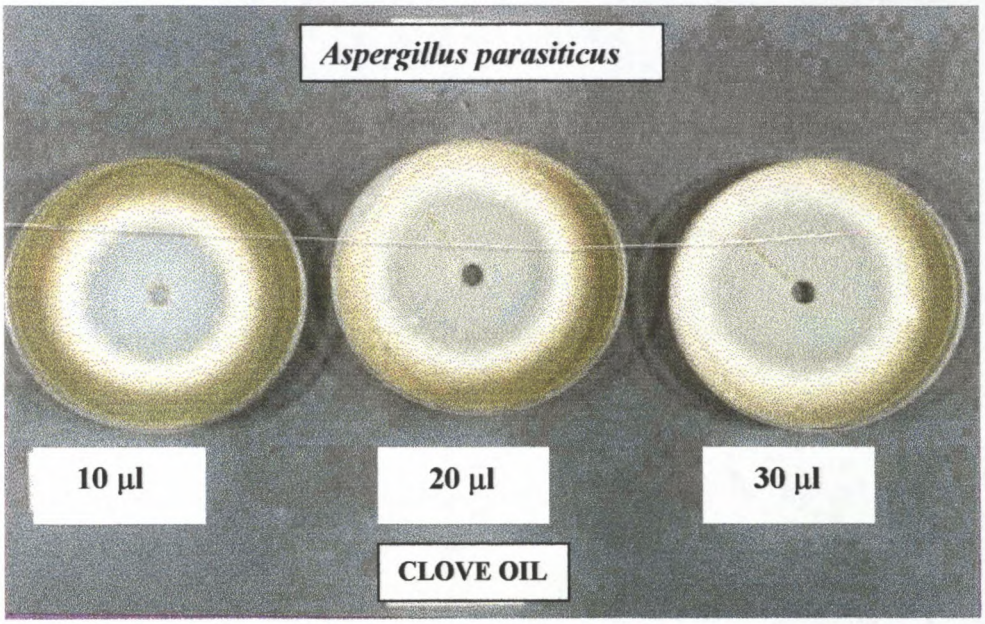


Figure 3.4: Agar overlay showing inhibition of *Aspergillus parasiticus* by 10, 20 and 30 µl clove oil.

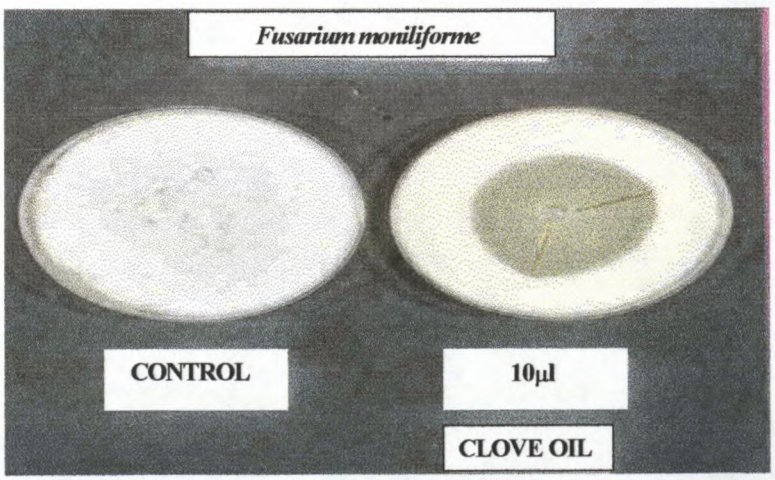


Figure 3.5: Agar overlay showing inhibition of *Fusarium moniliforme* by clove oil. *F. moniliforme* unchallenged by clove oil (control) and *F. moniliforme* challenged with 10 µl clove oil.

Table 3.2: Inhibition of *Aspergillus parasiticus* and *Fusarium moniliforme* using varying volumes of spice oils

SPICE OILS	ZONES OF INHIBITION (mm) ± SD									
	10 µl		20 µl		30 µl		40 µl		50 µl	
	A	**F**	**A**	**F**	**A**	**F**	**A**	**F**	**A**	**F**
Clove	10±0.71	14±0	15±0	15±0.77	16±0	CI	CI	CI	CI	CI
Cinnamon	8±0.71	8±0.63	11±0.89	11±0.71	14±0.89	13±0.77	CI	CI	CI	CI
Oregano	5±0	5±0.84	8±0.63	7±0.32	12±0.55	10±0	15±0	13±0.77	CI	CI
Mace	5±0.89	5±0	10±0	8±0.63	12±0.55	10±0.89	15±0	13±0.63	15±0.77	15±0
Nutmeg	2±0.63	4±0.71	4±0.63	6±0.45	6±0	8±0.55	8±0.89	9±0	8±0.89	10±0
Tumeric	1±0	1±0	3±0.45	2±0	3±0.63	3±0	3±0	3±0	5±0	5±0.71
Neem	0	0	0	0	0	0	0	0	0	0
Eucalyptus	0	0	0	0	0	0	0	0	0	0
Aniseed	0.5±0	2±0	1±0	3±0.63	2±0.77	6±0.63	4±0.84	8±0.77	6±0.63	10±0.63

CI = Complete inhibition
A = *Aspergillus parasiticus*
F = *Fusarium moniliforme*
SD = Standard deviation

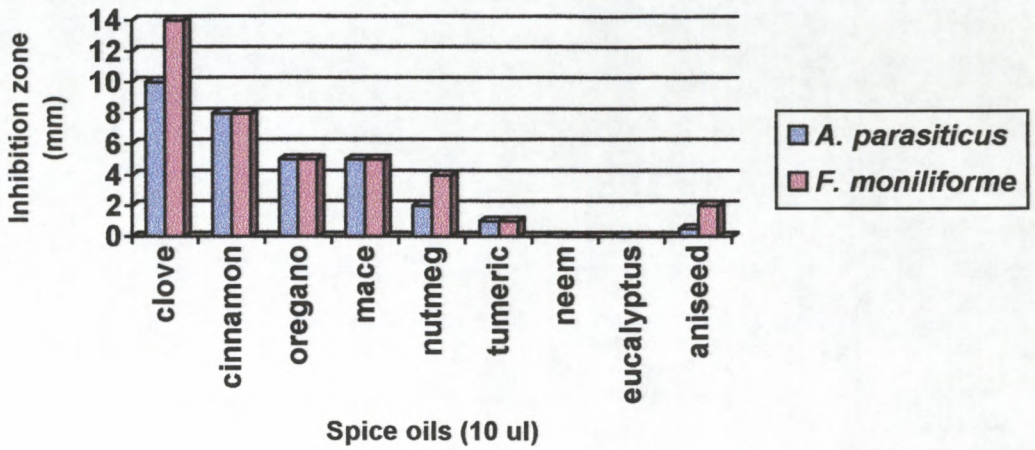


Figure 3.6: Comparison of the inhibition by spice oils of *Aspergillus parasiticus* and *Fusarium moniliforme*.

3.4. Inhibition of aflatoxin production by spice oils

SMKY broths incorporating varying concentrations of clove, mace, nutmeg, tumeric and eucalyptus oils were inoculated with *A. parasiticus* and incubated in order to test the inhibition of toxin production by the spice oils. The concentrated supernatants of the broth culture with spice oils, controls and aflatoxin standards were resolved on TLC plates (Appendix 4). *A. parasiticus* not challenged with spice oils produced all four aflatoxins viz. AFB_1, AFB_2, AFG_1 and AFG_2. Clove oil was the most potent inhibitor (Table. 3.3). Clove oil (0.5 ppm) inhibited all four aflatoxins whereas at the same concentration eucalyptus was not effective in inhibiting toxin production. Mace and nutmeg were only effective against AFG_2. At a two-fold increase in spice oil concentration (1 ppm), mace and nutmeg were effective in inhibiting AFB_1, AFB_2, AFG_1 and AFG_2. A four-fold increase in concentration (i.e from 0.5 to 2ppm). At a concentration of 2 ppm, all the spice oils inhibited AFB_1, AFB_2, AFG_1 and AFG_2, except for tumeric oil which was not effective against AFB_1 (Table 3.3).

Table 3.3: Inhibition of aflatoxins in broth culture at varying spice oil concentrations

	AFB_1	AFB_2	AFG_1	AFG_2
Control	+	+	+	+
Nutmeg (0.5 ppm)	+	+	+	-
Nutmeg (1.0 ppm)	-	-	-	-
Nutmeg (1.5 ppm)	-	-	-	-
Nutmeg (2.0 ppm)	-	-	-	-
Mace (0.1 ppm)	+	+	+	-
Mace (0.25 ppm)	+	+	+	-
Mace (1.0 ppm)	-	-	-	-
Eucalyptus (0.5 ppm)	+	+	+	+
Eucalyptus (1.0 ppm)	+	+	+	+
Eucalyptus (1.5 ppm)	+	+	+	+
Eucalyptus (2.0 ppm)	-	-	-	-
Clove (0.1 ppm)	+	-	-	+
Clove (0.25 ppm)	-	-	-	+
Clove (0.5 ppm)	-	-	-	-
Clove (2.0 ppm)	-	-	-	-
Tumeric (1.0 ppm)	+	+	-	+
Tumeric (1.5 ppm)	+	+	-	+
Tumeric (2.0 ppm)	+	-	-	-

Clove oil was the most effective spice oil on the inhibition of growth and toxin production of *A. parasiticus*. It was used in all subsequent experiments with *F. moniliforme* since these are co-occuring fungi.

3.5. Inhibition of *Fusarium moniliforme* by clove oil

Broth cultures (Alberts A broth) of *F. moniliforme* containing varying amounts of clove oil indicated that the growth of this fungus is inhibited by clove oil. In Fig. 3.7 the concentration dose-dependent inhibition is clearly visible. The clearing in the flasks containing higher concentrations of the oil shows inhibition of growth (disappearance of the white fungal mass) as well as inhibition of toxin production (disappearance of the pinkish pigment). Toxin production is inhibited first at 0.15 ppm whereas growth is still evident at 0.2 ppm.

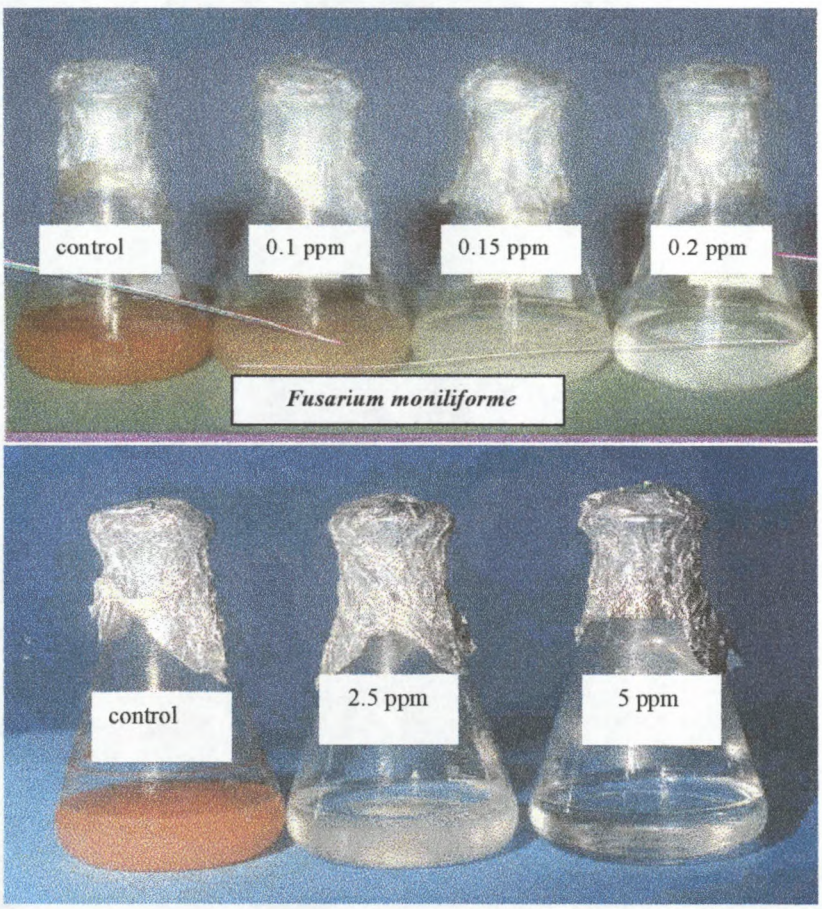


Figure 3.7: Inhibition of the growth of *Fusarium moniliforme* in broth cultures by clove oil showing a decrease in growth with increasing concentration of clove oil. Pink colour of broth indicates toxin presence and white indicates mycelial mass of *F. moniliforme*.

3.6. Inhibition of aflatoxin and fumonisin B_1 production by clove oil

The effect of clove oil on FB_1 and aflatoxin production was analysed by TLC and HPLC.

3.6.1. Thin layer chromatography

3.6.1.1. *Aspergillus parasiticus*

The minimum inhibitory concentration (MIC) of clove oil that inhibited the production of AFB_1, AFB_2, AFG_1 and AFG_2 was 0.15 ppm (Table 3.4). At 0.1 ppm only AFB_2, AFG_1 and AFG_2 were inhibited and at 0.03 ppm only AFG_2 was inhibited. TLC results are shown in Appendix 4.

Table 3.4: Minimum inhibitory concentration of clove oil for aflatoxin production by *Aspergillus parasiticus* in SMKY broth culture determined by thin layer chromatography

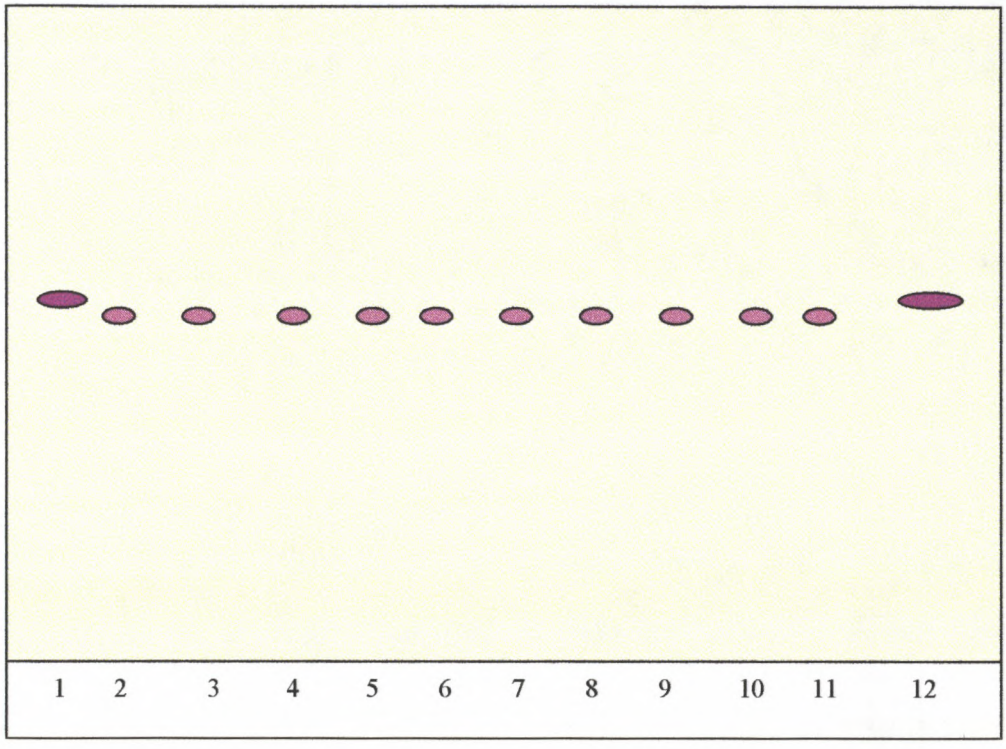
	0.03	**0.05**	**0.1**	**0.15**	**0.2**	**0.25**
AFB_1	+	+	+	-	-	-
AFB_2	+	-	-	-	-	-
AFG_1	+	-	-	-	-	-
AFG_2	-	-	-	-	-	-

\+ = Not Inhibited
– = Inhibition

3.6.1.2. *Fusarium moniliforme*

A TLC plate showing the effect of varying concentrations (0.2–2.0 ppm) of clove oil on fumonisin production in maize patty cultures is shown in Fig. 3.8. At all concentrations of clove oil used, FB_1 was not completely inhibited since a spot corressponding to the R_f value of FB_1 is still visible in all the lanes. The higher concentrations of clove oil did inhibit FB_1 production to a

certain extent since the intensity of the spots decreased with increasing clove oil concentrations. This was later confirmed by HPLC analyses.



Lane 1 and 12	:	FB_1 standard	R_f	=	0.45
Lane 2 and 3	:	control	R_f	=	0.44
Lane 4 and 5	:	0.2 ppm clove oil	R_F	=	0.43
Lane 6 and 7	:	0.5 ppm clove oil	R_F	=	0.43
Lane 8 and 9	:	1.0 ppm clove oil	R_F	=	0.43
Lane 10 and 11	:	2.0 ppm clove oil	R_F	=	0.43

Figure 3.8: Diagrammatic representation of a TLC plate showing fumonisins produced by *Fusarium moniliforme* grown in the presence of varying concentrations of clove oil.

3.6.2. High pressure liquid chromatography

3.6.2.1. *Aspergillus parasiticus*

HPLC analysis was used to quantify aflatoxin levels in broth cultures of *A. parasiticus* containing various concentrations of clove oil. Chromatograms of the aflatoxin standards produced four well resolved peaks (Fig. 3.9). The retention times for the different standards were 8.45 for AFG_2 , 12.04 for AFG_1, 16. 83 for AFB_2 and 25.89 for AFB_1. The chromatogram of the control, i.e. SMKY broth culture not challenged with clove oil showed several peaks (Fig. 3.10). Two major peaks were observed, the peak that eluted at 2.08 was that of the derivatizing agent, TFA, and the second major peak at retention time 12.05 corressponded to that of AFG_1. Several other peaks present are most probably due to constituents in the broth or other metabolites produced by the fungus. All four aflatoxins were produced but in varying concentrations, with AFG_1 produced in very large quantity compared to the other three aflatoxins which were 43 ppm for AFB_1, 34 ppm for AFB_2, 200 ppm for AFG_1, and 77 ppm for AFG_2.

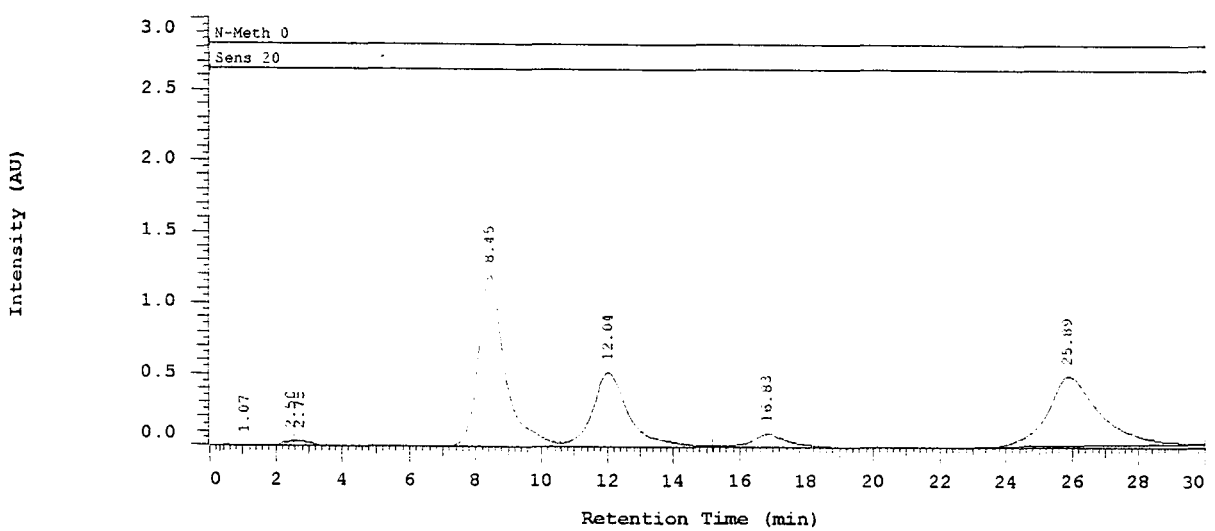


Figure 3.9: HPLC chromatogram of aflatoxin standards showing retention times for AFG_2, AFG_1, AFB_2 and AFB_1 respectively.

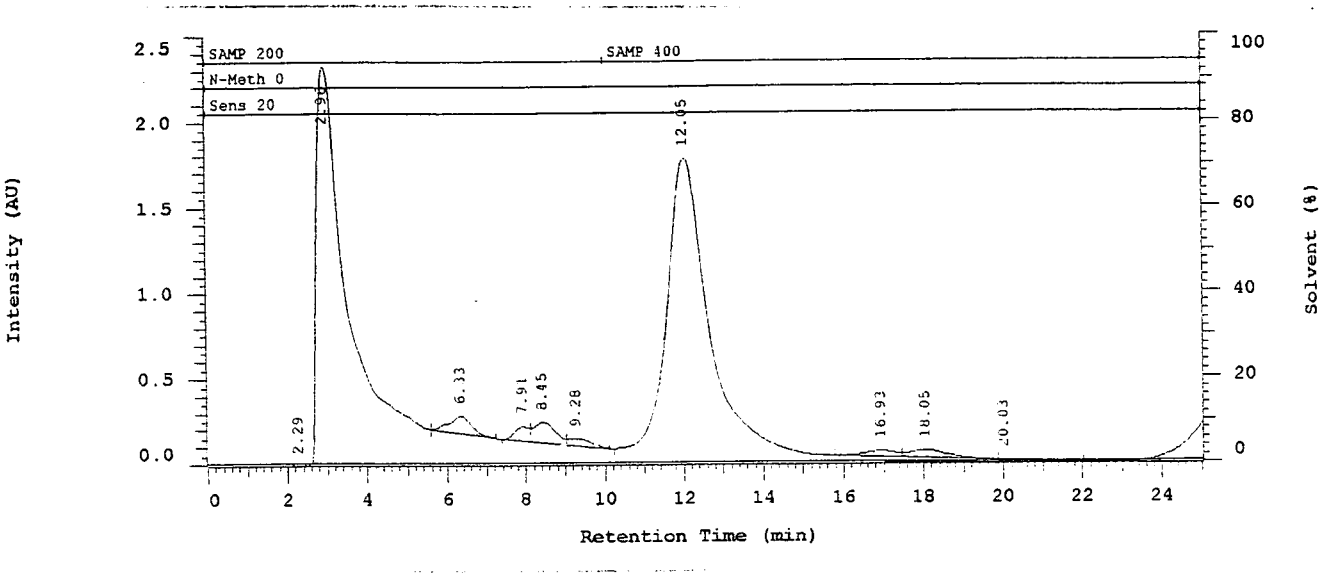


Figure 3.10: HPLC chromatogram of aflatoxins AFG_2, AFG_1, AFB_2 and AFB_1 produced by *Aspergillus parasiticus* grown in SMKY broth containing no spice oil (control).

Fig. 3.11. and Fig. 3.12 are the chromatograms for *A. parasiticus* challenged with 0.03 ppm and 0.05 ppm clove oil respectively. The peak at retention time 9.68 corresponds with that for AFG_1 which was confirmed by running the AFG_1 standard which also had the same retention time. We found that all the toxins eluted out earlier for the next few runs. This was most probably due to the increased column pressure. The retention time of AFB_1 has changed from 25.89 to 21.28. At 0.03 ppm clove oil a decrease in aflatoxin concentration was observed. The inhibitory effect was varied for the different aflatoxins. Fig. 3.13. is the chromatogram for *A. parasiticus* challenged by 0.15 ppm clove oil. The only major peak corresponds to the AFG_1 toxin. The other toxins have been completely inhibited.

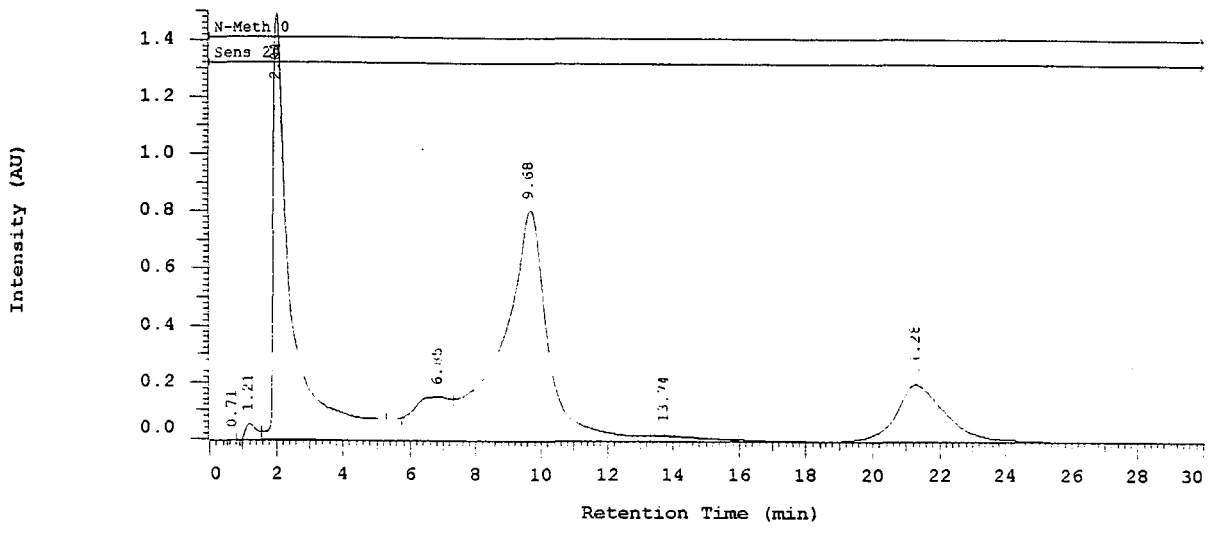


Figure 3.11: HPLC chromatogram of aflatoxins AFG_2, AFG_1, AFB_2 and AFB_1 produced by *Aspergillus parasiticus* grown in of SMKY broth containing 0.03 ppm clove oil.

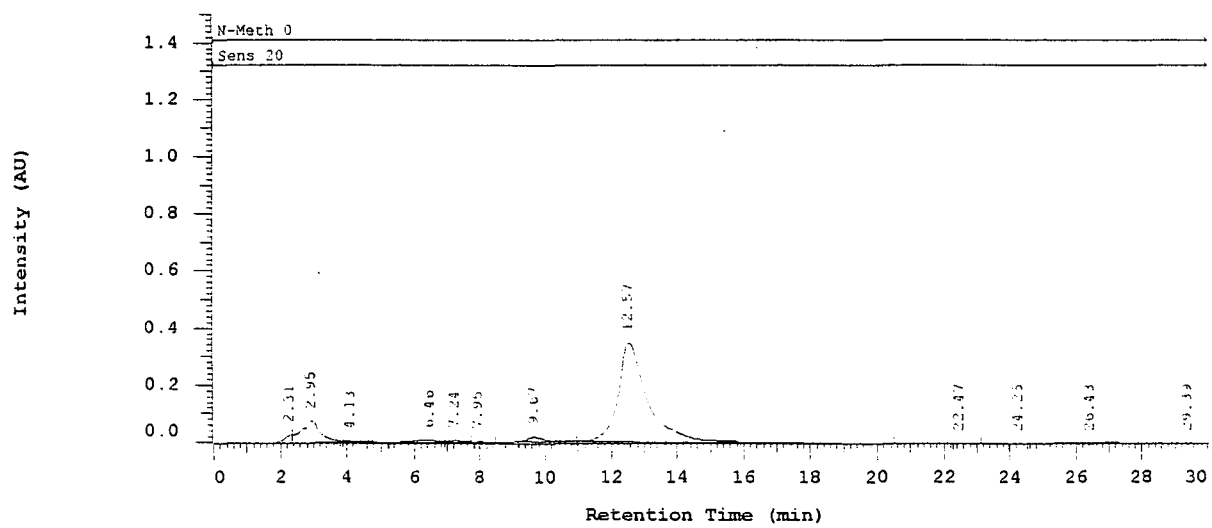


Figure 3.12: HPLC chromatogram of aflatoxins AFG_1, AFB_2 and AFB_1 produced by *Aspergillus parasiticus* grown in SMKY broth containing 0.05 ppm clove oil.

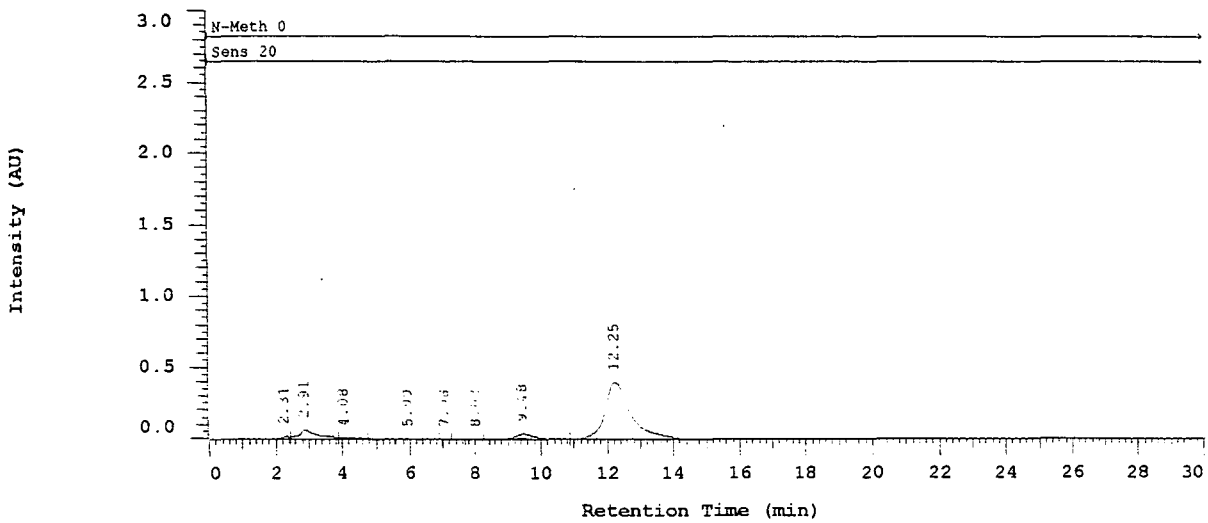


Figure 3.13: HPLC chromatogram of aflatoxin AFG_1 produced by *Aspergillus parasiticus* grown in SMKY broth containing 0.15 ppm clove oil.

The level of the aflatoxins produced by *A. parasiticus* challenged by varying concentrations of clove oil as well as the percentage reduction of the aflatoxins are summarized in Table 3.5. *A. parasiticus* unchallenged by clove oil produced varying amounts of the different aflatoxins: approximately a third less AFG_2 and 5 times less AFB_1 and AFB_2 than AFG_1. The lowest concentration of clove oil (i.e. 0.03 ppm) reduced AFB_1 by 50%, AFB_2 by 14%, AFG_1 by 41% and AFG_2 by 77%. AFG_2 production was the most sensitive toxin to clove oil and was completely inhibited by 0.05 ppm. There was a substantial decrease in AFB_2 levels: 0.03 ppm reduced it by 14% and 0.05 ppm reduced it by 76%. At 0.1 ppm clove oil, AFB_1, AFB_2 and AFG_2 were completely inhibited. At the same concentration, AFG_1 was reduced by ± 66% (from 200 ppm to 67.96 ppm) and is inhibited completely at 0.2 ppm. AFG_1 had the highest initial concentration (200 ppm) and required the highest concentration (0.2 ppm) of clove oil for

AFG_1 appears to be more resistant to the effects of clove oil and is only inhibited at 0.2 ppm clove oil. AFG_2 is the most sensitive of the aflatoxins and is inhibited at 0.05 ppm.

Table 3.5: Concentration and reduction (%) of aflatoxin production by *Aspergillus parasiticus* grown in the presence of clove oil

Clove oil	**AFB_1**		**AFB_2**		**AFG_1**		**AFG_2**	
	Ppm	**%**	**ppm**	**%**	**ppm**	**%**	**ppm**	**%**
Control	43	0	34	0	200	0	77	0
0.03 ppm	20	52.24	29	14.22	118	40.99	18	76.66
0.05 ppm	16	66.66	8	76.16	109	45.06	0	100
0.10 ppm	0	100	0	100	67	66.02	0	100
0.15 ppm	0	100	0	100	37	82.24	0	100
0.20 ppm	0	100	0	100	0	100	0	100
0.25 ppm	0	100	0	100	0	100	0	100

Aflatoxin concentrations are mean values of 2 results.

Table 3.6: Minimum inhibitory concentration of clove oil for aflatoxin production by *Aspergillus parasiticus* in SMKY broth

	0.03 ppm	**0.05 ppm**	**0.1 ppm**	**0.15 ppm**	**0.2 ppm**	**0.25 ppm**
AFB_1	+	+	-	-	-	-
AFB_2	+	+	-	-	-	-
AFG_1	+	+	+	+	-	-
AFG_2	+	-	-	-	-	-

+ = Not inhibited – = Inhibition

3.6.2.2. *Fusarium moniliforme*

F. moniliforme produced 2.75 ppm FB_1 in maize patty cultures. FB_1 production was inhibited by clove oil in a dose dependant response. A clove oil concentration of 2 ppm reduced FB_1 levels from 2.75 ppm to 0.6 ppm which is a 78% reduction. 0.5 ppm clove oil resulted in 45% reduction. At 0.2 ppm there was only a 22% reduction (Table 3.7)

Table 3.7: Inhibition of fumonisin B_1 production by *Fusarium moniliforme* by clove oil

CLOVE OIL	FB_1 (ppm)	% REDUCTION
CONTROL	2.75±	0
0.2 ppm	2.15±	22
0.5 ppm	1.5±	45
1.0 ppm	0.85±	69
2.0 ppm	0.6±	78

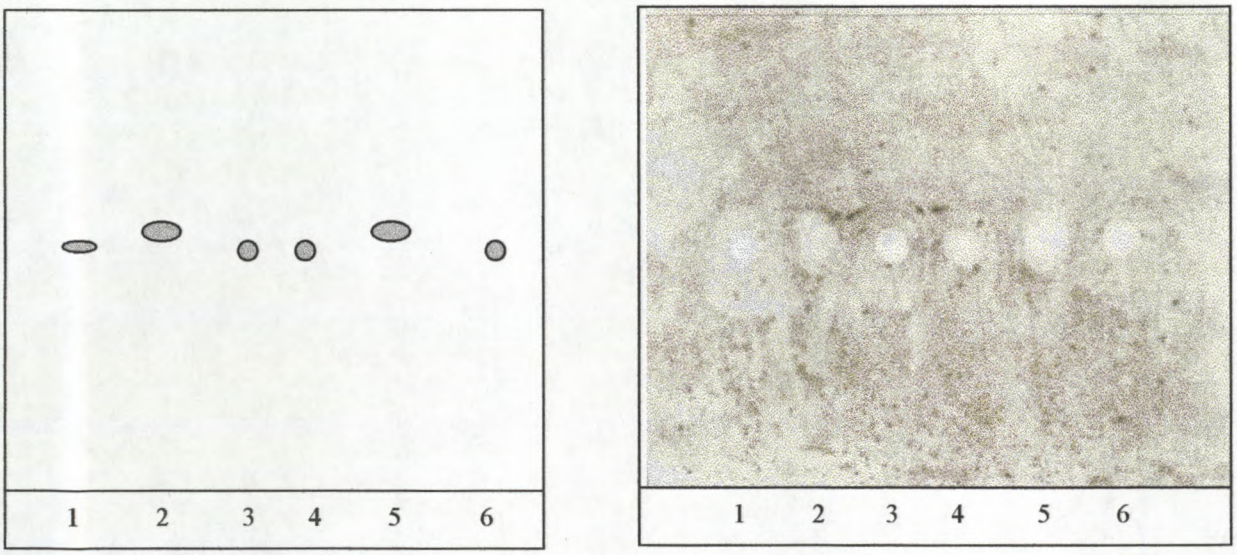
Ten µl of clove oil produced 14 mm and 10 mm zones of inhibition for *F. moniliforme* and *A. parasiticus*, respectively. Clove oil therefore produced a 40% greater inhibition for *F. moniliforme* than *A. parasiticus* (Table 3.8). However 0.2 ppm clove oil completely inhibited production of all four aflatoxins and only reduced fumonisin B_1 by 22%.

Table 3.8: Relationship between fungal growth and toxin production of *Aspergillus parasiticus* and *Fusarium moniliforme*

	A. parasiticus	***F. moniliforme***
Inhibition of fungal growth (10 µl clove oil)	10 mm	14 mm
Inhibition of toxin production (0.2 ppm clove oil)	Complete inhibition of AFB_1, AFB_2, AFG_1, AFG_2	FB_1 reduced by 22%

3.7. Components of clove oil inhibitory to *Aspergillus parasiticus* and *Fusarium moniliforme*

Bioautography of eugenol and cinnamic aldehyde, the active components of clove oil, was performed to determine if these components were active against *A. parasiticus* and *F. moniliforme*. Fig 3.14A is a schematic diagram of the TLC demonstrating the separation of clove oil, cinnamic aldehyde and eugenol. These compounds produced a pale colour on the TLC plate and did not provide sufficient contrast in a photographic representation. Clove oil and eugenol had the same R_f values of 0.53 and cinnamic aldehyde had a R_f of 0.55. Fig. 3.14B is the bioautographic plate and shows clear inhibition of *A. parasiticus*. Eugenol was more effective than cinnamic aldehyde in inhibiting *A. parasiticus*.

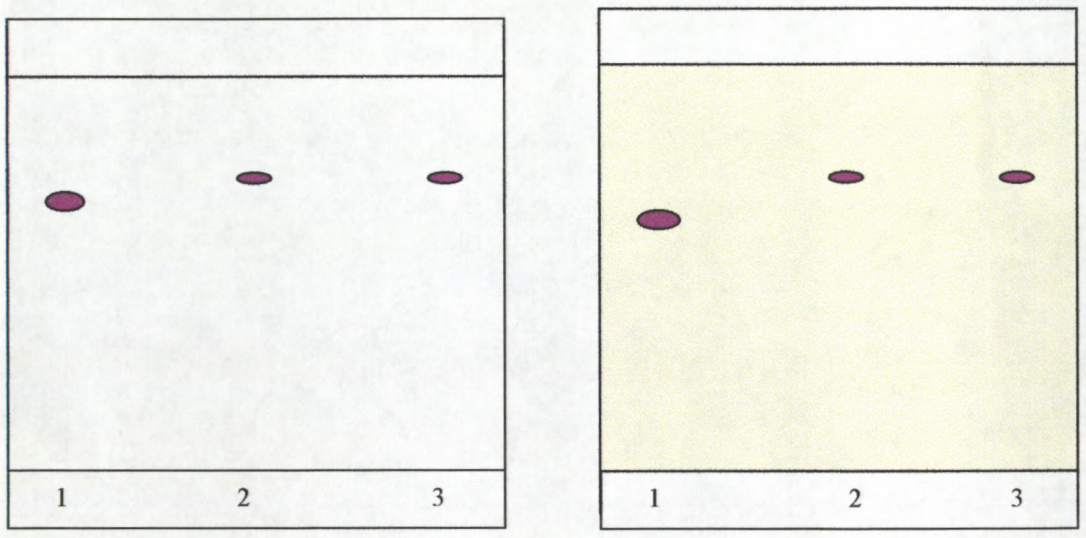


A B

1 & 4	=	Clove oil	R_f	=	0.53
2 & 5	=	Cinnamic aldehyde	R_f	=	0.55
3 & 6	=	Eugenol	R_f	=	0.53

Figure 3.14: A: TLC plate of clove oil and two of its active components, viz., cinnamic aldehyde and eugenol. B: Bioautography showing inhibition of *Aspergillus parasiticus* by clove oil and its active components. Clearings indicates inhibition.

Fig. 3.15.A and B are schematic representation of TLC and bioautography plates showing inhibition of *F. moniliforme* by clove oil and its active components. Since *F. moniliforme* produces white mycelial mass, zones of inhibition which were visible to the naked eye were not visible in a photograph or scans of the plate. Both cinnamic aldehyde and eugenol successfully inhibted growth of *F. moniliforme*. It was not possible to determine whether eugenol was more effective against *F. moniliforme*.



A B

1	= Clove oil	R_f	=	0.67
2	= Cinnamic aldehyde	R_f	=	0.69
3	= Eugenol	R_f	=	0.67

Figure 3.15: A: TLC plate of clove oil and two of its active components, viz., cinnamic aldehyde and eugenol. B: Bioautography showing inhibition of *Fusarium moniliforme* by clove oil and its active components. Yellow colour indicates growth of *F. moniliforme*.

3.8. The effect of clove on fumonisin production in grain

Fig. 3.16 shows the inhibition of the growth of *F. moniliforme* by whole cloves in a dose-dependant response. 10 mg/g has minimal effect on growth. Inhibition of toxin production is also minimal at this concentration. At 50 mg/g clove, very little growth is evident and at 100 mg/g, there is no visible growth.

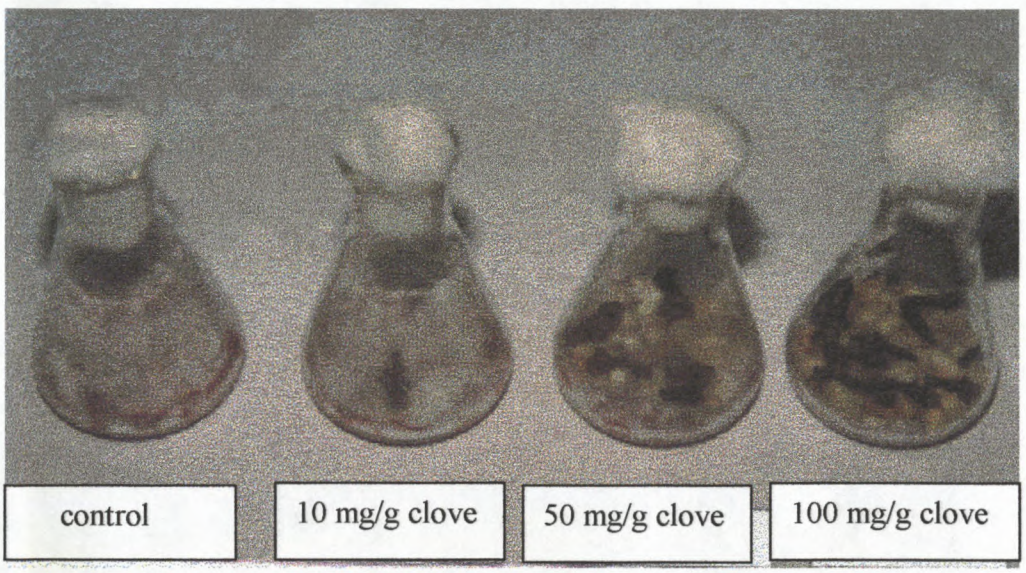


Figure 3.16: Inhibition of the growth of *Fusarium moniliforme* in samp by whole cloves. Flask with 1 g of clove shows no visible growth of *F. moniliforme*. Growth is indicated by a white fuzzy mass. The cloves are black.

HPLC results for FB_1 production by *F. moniliforme* in samp containing either ground or whole cloves are summarised in Table 3.9. At the lowest concentration (10 mg/g) of clove tested, ground clove was more effective than whole clove, with 14 and 2.5% reduction, respectively. At

a five-fold higher concentration (50 mg/g) there was a substantial decrease in FB_1 levels for both ground and whole cloves. At this concentration whole clove was more effective (83% reduction) compared to ground clove (71% reduction). At a two-fold higher concentration, FB_1 inhibition increased to 92% for whole cloves and 87% for ground cloves.

Table 3.9: The effect of whole and ground cloves on the reduction of FB_1 levels in samp

	CLOVE CONCENTRATION (mg/g)							
	Control		10		50		100	
	W	G	W	G	W	G	W	G
FB_1 (ppm)	6.05	4.95	5.9	4.25	1.0	1.43	0.5	0.65
% Reduction	0	0	2.5	14	83	71	92	87

W = Whole clove
G = Ground clove

3.8.1. Cost evaluation

In order to determine the costs of control or inhibition of fungal growth and toxin production using clove oil the following pricing was carried out. Clove was purchased at approximately R40/kg or R0.40/g. In the pilot study in section 3.8. 100 mg of clove incorporated into 1 g of samp, produced a 92% inhibition of FB_1. Therefore for the minimal amount of R0.04/ 1 g of samp one could obtain a 92% reduction of FB_1.

CHAPTER FOUR: DISCUSSION

There has been increasing interest in using naturally occurring compounds, especially essential oils, to limit growth and toxin production by fungi. The fungistatic effects of essential oils have been previously indicated by many authors (Mahmoud, 1994; Farag *et al.*, 1989a) and it has been shown that some constituent in each oil plays an important role in its antifungal action. Although the effect of these spices have been extensively studied for aflatoxins, they have not been exploited for other mycotoxins.

To exploit the potential of spice oils as mycotoxin control agents, spices were distilled from the whole spices and tested against some of the commonly occurring mycotoxin producing fungi. An initial screen to evaluate whether spice oils could influence mycotoxin production was carried out with clove, oregano, eucalyptus and tumeric. These were tested against *A. oryzae*, which produces ochratoxin A; *A. versicolor*, a sterigmatocystin-producing strain; *A. flavus*, an aflatoxin B_1 and B_2 producing strain; *A. parasiticus*, an aflatoxin B_1, B_2, G_1 and G_2 producing-strain and *F. moniliforme* which produces fumonisin B_1 (Fig. 3.1). The results of these studies showed that clove and oregano oils inhibited fungal growth. It was thus feasible to test them against local strains, particularly against *A parasiticus* and *F. moniliforme* which co-occur (Marin *et al.*, 1998) and of are of considerable interest locally.

Experiments were also designed to find a control measure that could be applied by local, rural and subsistence farmers to control mycotoxins. A strain of *A. parasiticus* that produced all four aflatoxins was sought. Local products, viz., beans, pet food, maize and peanuts were screened. Various mycotoxin-producing fungal species were isolated i.e., *A. flavus*, *A.*

tamarii and *A. niger* and *A. parasiticus.* The *A. parasiticus* strain, which was isolated from pet food purchased from a local supermarket, was used in this study, as it produced AFB_1, AFB_2, AFG_1 and AFG_2. The presence of mycotoxins in local foods is of concern since they have major health implications for humans and animals.

Nine spice oils were selected and tested against *A. parasiticus* and *F. moniliforme,* two commonly occurring fungi that is of local significance. The results of this study showed that the essential oils of clove, cinnamon, oregano, mace, nutmeg, tumeric and aniseed oil were effective in inhibiting fungi, whilst neem and eucalyptus had no effect. Clove oil was found to be the most inhibitory oil on fungal growth and was twice as effective as mace and oregano. The toxin- inhibiting potential of clove, nutmeg, mace, tumeric and eucalyptus oils was determined by incorporating various concentrations of the oils in broth and determining their effect on toxin production. TLC results showed that 2 ppm of five oils; viz. clove, nutmeg, mace, tumeric and eucalyptus oils completely suppressed growth of *A. parasiticus*, and effectively prevented the formation of aflatoxins (Table 3.3). Determination of the minimum inhibitory concentration of these oils revealed that mace and nutmeg were effective at 1 ppm while clove was effective at a dose as low as 0.5 ppm. Comparisons of the oils show that clove oil was the most effective spice oil in reducing growth of *A. parasiticus* and *F. moniliforme* (Table 3.2) and also in reducing aflatoxin production (Table 3.3). Clove oil is more inhibitory on growth of *F. moniliforme* than *A. parasiticus.* However aflatoxins are more sensitive to clove oil than fumonisins (Table 3.8). These results are similar to those found by Farag *et al.* (1989), who found that some essential oils caused complete inhibition of both mycelial growth and aflatoxin production of *A. parasiticus.* Their study also indicated that thyme oil inhibited mould growth and aflatoxin production at a concentration of 0.2

mg/ml. In their study, clove oil was effective at a concentration of 0.4 mg/ml, which is 200 times more concentrated than the inhibitory concentrations obtained in this study. This could be due to the fact that 100% pure clove oil was used in this study. These results also followed the pattern of Ueda *et al.* (1982) who evaluated the antifungal properties of extracts of 14 spices against several moulds with the paper disc method and found clove oil to be the most effective followed by cinnamon and oregano.

Current control measures are aimed at controlling fungal growth and subsequent mycotoxin formation in stored grains by physical methods, viz., aeration, cooling, modified atmosphere (Paster *et al.*, 1988) or by chemical methods, viz., ammoniation, acids and bases and food preservatives. These methods require sophisticated equipment and expensive chemicals or reagents. Relatively drastic conditions are necessary when using acids and bases to convert large amounts of AFB_1 and AFG_1 to AFB_{2a} and AFG_2, respectively. Pons *et al.* (1972) calculated that 6 hours at 100°C is required to convert 95% of AFB_1 in an aqueous solution adjusted to pH 3 to its hydroxyl analogue. Hence, it is not a viable alternative for detoxifying agricultural commodities. Chemical degradation of AFB_1 in most ammoniation experiments is supported by toxicological evidence from feeding trials with laboratory and farm animals. Pathological and histological examinations of ducklings fed with ammoniated meals revealed no significant signs of aflatoxicosis (Pons *et al.*, 1972). Moreck *et al.* (1980) evaluated the effect the food preservative sodium bisulphite had on naturally contaminated maize. An initial concentration of 200 ppb of AFB_1 was degraded by over 90% by 2% bisulphite. It has not yet been demonstrated that aflatoxin contaminated commodities treated with bisulphite are biologically safe and retain their nutritional and functional properties. All the above treatments are not feasible when one considers the rural subsistence farmers since they require

expensive chemicals, technology and technical expertise to monitor temperature, pressure, etc.

There are also no effective control measures to control fumonisins. Ammoniation has been successfully used to decontaminate aflatoxin-contaminated foodstuffs (Brekke *et al.*, 1977). With this treatment, however, although the fumonisin content was reduced by 90%, the effective loss was found to be <30% when tested for toxicity in rats (Voss *et al.*, 1989). Similarly, alkali treatment of maize with $Ca(OH)_2$ was found to convert FB_1 to its hydrolysed aminopentol form, but this compound had the same carcinogenic potential as fumonisin B_1 (Hendrich *et al.*, 1993). The only biological treatment evaluated for the control of fumonisins has been in beer, which underwent fermentation. Even these had to be further detoxified before they could be used as animal feeds (Bothast *et al.*, 1992). The results of various physical methods of controlling fumonisins have also been investigated. Sydenham *et al.* (1994) reported that physically removing the fines or screenings from bulk shipments of maize reduced the fumonisin content by 26-29%. Bennett and Richard (1995) showed that starch prepared by wet milling of maize reduced fumonisin content, however , most of the fumonisins were subsequently found in the gluten and fibre fractions. This toxin poses a serious threat since heat treatments of this toxin is ineffective as temperatures of 62^0C for 30 min is inadequate, and temperatures as high as 190-220^0C for 25 minutes are required to produce losses of 75% of the toxin. The only successful report on the control of fumonisins is a report by Scott and Lawrence (1994) that indicates that steeping maize for 6 h in 2% SO_2 was an effective treatment for decreasing fumonisin concentrations in solution. This study offers a simple solution since 2 ppm clove oil resulted in a reduction of FB_1 levels from 2.75 ppm to 0.6 ppm, which is a 78% reduction. Half the concentration (1 ppm), reduced FB_1

levels by 69%, i.e., from 2.75 ppm to 0.85 ppm (Table 3.11). The other advantage of using clove oil in storage grain is that it scored highly in organoleptic tests undertaken by Chatterjee (1990).

The implications of the results of this study in context with other control measures used to prevent aflatoxins indicate that none of the control measures whether physical (aeration, cooling, modified atmospheres) or chemical (acids and bases, ammoniation, food preservatives) are optimum. The disadvantages of some of the control measures are outlined in Table 4.1. For fumonisins, physical treatments such as heat and removal of screenings had been unsuccessful due to the highly heat resistant nature of fumonisins. Chemicals using ammoniation, treatment with calcium hydroxide [$Ca(OH)_2$] and steeping in sulphur dioxide [SO_2] are effective to some extent, but very expensive (Table 4.1). The results of this study shows that cloves can be used to control both fumonisin and aflatoxin contamination as it is inexpensive, does not require expensive sophisticated equipment and is non-toxic. These parameters indicate that clove can be used to control mycotoxins by the rural community and commercially in the storage of grain, where post-harvest mycotoxin contamination is a significant problem.

Spices and herbs have played an important role in human life from prehistoric times. They have been used not only for flavouring food but also for their antioxidant, preservative and medicinal properties (Nakatani, 1994). Essential oils of thyme, sage, clove, cumin, rosemary, garlic and cardamom possess antioxidant and antimicrobial activities and the activity depends not only on their oil components but also on the chemical structure of these components

(Farag *et al.*, 1989a, 1989; Daw *et al.*, 1995; Kim *et al.*, 1995; Hassan 1996). The great majority of naturally occurring antioxidants and preservatives isolated from spices and herbs

Table 4.1: Advantages and disadvantages of current control measures for aflatoxins and fumonisins

AFLATOXINS	**ADVANTAGES/ DISADVANTAGES**
1. Aeration, cooling, modified atmosphere	Sophisticated equipment (Paster *et al.*, 1988).
2. Acids and bases	Expensive reagents and equipment (Pons *et al.*, 1972).
3. Ammoniation	Non-toxic, but reagents expensive (Paster *et al.*, 1988).
4. Food preservatives	Not tested if biologically safe and if retains nutritional and functional properties (Moreck *et al.*, 1980).
5. *Naturally occurring substances: clove*	*Simple to use, not toxic, cost effective, no equipment required 0.2 ppm prevents AFB_1, AFB_2, AFG_1 and AFG_2 production.*
FUMONISINS	**ADVANTAGES/ DISADVANTAGES**
1. Ammoniation	Expensive reagents, fumonisins reduced by 90% but loss <30% (Voss *et al.*, 1989).
2. Treatment with $Ca(OH)_2$	By-product carcinogenic; expensive reagents (Hendrich *et al.*, 1993).
3. Fermentation with beer	Sophisticated equipment; needed to be further detoxified (Bothast *et al.*, 1992).
4. Removal of screenings	Toxin level reduced by 26-29% (Sydenham *et al.*, 1994).
5. Heat treatment	Extreme high temp. (190° – 220° for 25 min) required for 25% reduction in toxin concentration.
6. Steeping maize in 2% SO_2	Effective, but expensive reagents (Scott and Lawrence, 1994).
7. *Naturally occurring substances : clove*	*Simple to use, non-toxic, cost effective, no equipment required, 2 ppm clove oil reduced FB_1 by 78%, 100 mg/g whole clove reduced FB_1 by 92% and 100 mg/g ground clove reduced FB_1 by 87%.*

are phenolic compounds including coumarins, flavonoids and essential oils (Farag *et al.*, 1989a; El-Baroty, 1994; Nakatani, 1994; Kim *et al.*, 1995). According to Mahmoud (1994), all oils that completely suppressed the growth of *A. flavus* and prevented the formation of aflatoxins had aliphatic alcohols, aromatic aldehydes and phenolic ketones as essential oil constituents. Among these constituents, carvacol was most effective and like eugenol, belongs to the phenol group. This is suggested to be attributed to the presence of an aromatic nucleus and phenolic hydroxyl group which is known to be reactive and forms hydrogen bonds with active sites of target enzymes (Farag *et al.*, 1989b). Eugenol constitutes 93-95% of the essential oil of clove (Bullerman *et al.*, 1977; Farrel, 1985). Cinnamic aldehyde, a constituent of the essential oil of clove, is an aromatic compound and the inhibitory activity of clove oil could also be attributed to the aromatic ring structure of this compound. Although much research is devoted to the detection and occurrence of these toxins, to date there are no effective control measures that can prevent toxins being produced or eliminate the toxin from foods before they are consumed. The results of this study offer a safe, simple and effective control measure for fumonisin and aflatoxin contamination. These oils are safe for humans (Farag *et al.*, 1991).

Clove oil (0.2 ppm) was effective in eliminating all four aflatoxins, however, at 0.1 ppm only AFB_1, AFB_2 and AFG_2 were eliminated as shown in HPLC results outlined in Table 3.5. Clove oil (0.1 ppm) completely inhibited AFB_1 (0.043 μg/g), AFB_2 (0.034 μg/g) and AFG_2, (0.077 μg/g). However, the same concentration of clove oil, reduced AFG_1 by 66%. At 0.2 ppm clove oil inhibited the production of AFG_1 completely.

Bhatnagar *et al.* (1991) showed that AFB_1 and AFB_2 are synthesised in fungal mycelia by separate pathways, indicating a branch point in the pathway. It has also been suggested by Dutton (1988) that AFB_1 and AFB_2 are converted to AFG_1 and AFG_2, respectively by a monooxygenase. In this study at 0.05 ppm clove oil, AFB_2 was detected when no AFG_2 was found, indicating that AFG_2 is a derivative of AFB_2. This is in accordance with Dutton's observation that AFB_2 is a precursor of AFG_2. However, the same explanation cannot be applied to AFB_1 and AFG_1 as AFG_1 was detected long after AFB_1 synthesis was stopped, as AFB_1 is suggested to be the pre-cursor of AFG_2 (Table 3.4). This requires further investigation.

To relate the findings of this study on a practical scale that can be applied in Third World countries that lack resources, whole clove and ground clove were tested for their inhibitory potential in samp, the staple food of the major population of Kwa-Zulu Natal. Results show that whole clove (100 mg/g) and ground clove (100 mg/g) were capable of reducing FB_1 from 6 ppm to 0.5 ppm (92%) and from 4.95 ppm to 0.6 ppm (87%), respectively. Half the concentration (50 mg/g) of whole cloves and ground cloves reduced FB_1 levels to 1.0 ppm (83%) and 1.4 ppm (71%), respectively. The cost of utilizing cloves as a control measure was estimated to be R0.04/ 1 g samp sample in order to obtain a 92% reduction of FB_1.

Clove oil used in this study is 100% pure and offers an effective form of control as it can be used at low concentrations. Spices are normally used as flavouring agents in levels of 0.5 to 1.0% in finished products (Furia, 1968). Cloves contain approximately 17% essential oil, which is made up of 95% eugenol (Bullerman *et al*, 1977). Results of this study show that if clove oil is used at a level of 0.5%, this would result in a level of eugenol sufficient to provide

effective fungistatic action and thereby control mycotoxins. However, this is expensive and may not be easily accessible/affordable to rural farmers. Whole cloves and ground cloves are cheap, in abundance and easy to use. Cloves (whole and ground) may be mixed with stored grain in varying proportions, as it is not toxic. Although 100 mg/g of clove appears to be high for practical application, it should be recognised that the study was performed in a system highly favourable to mould growth and toxin production. Hence, in a food storage system with a less conducive environment for mould growth due to substrate composition, processing and storage conditions of lower levels of clove could be used.

Fusarium spp. does not colonise maize in isolation. To become established, they need to compete effectively against other colonisers (Marin *et al.*, 1998). Recently, many researchers have focused on co-occurrence of toxins. Most fungal species can produce two to four mycotoxins. However, some species produce as many as 12 toxins, e.g. *A. flavus*, *A. fumigatus* and *F. moniliforme* produce 9, 10 and 9 toxins respectively (Frisvad and Samson, 1991). Maize grains are contaminated by a variety of toxigenic fungi, *Fusarium* and *Aspergillus* being the most frequent ones (Etcheverry *et al.*, 1998). In Argentina maize samples were found to be infected with *Fusarium* mycotoxins and aflatoxins, where fumonisins B_1, B_2, B_3 and ZEA were found in all samples (Gonzalez *et al.*, 1999). Other toxins that have been found to co-occur are the 8-keto trichotecenes, ZEA and fumonisins (Sohn *et al.*, 1999).

The results of this study shows that clove oil is capable of inhibiting many of these mycotoxin-producing fungi, viz. *A. oryzae*, *A. flavus*, *A. versicolor*, *F. moniliforme*, and *A. parasiticus* (Fig. 3.1). The importance of these results is that these commonly co-occurring

mycotoxigenic fungi may be controlled with clove oil as this spice oil has successfully inhibited the growth of both *A. parasiticus* and *F. moniliforme* and prevented the formation of aflatoxins and fumonisins. The simplicity of the technique lends itself to controlling co-occurring fungi.

In conclusion, this study indicates that clove oil, if used in sufficient quantities, is effective in inhibiting the growth of *A. parasiticus* and *F. moniliforme*. This fungistatic activity may have potential for use in the prevention of aflatoxin and fumonisin production.

CHAPTER FIVE: REFERENCES

Alberts, J. F., Gelderblom, W. C. A. and Marasas, W. F. O. (1993). Evaluation of the extraction and purification procedures of the maleyl derivitization HPLC techique for the quantification of the fumonisin B_1 mycotoxins in corn cultures. *Mycotoxin Res.* **9**:2-12.

Alberts, J. F., Gelderblom, W. C. A., Marasas, W. F. O. and Rheeder, J. P. (1994). Evaluation of liquid media for fumonisin production by *Fusarium moniliforme*. *Mycotoxin Res.* **10**: 107-115.

Alderman, G. G. and Marth, E. H. (1976). Inhibition of growth and aflatoxin production of *Aspergillus parasiticus* by citrus oils. *Z. Lebensm. Unters. Forsch.* **160**:353-358.

Alcroft, R., Carnaghan, R. B. A., Sargeant, K. and O'Kelly, J. (1961). A toxic factor in Brazilian groundnut meal. *Vet. Rec.* **73**:428-429

Alvarez-Barrea, V., Pearson, A. M., Price, J. F., Gray, J. I. and Aust, S. D. (1982). Some factors influencing aflatoxin production in fermented sausages. *J. Food Sci.* **47**:1773-1775.

Applebaum, R. S. and Marth, E. H. (1981). Biogenesis of the C_{20} polyketide, aflatoxin. A review. *Mycopathologica* **76**:103-114.

Arseculeratne, S. N., Samarajeewa, U. and Wellinga, L. V. (1976). Inhibition of aflatoxin accumulation in smoked substrates. *J. Appl. Bacteriol.* **41**:223-233.

Asoa, T., Büchi, G., Abdel-Kader, M. M., Chang, S. B., Wick, E. L. and Wogan, G. N. (1965). The structures of aflatoxin B_1 and G_1. *J. Am. Chem. Soc.* **87**:882-886.

Azzouz, M. A. and Bullerman, L. B. (1982). Comparative antimycotic effects of selected herbs, spices, plant components and commercial antifungal agents. *J. Food Prot.* **45**:1298-1301.

Bacon, C. W. and Hinton, D. M. (1996). Fusaric acid and pathogenic interaction of corn and non-corn, isolates of *Fusarium moniliforme*, a nonobligate pathogen of corn. *In: Fumonisins in Food; Advances in Experimental Medicine and Biology*. Jackson, L. S., DeVries, J. W. and Bullerman, L. B. (eds). Plenum Press, New York. Vol. 392. pp. 1-18.

Bacon, C. W. , Bennett, R. M., Hinton, D. M. and Voss, K. A. (1992). Scanning electron microscopy of *Fusarium moniliforme* within asymptomic corn kernels and kernels associated with equine leukoencephalomalacia. *Plant Dis.* pp. 144-148.

Bahk, J. and Marth, E. H. (1983a). Aflatoxin production is inhibited by selected herbal drugs. *Mycopathologica* **83**:129-134.

Bahk, J. and Marth, E. H. (1983b). Growth and synthesis of aflatoxin by *Aspergillus parasiticus* in the presence of ginseng products. *J. Food Prot.* **46**:210-215.

Bahk, J. R., Kwang-Sik, I. and Lee, J. K. (1985). Effects of crude saponin on growth and aflatoxin production by *Aspergillus parasiticus*. *Korean J. Microbiol.* **23**:259-264. [Chem. Abstr. 104,105769b(1986)]

Baranowski, J. D. and Nagel, C. W. (1983). Inhibition of *Pseudomonas fluorescens* by hydroxy-cinnamic acids and their alkyl esters. *J. Food Sci.* **47**:1587.

Baranowski, J. D., Davidson, P. M., Nagel, C. W. and Branen, A. L. (1980). Inhibition of *Saccharomyces cerevisiae* by naturally occurring hydroxy-cinnimates. *J. Food Sci.* **45**:592.

Basilico, M. Z. and Basilico, J. C. (1999). Inhibitory effects of some spice essential oils on *Aspergillus ochraceus* NRRL 3174 growth and ochratoxin A production. *Lett. in Appl. Microbiol.* **29**:238-241.

Batt, C., Solberg, M. and Ceponis, M. (1980). Inhibition of aflatoxin production by carrot root extract. *J. Food Sci.* **45**:1210-1213.

Batt, C., Solberg, M. and Ceponis, M. (1983). Effect of volatile components of carrot seed oil on growth and aflatoxin production by *Aspergillus parasiticus*. *J. Food Sci.* **48**:762-768.

Bean, G. A. and Rambo, A. (1975). Use of dimethyl sulfoxide to control aflatoxin production. *Ann. N.Y. Acad. Sci.* **243**:238-245.

Bean, G. A. and Southall, A. (1983). Effect of pyriazinone herbicides on growth and aflatoxin release by *Aspergillus flavus* and *Aspergillus parasiticus*. *Appl. Environ. Microbiol.* **46**:503-505.

Beardall, J. M. and Miller, J. D. (1994). Natural occurrence of mycotoxins other than aflatoxins in Africa, Asia and South America. *Mycotoxin Res.* **10**:21-40.

Bennett, G. A. and Richard, J. L. (1995). Effect of thermal processing on the stability of fumonisins. *In: Fumonisins in Foods: Advances in Experimental Medicine and Biology.* Jackson, L. S., DeVries, J. W. and Bullerman, L. B. (eds). Plenum Press : New York. pp. 345-353.

Bennett, J. W. and Christensen, S. B. (1983). New perspectives on aflatoxin biosynthesis. *Adv. Appl. Microbiol.* **29**:53-92.

Bennett, J. W., Lee, L. S. and Gaar, G. G. (1976). Effect of acetone on production of aflatoxins and versicolorin pigments by resting cell cultures of *Aspergillus parasiticus*. *Mycopathologica* **58**:9-12.

Beuchat, L. R. (1976). Sensitivity of *Vibrio parahaemolyticus* to spices and organic acids. *J. Food Sci.* **41**:899.

Bhat, R. V. and Miller, J. D. (1991). Mycotoxins and food supply. *Food Nutr. Agric.* **1**:27-31.

Bhatnagar, D., Cleveland, T. E. and Kingston, D. G. I. (1991). Enzymological evidence for separate pathways for AFB_1 and AFB_2. *Biochem.* **30**:4343-4350.

Bilgrami, K. S., Misra, R. S., Sinha, K. K. and Singh, P. L. (1979). Prevention of aflatoxin production in liquid and solid substrates through some plant extracts. *Indian J. Exp. Biol.* **17**:1151.

Bilgrami, K. S., Misra, R. S., Sinha, K. K. and Singh, P. L. (1980). Effect of some wild and medicinal plant extracts on aflatoxin production and growth of *Aspergillus flavus* in liquid culture. *J. Indian Bot. Soc.* **59**:123-126.

Bilgrami, K. S., Sinha, K. K. and Singh, P. (1981). Inhibition of aflatoxin production by ferulic acid on some cereals and oil seeds. *Curr. Sci.* **50**:997-998.

Bilgrami, K. S., Sinha, K. K. and Singh, P. (1982). Prevention of aflatoxin production on some cereals and oil seeds by *o*-vanillin. *Curr. Sci.* **51**:138.

Biollaz, M. G., Büchi, G. and Milne, G. (1968). Biosynthesis of aflatoxins. *J. Am. Chem. Soc.* **90**:5017-5019.

Biollaz, M. G., Büchi, G. and Milne, G. (1970). The biosynthesis of the aflatoxins. *J. Am. Chem. Soc.* **92**:1035-1043.

Blount, W. P. (1961). Turkey X disease. *J. Brit. Turkey. Fed.* **9**:52-77.

Bothast, R. J., Bennett, G. A., Vancauwenberge, J. E. and Richard, J. L. (1992). Fate of fumonisin B_1 in naturally contaminated maize during ethanol fermentation. *Appl. Environ. Microbiol.* **58**:233-236.

Brekke, O. L., Sinnhube, R. O., Peplinski, A. J., Wales, H. H. and Putman, G. B. (1977). Aflatoxin in corn; ammonia inactivation and bioassay with rainbow trout. *Appl. Environ. Microbiol.* **34**:34-37.

Buchanan, R. L. and Ayres, J. C. (1976). Effect of sodium acetate on growth and aflatoxin production by *Aspergillus parasiticus* NRRL 2999. *J. Food Sci.* **41**:128-132.

Buchanan, R. L. and Shepherd, A. J. (1981). Inhibition of *Aspergillus parasiticus* by thymol. *J. Food Sci.* **46**:976-977.

Bullerman, L. B. (1974). Inhibition of aflatoxin production by cinnamon. *J. Food Sci.* **39**:1163-1165.

Bullerman, L. B. (1983). Effects of potassium sorbate on growth and aflatoxin production by *Aspergillus parasiticus* and *Aspergillus flavus*. *J. Food Prot.* **46:**940-942.

Bullerman, L. B., Lieu, F. Y. and Seiler, S. A. (1977). Inhibition of growth of aflatoxin production by cinnamon and clove oils, cinnamic aldehyde and eugenol. *J. Food Sci.* **42**:1107-1109, 1116.

CAST (1989). *Mycotoxins: economic and health risks.* Report 116. Council for Agriculture Science and Technology, Ames, IA.

Cavallito, C. J. and Bailey, J. H. (1944). *J. Am. Chem. Soc.* **66:**1150.

Cawood, M. E., Gelderblom, W. C. A., Vleggar, R., Behrend, Y., Thiel, P. G. and Marasas, W. F. O. (1991). Isolation of the fumonisin mycotoxins: a quantitative approach. *J. Agric. Food Chem.* **39**:1958-1962.

Chain, A. (1941). *Lancet.* **241**:177

Charmley, L. L., Rosenberg, A. and Trenholm, H. L. (1994). Factors responsible for economic losses due to *Fusarium* mycotoxin contamination of grains, foods and feedstuffs. *In: Mycotoxins in Grain.* J. D. Miller and Trenholm, L.L (eds). Eagan Press: St. Paul. pp. 471-486.

Chatterjee, D. (1990). Inhibition of fungal growth and infection in maize grains by spice oils. *Lett. Appl. Microbiol.* **11**:148-151.

Chipley, J. R. and Uraih, N. (1980). Inhibition of *Aspergillus* growth and aflatoxin release by derivatives of benzoic acid. *Appl. Environ. Microbiol.* **40**:352-357.

Chipley, J. R., Story, L. D., Todd, P. T. and Kabara, J. J. (1981). Inhibition of *Aspergillus* growth and extracellular aflatoxin accumulation by sorbic acid and derivatives of fatty acids. *J. Food Saf.* **2**:109-120.

Chu, F. S. and Li, G. Y. (1994). Simultaneous occurrence of fumonisin B_1 and other mycotoxins in moldy corn collected from the People's Republic of China in regions with high incidence of eosophageal cancer. *Appl. Environ. Microbiol.* **60**:847-852.

Colvin, B. M. and Harrison, L. R. (1992). Fumonisin-induced pulmonary edema and hydrothorax in swine. *Mycopathologia* **117**:79-82.

Conner, D. E. (1983). Inhibitory effects of essential oils and oleoresins from plants on food spoilage yeasts. M. Sc. Thesis. University of Georgia, Athens.

Conner, D. E. (1993). Naturally occuring compounds. *In: Antimicrobials in Foods,* 2nd edition. Davidson, P. M. and Branen, A. L. (eds). Marcel Dekker, Inc. : New York. pp. 441-468.

Conner, D. E. and Beuchat, L. R. (1984). Effects of essential oils from plants on growth of food spoilage yeasts. *J. Food Sci.* **49**:429.

Conner, D. E., Beuchat, L. R., Worthington, R. E. and Hitchcock, H. L. (1984). Effects of essential oils and oleoresins of plants on ethanol production, respiration and sporulation of yeasts. *Int. J. Food Microbiol.* **1**:63.

Davis, N. D. and Diener, U. L. (1967). Inhibition of aflatoxin synthesis by p-aminobenzoic acid, potassium sulphate and potassium flouride. *Appl. Microbiol.* **15**:1515-1518.

Daw, Z. Y., El-Baroty, G. E. and Mahmoud, A. M. (1995). Inhibition of *Aspergillus parasiticus* growth and aflatoxin production by some essential oils. *J. Afric. Crop Sci.* **3**:511-517.

Diener, U. L. and Davis, N. D. (1966). Aflatoxin production by *Aspergillus flavus*. *Phytopathol.* **56**:1390-1393.

Doko, M. B., Siame, A. B., Mpunchane, S., Brown, N., Sydenham, E. W. and Canet, C. (1996). Natural co-occurrence of fumonisins in cereals and cereal based foods collected from eastern and southern Africa. *J. Agric. Food Chem.* **44**:3240-3243.

Draughon, F. A. and Ayres, J. C. (1981). Inhibition of aflatoxin production by selected insecticides. *Appl. Environ. Microbiol.* **41**:972-976.

Dutton, M. F. (1985). Characterization of metabolites from a strain of *Aspergillus flavus* accumulating aflatoxin B_2. *Afr. J. Chem.* **38**:107-109.

Dutton, M. F. (1988). Enzymes and aflatoxin biosynthesis. *Microbiol. Rev.* **52**:274-295.

Dutton, M. F. and Heathcote, J. G. (1966). Two new hydroxyaflatoxins. *Biochem. J.* **101**:21-22.

Dwivedi, S. K. and Singh, K. P. (1999). Fungitoxicity of some higher plant products against *Macrophomina phaseolina* (tassi) Giod. *Flav. Frag. J.* **13**:397-399.

El-Baroty, G. S. (1994). Antioxidant and antilipolytic effect of natural phenolic acid on Egyptian olive by-products. *Texas S. Univ. Res. J.* **4**:22-35.

El- Baroty, G. S. (1997). Antimicrobials and antioxidant activities of some essential oil. *J. of Agric. Sci. Mansoura Univ.* **22**:1223-1233.

El-Gazzar, F. E., Rusul, G. and Marth, E. H. (1986). Growth and aflatoxin production by *Aspergillus parasiticus* in the presence of sodium chloride. *J. Food Prot.* **49**:461-466.

Ehrlich, K. and Ciegler, A. (1984). Effect of phytate on aflatoxin formation by *Aspergillus parasiticus* and *Aspergillus flavus* in synthetic media. *Mycopathologia* **87**:99-103.

Ehrlich, K. and Ciegler, A. (1985). Effect of phytate on aflatoxin formation by *Aspergillus* grown on different grains. *Mycopathologia* **92**:3-6.

Etcheverry, M., Magnoil, C., Dalcero, A., Chulze, S. and Lecumberry, S. (1998). Aflatoxin B_1, zearalenone and deoxynivalenol production by *Aspergillus parasiticus* and *Fusarium graminearum* in interactive cultures on irradiated corn kernels. *Mycopathologia* **142**:37-42.

FAO/UN. (1979). *Recommended Practices for the Prevention of Mycotoxins in Foods, Feeds and their Products.* FAO Food and Nutrition Paper. Food and Agriculture Organization of the United Nations. Rome.

FAO/WHO/UNEP. (1987). *Report of the Second Joint International Conference on Mycotoxins* held in Bangkok, Thailand from 28 Sept. to 2 Oct. 1987.

Farag, R. S., Abo-Raya, S. H., Desoky, G. E. and El-Baroty, G. S. (1991). Safety evaluation of thyme and clove essential oils as natural antioxidants. *Afric. J. Agric. Sci.* **18**:169-176.

Farag, R. S., Badei, A. Z. and El-Baroty, G. S. (1989a). Antioxidant activity of some spice essential oils on linoleic acid oxidation in aqueous media. *J. Am. Chem. Oil Soc.* **66**:792-799.

Farag, R. S., Daw, Z. Y. and Abo-Raya, S. H. (1989b). Influence of some spice essential oils on *Aspergillus parasiticus* growth and production of aflatoxins in a synthetic medium. *J. Food Sci.* **54**:74-76.

Farag, R. S., Daw, Z. Y., Hewedi, F. M. and El-Baroty, G. S. A. (1989c). Antimicrobial activity of some Egyptian spice essential oils. *J. Food Prot.* **52**:665.

Farrell, K. T. (1985). *Spices, Condiments and Seasonings*, AVI Publishing : Westport, CT.

Flanigan, B. and Hui, S. C. (1976). The occurrence of aflatoxin producing strains of *Aspergillus flavus* in the mould floras of ground spices. *J. Appl. Bacteriol.* **41**:411-418.

Floyd, J. C., Bennett, J. W., Dunn, J. J. and Fine, J. S. (1982). Growth of high aflatoxin B_2 mutants on defined and complex media and with ethoxyquin. *Microbios.* **35**:21-30.

Foudin, A. S., Leaich, L. L. and Ayres, J. C. (1978). Effects of ethoxyquin on aflatoxin producing strains of *Aspergillus parasiticus* and *Aspergillus flavus.* *J. Food Sci.* **43**:731-734.

Frisvad, J. C. and Samson, R. A. (1991). Filamentous fungi in foods and feeds: ecology, spoilage and mycotoxin production. *In: Handbook of Applied Mycology: Foods and Feeds.* Vol. 3. Arora, D. K., Mukerji, K. G. and Marth, E. H. (eds). Marcel Dekker : New York. pp. 31-68.

Fung, D. Y. C., Taylor, S. and Kahan, J. (1977). Effects of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) on growth and aflatoxin production of *Aspergillus flavus. J. Food Saf.* **1**:39-51.

Furia, T. E. (ed) (1968). *Handbook of Food Additives.* The Chemical Rubber Company: Cleveland.

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

Gelderblom, W. C. A., Semle, E., Marasas, W. F. O. and Faber, E. (1992). The cancer initiating potential of the fumonisin B mycotoxins. *Carcinogenesis* **13:**433-437.

Ghosh, J. and Häggblom, P. (1985). Effect of sublethal concentrations of propionic or butyric acid on growth and aflatoxin production by *Aspergillus flavus. Int. J. Food. Microbiol.* **2**:323-330.

Gold, L. S., Sawyer, C. B., Magaw, R., Backman, G. M., de Veciana, M., Levinson, R., Hooper, N. K., Havender, W. R., Bernstein, L., Peto, R., Pike, M. C. and Ames, B. N. (1984). A carcinogenic potency database of the standardised results of animal bioassays. *Environ. Health Persp.* **58**:9-13.

Gonzalez, H. H. L., Martinez, E. J., Pacin, M., Resnik, S. L. and Sydenham, E. W. (1999). Natural occurrence of fumonisins, deoxynivalenol, zearalenone and aflatoxins in field trail corn in Argentina. *Food Addit. Contam.* **16**:565-569.

Gueldner, R. C., Wilson, D. M. and Heidt, A. R. (1985). Volatile compounds inhibiting *Aspergillus flavus. J. Agric. Food Chem.* **33**:411-413.

Gunasekaran, M. (1981). Optimum culture conditions for aflatoxin B_2 production by a human pathogenic strain of *Aspergillus flavus*. *Mycologia* **73**:697-704.

Hassan, M. N. (1996). Cardamom and mastich oils as natural preservation of butter. *Zagazig J. Agric. Res.* **23**:829-842.

Hartley, R. D., Nesbitt, B. F. and O'Kelly, J. (1963). Toxic metabolites of *Aspergillus flavus*. *Nature* (London) **198**:1056-1058.

Hendrich, S., Miller, K. A., Wilson, T. M. and Murphy, P. A. (1993). Toxicity of *Fusarium proliferatum* fermented nixtamilized corn based diets fed to rats: effect of nutritional status. *J. Agric. Food Chem.* **41**:357-360.

Hirasa, K. and Takemasa, M. (1998). Antimicrobial and antioxidant properties of spices. *In: Spice Science and Technology.* Marcel Dekker : New York. pp. 163-200.

Hitokoto, H., Morozumi, S., Wauke, T., Sakai, S. and Kurata, H. (1980). Inhibitory effects of spices on growth and toxin production of toxigenic fungi. *Appl. Environ. Microbiol.* **39**:818-822.

Hitokoto, H., Morozumi, S., Wauke, T., Sakai, S. and Ueno, I. (1978). Inhibitory effects of condiments and herbal drugs on the growth and toxin production of toxigenic fungi. *Mycopathologia* **66**:161-167.

Hollinger, K. and Ekpergin, H. E. (1999). Mycotoxicosis in food producing animals. *Vet. Clin. North Am.- Food. Anim. Pract.* **15**:133.

Holmquist, G. U., Walker, H. W. and Stahr, H. M. (1983). Influence of temperature, pH, water activity and antifungal agents on growth of *Aspergillus flavus* and *Aspergillus parasitcus*. *J. Food Sci.* **48**:778-782.

Homans, A. L. and Fuchs, A. (1970). Direct bioautography of TLC as a method for detection of fungitoxic substances. *J. Chromatograph* **51**:325-327.

IARC. (1993). Monograph 56. *Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins.* International Agency for Research on Cancer : Lyon.

Jayashree, T. and Subramanyam, C. (1999). Antiaflatoxigenic activity of eugenol is due to inhibition of lipid peroxidation. *Lett. Appl. Microbiol.* **28**: 179-183.

Jung, S. J. and Suh, M. J. (1985). Effects of steroidal carbamate derivatives on the aflatoxin productivity by *Aspergillus flavus. Korean J. Mycol.* **13:**243-247.

Jurd, L., King, A. D. Jr., Mihara, K. and Stanley, W. L. (1971). Antimicrobial properties of natural phenols and related comounds. *I. Obtusastyrene. Appl. Microbiol.* **21**:507-510.

Karapinar, M. (1985). The effect of citrus oils and some spices on growth and aflatoxin production by *Aspergillus parasiticus* NRRL 2999. *Int. J. Food Microbiol.* **2**:239-245.

Kellerman, T. S., Marasas, W. F. O., Thiel, P. G., Gelderblom, W. C. A., Cawood, M. E. and Coetzer, J. A. W. (1990). Leukoencephalomalacia in two horses induced by oral dosing of fumonisin B_1. *Onderstepoort J. Vet. Res.* **57**:269-275.

Khan, S. N., Maggon, K. K. and Venkitasubramanian, T. A. (1978). Inhibition of aflatoxin biosynthesis by tolnaftate. *Appl. Environ. Microbiol.* **36**:270-273.

Kim, J. M., Marshall, M. R., Cornell, J. A., Preston, J. A. and Wei, C. I. (1995). Antibacterial activity of carvacrol, citral and geraniol against *Salmonella typhimurium* in culture medium and on fish cubes. *J. Food Sci.* **60**:1364-1368.

King, A. D. Jr., Hocking, A. D. and Pitt, J. I. (1979). Dichloran-rose bengal medium for enumeration and isolation of moulds from foods. *Appl. Environ. Microbiol.* **37**:859-964.

Klich, M. A. and Pitt, J. I. (1992). *A Laboratory Guide to Common Aspergilli Species and their Telomorphs.* Commonwealth Scientific and Industrial Research Organisation.

Kurita, N., Miyaji, M., Kurane, R., Takahara, Y. and Ischimura, K. (1979). Antifungal activity and molecular orbital energies of aldehyde compounds from oils of higher plants. *Agric. Biological Chem.* **43**:2365-2368.

Kurosaki, F. and Nishi, A. (1983). Isolation and antimicrobial activity of phytoalexin 6-meth-oxymellein from cultured carrot cells. *Phytochem.* **22**:669.

Laurent, D., Lanson, M., Goasdoué, N., Kohler, F., Pellegrin, F. and Platzer, N. (1990). Étude en RMN ^{1}H et ^{13}C de la macrofusine, toxine isolée de maïs infesté par *Fusarium moniliforme* Sheld. *Microbiol. Alim. Nutr.* 7:9-16.

Leven, M., Van der Berge, D. A., Mertens, F., Vlietinck, A. and Lammens, E. (1979). Screening of higher plants for biological activities. *Planta Medica J. of Medicinal Plant Res.* **36**: 311-321.

Lin, C. C. S. and Fung, D. Y. C. (1983). Effect of BHA, BHT, TBHQ and PG on growth and toxigenesis of selected aspergilli. *J. Food Sci.* **48**:576-580.

Llewellyn, G. C., Burkett, M. L. and Eadie, T. (1981a). Potential mould growth, aflatoxin production and antimycotic activity of selected natural spices and herbs. *J. Assoc. Anal. Chem.* **64**:955-960.

Llewellyn, G. C., Dixon, E. C., Eadie, T., Dashek, W. V. and O'Rear, C. E. (1981b). Aflatoxin formation on whole and ground cumin and anise seeds. *J. Am. Oil Chem. Soc.* **58**:9585A-988A.

Lu, Z., Dantzer, W. R., Hopmans, E. C., Prisk, V., Cunnick, J. E., Murphy, P. A. and Hendrich, S. (1997). Reaction with fructose detoxifies fumonisin B_1 while stimulating liver associated natural killer cell activity in rats. *J. Agric. Food Chem.* **45**:803-809.

Lubulwa, A. S. G. and Davis, J. S. (1994). Estimating the social costs of impact of fungi and aflatoxins in maize and peanuts. *In: Stored products protect.* Highley, E., Wright, E. J., Banks, H. J. and Champ, B. R. (eds). CAB International : UK. pp. 1017-1042.

Mabrouk, S. S., El-Shayeb, N. M. A., El-Rafai, A. H. Sallam, L. A. R. and Hamby, A. A. (1985). Inhibitory activities of some marine algae on aflatoxin accumulation. *Appl. Microbiol. Biotechnol.* **22**:152-155.

Madhyastha, M. S. and Bhat, R. V. (1984). *Aspergillus parasiticus* growth and aflatoxin production on black and white pepper and the inhibitory action of their chemical constituents. *Appl. Environ. Microbiol.* **48**:376-379.

Mahmoud, A. L. E. (1994). Antifungal action and antiaflatoxigenic properties of some essential oil constituents. *Lett. Appl. Microbiol.* **19**:110-113.

Mallozi, M. A. B., Campacci, C. A. and Chiba, S. (1985). Acao do acido propionico na producao de aflatoxina e no crescimento de *Aspergillus flavus*. *Summa Phytopathol.* **11**:220-229.

Marasas, W. F. O. (1996). Fumonisins: history, world-wide occurrence and impact. *In: Fumonisins in Food.* L. S. Jackson, J. W. DeVries, and L. B. Bullerman (eds). Plenum Press : New York. pp. 1-18.

Marasas, W. F. O., Jaskiewicz, K., Venter, F. S. and Schalkwyk, D. J. (1988). *Fusarium moniliforme* contamination of maize in oesophageal cancer areas in Transkei. *South Afric. Medical J.* **74**:110-114.

Marin, S., Sanchis, V., Rull, F., Ramos, A. J. and Magan, N. (1998). Colonization of maize grain by *Fusarium moniliforme* and *Fusarium proliferatum* in the presence of competing fungi and their impact on fumonisin production. *J. Food Prot.* **61**:1489-1496.

Marshall, D. L. and Bullerman, L. B. (1986). Effect of sucrose esters in combination with selected mould inhibitors on growth and aflatoxin production by *Aspergillus parasiticus*. *J. Food Prot.* **49**:378-382.

Martindale, W. H. (1910). *Perfum. Essential Oil Rec.* 1:266.

Maruzzella, J. C. and Liguori, L. (1958). The *in vitro* antifungal activity of essential oils. *J. Am. Pharm. Assoc.* **47:**250.

Mayura, K., Basappa, S. C. and Sreenivas Murthy, V. (1985). Studies on some factors affecting aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus*. *J. Food. Sci. Technol.* **22**:126-129.

Mesterházy, Á., (ed). (1997). Proceedings of the 5th European *Fusarium* seminar. *Cereal Res. Commun.* **25**:231-857.

Miller, J. D. (1991). Significance of mycotoxins for human health and nutrition. *ACIAR Proc.* **36**:126-135.

Miller, J. D. (1993). *The toxicological significance of mixtures of fungal toxins.* African Newsletter Occupational Health and Safety. Institute of Occupational Health : Helsinki. **3**:32-38.

Miller, J. D. (1998). Mycotoxins and phycotoxins – development in chemistry, toxicology and food safety. *In: Global Significance of Mycotoxins.* Alaken IN : Colorado. pp. 3-15.

Moreck, K. E. P. A., McElfresh, A., Wohlman, A. and Hilton, B. W. (1980). Aflatoxin destruction in corn using sodium bisulphite, sodium hydroxide and aqueous ammonia. *J. Food Prot.* **43**:571-574.

Morozumi, S. (1978). Isolation, purification and antibiotic activity of *o*-methoxycinnamic-aldehyde from cinnamon. *Appl. Environ. Microbiol.* **43**:895-898.

Munkvold, G. P. and Desjardins, A. E. (1997). Fumonisins in maize. *Plant Dis.* **81**:556-565.

Nakatani, N. (1994). Antioxidantive and antimicrobial constituents of herbs and spices. *In: Spices, Herbs and Edible Fungi.* Charalmbous, G. (ed). El-Sevier Sci. B. V. Amsterdam, London and New York. pp. 251-271.

Nesbitt, B. F., O'Kelly, J., Sargeant, K. and Sheridan, A. (1962). Toxic metabolites of *Aspergillus flavus*. *Nature* (London) **195**:1062-1063.

Noda, K., Isozaki, S. and Taniguchi, H. (1985). *Nippon Shokuhin Kogyo Gakkaishi.* **32**:791.

Norred, W. P., Wang, E., Yoo, H., Riley, R. T. and Merill, A. H., Jr. (1992). In vitro toxicology of fumonisins and the mechanistic implications. *Mycopathologia* **117**:73-78.

Obioha, W. I., Stahr, H. M. and Kraft, A. A. (1983). Effect of sodium nitrate on aflatoxin production in pork sausage at different temperatures and the effect of nitrite on growth of *Aspergillus parasiticus* in culture. *J. Agric. Food Chem.* **31**:1039-1042.

Paster, N., Juven, B. J. and Harshemesh, H. (1988) Antimicrobial activity and inhibition of aflatoxin B_1 formation by olive plant tissue constituents. *J. Appl. Bacteriol.* **64**: 293-297.

Patel, S., Hazel, C. M., Winterton, A. G. M. and Gleadle, A. E. (1997). Surveillance of fumonisins in UK maize-based foods and cereals. *Food Addit. Contam.* **14**:187-191.

Peraica, M., Radic, B., Lucic, A. and Pavlovic, M. (1999). Toxic effects of mycotoxins in humans. *Bull. World Health Org.* 77: 754-766.

Pons, W. A. Jr., Cucullu, L. S., Lee, L. S., Janssen, H. J. and Goldblatt, L. A. (1972). Kinetic study of acid-catalysed conversion of aflatoxins B_1 and G_1 to B_{2a} and G_{2a}. *J. Am. Oil Chem. Soc.* **49**:124-128.

Prasad, T. (1983). *Precursors and biogenetic schemes of aflatoxin synthesis.* A review. *Proc. Symp. Mycotoxin in Food and Feed* : Bhagalpur. pp. 173-197.

Quain, G. S., Ross, R. K., Yu, M. C., Yuan, J. M., Gao, Y-T., Henderson, B. E.,Wogan, G. N. and Groopman, J. D. (1994). A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in Shangai, Peoples Republic of China. *Cancer Epidemiol., Biomarkers and Prevention.* **3**:3-10.

Rai, M. K., Qureshi, S. and Pandey, A. K. (1999). *In vitro* susceptibility of opportunistic *Fusarium* spp. to essential oils. *Mycoses* **42**: 1-2.

Rama Devi, G. and Polasa, H. (1984). Inhibition of growth and toxin production by lauric acid derivatives in *Aspergillus* species. *Curr. Sci.* **53**:811-813.

Rama Devi, G. and Polasa, H. (1985). Diacetyl - a growth and toxin inhibitor of aspergilli. *J. Stored Prod. Res.* **21**:195-197.

Ramjee, G. (1990). Some studies on fungal incidence, aflatoxin and kwashiorkor. M. Sc. Thesis, University of Natal–Durban.

Reiss, J. (1982). Development of *Aspergillus parasiticus* and formation of aflatoxin B_1 under the influence of conidiogenesis affecting compounds. *Arch. Microbiol.* **133**:236-238.

Rheeder, J. P., Marasas, W. F. O., Thiel, P. G., Sydenham, E. W. and van Schalkwyk, D. J. (1992). *Fusarium moniliforme* and fumonisins in corn related to human oesphageal cancer in Transkei. *Phytopathol.* **82**:353-357.

Riley, R. T., Norred, W. P. and Bacon, C. W. (1993). Fungal toxins in foods: recent concerns. *Ann. Rev. Nutr.* **13**:167-189.

Riley, R. T., Wang, E., Schroeder, J. J., Smith, E. R., Plattner, R. D., Abbas, H., Yoo, H-S., Chamberlain, W. J. and Norred, W. F. (1996). Evidence for disruption of sphingolipid metabolism as a contributing factor in the toxicity and carcinogenicity of fumonisins. *Natural Toxins* **4**:3-15.

Rosales, A. M. and Mew, T. W. (1997). Suppression of *Fusarium moniliforme* in rice by rice-associated antagonistic bacteria. *Plant Dis.* **81**:49-52.

Rumbeiha, W. K. and Oehme, F. W. (1997). Fumonisin exposure in Kansas through consumption of maize-based market foods. *Vet. Human Toxicol.* **39**:220-225.

Salomao de Cassia, R. and Purchio, A. (1982). Atividade de sub-stancias de origem vegetal na inhibicao do crescimento e na producao de aflatoxinas por *Aspergillus flavus* e *Aspergillus parasiticus*. *Rev. Microbiol. Sao Paulo* **13**:394-401.

Sargeant, K., Sheridan, A., O'Kelly, J. and Carnaghan, R. B. A. (1961). Toxicity associated with certain samples of ground nuts. *Nature* (London) **192**:1095-1097.

Schroeder, H. W. and Carlton, W. W. (1973). Accumulation of only aflatoxin B_2 by a strain of *Aspergillus flavus*. *Appl. Microbiol.* **25**:146-148.

Scott, P. M. and Lawrence, G. A. (1994). Stability and problems in recovery of fumonisins added to corn-based foods. *J. AOAC Int.* **77**:541-545.

Seenappa, M. and Kempton, A. G. (1980). *Aspergillus* growth and aflatoxin production on black pepper. *Mycopathologia* **70:**135-137

Sharma, A., Tewari, G. M., Shrikande, A. J., Padwal-Desai, S. R. and Bandypadhyay, C. (1979). Inhibition of aflatoxin producing fungi by onion extracts. *J. Food. Sci.* **44**:1545-1547.

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

Sibanda, L., Marovatsanga, L. V. and Restka, J. J. (1997) Review of mycotoxin work in sub-Saharan Africa. *Food Contr.* **8**:21-29.

Sinha, K. K. and Singh, P. (1981). Effect of some phenolics on aflatoxin production and growth of *Aspergillus parasiticus. Indian Phytopathol.* **34**:530-531.

Sohn, H. B., Seo, J. A. and Lee, Y. W. (1999). Co-occurrence of *Fusarium* mycotoxins in mouldy and healthy corn from Korea. *Food Addit. Contam.* **16**:153-158.

Stewart, R. G., Wyatt, R. D. and Ashmore, M. D. (1977). The effect of various antifungal agents on aflatoxin production and growth characteristics of *Aspergillus parasiticus* and *Aspergillus flavus* in liquid medium. *Poultry Sci.* **56**:1630-1635.

Steyn, P. S., Vleggaar, R. and Wessels, P. L. (1980). The biosynthesis of aflatoxin and its congeners. *In: The Biosynthesis of Mycotoxins.* Steyn, P. S. (ed.) A study in secondary metabolism. Academic Press : New York. pp. 105-155

Swaminanthan, B. and Koehler, P. E. (1976). Isolation of an inhibitor of *Aspergillus parasiticus* from white potatoes (*Solanum tuberosum*). *J. Food Sci.* **41**:313-319.

Sydenham, E. W., Shephard, G. S., Thiel, P. G., Marasas, W. F. O. and Stockenstrom, S. (1991). Fumonisin contamination of corn-based human foodstuffs. *J. Agric. Food Chem.* **39**:2014-2018.

Sydenham, E. W., Thiel, P. G., Marasas, W. F. O., Sherphard, G. S., van Schalkwyk, D. J. and Kock, K. R. (1990). Natural occurrence of some *Fusarium* mycotoxins in maize from low and high oesophageal cancer prevelance areas of the Transkei, Southern Africa. *J. Agric. Food Chem.* **38**:1900-1903.

Sydenham, E. W., van der Westhuizen, L., Stockenstrom, S., Shephard, G. S. and Thiel, P. G. (1994). Fumonisin-contaminated maize: physical treatment for the partial decontamination of bulk shipments. *Food Addit. Contam.* **11**:25-32.

Takahashi, D. M. and Beebe, R. M. (1979). Analysis of food and beverages. *In: Reversed Phase High Performance Liquid Chromatographic Procedure for Determining Aflatoxins in Foods and Beverages using Fluorescence Detection.* Vol.1. Academic Press. pp. 100- 129.

Thiel, P. G. (1998). The role of natural toxins in environmental pollutions. *South Afric. J. Sci.* **94**:419-422.

Thiel, P. G., Marasas, W. F. O., Sydenham, E. W., Shephard, G. S. and Gelderblom, W. C. A. (1992). The implications of naturally occurring levels of fumonisins in corn for human and animal health. *Mycopathologia* **117**:3-9.

Thiel, P. G., Meyer, C. J. and Marasas, W. F. O. (1982). Natural occurrence of moniliformin together with deoxynivalenol and zearalenone in Transkein corn. *J. Agric. Food Chem.* **30**:308-312.

Thiel, P. G., Sydenham E. W., Shephard, G. S. and Schalkwyk, D. J. (1993). Study of the reproducibility of a liquid chromatographic method for the determination of fumonisins B_1 and B_2 in corn: IUPAC Collaborative Study. *J. AOAC. Int.* **76**:361-366.

Tiwari, R. P., Mittal, V., Bhalla, T. C., Saini, S. S. and Vadehra, D. V. (1986). Effect of fatty acids on aflatoxin production by *Aspergillus parasiticus*. *Folia Microbiol.* **31**:120-123.

Thompson, D. P. (1990). Effect of essential oils on spore germination of *Rhizopus, Mucor* and *Aspergillus* species. *Mucologia* **78**:482-485.

Tsai, W. Y. J., Shao, K. P. P. and Bullerman, L. B. (1984). Effects of sorbate and propionate on growth and aflatoxin production of sub-lethally injured *Aspergillus parasiticus*. *J. Food Sci.* **49**:86-90.

Turner, R. B., Lindsey, D. L., Davis, D. D. and Bishop, R. D. (1975). Isolation and identification of 5,7-dimethoxyisoflavone, an inhibitor of *Aspergillus flavus* from peanuts. *Mycopathologia* **57**:39-40.

Ueda, S., Yamashita, H., Nakajima, M. and Kuawahara, S. (1982) *Nippon Shokuhin Kogyo Gakkaishi* **29**:111

Ueno, Y., Aoyama, S., Sugiura, Y., Wang, D-S., Lee, U-S., Hirooka, E. Y., Hara, S., Karki, T., Chen, G. and Yu, S-Z. (1993). A limited survey of fumonisins in maize and maize based products in Asian countries. *Mycotoxin Res.* **9**:27-34.

Uraih, N. and Chipley, J. R. (1976). Effects of various acids and salts on growth aflatoxin production by *Aspergillus flavus* NRRL 3145. *Microbios* **17**:51-59.

Uraih, N. and Ogbadu, L. (1982). Influence of woodsmoke on aflatoxin production by *Aspergillus flavus*. *Eur. J. Appl. Microbiol. Biotechnol.* **14**:51-53.

Vesonder, R. F., Labeda, D. P. and Peterson, R. E. (1992). Phytotoxic activity of selected water soluble metabolites of *Fusarium* against *Lemna minor* L. (Duckweed). *Mycopathologia.* **118**:185-189.

Vesonder, R., Peterson, R., Plattner, R. and Weisleder, D. (1990). Fumonisin B_1: Isolation from corn culture, and purification by high performance liquid chromatography. *Mycotoxin Res.* **6:**85-88.

Visconti, A. (1996). Fumonisins in maize genotypes grown in various geographic areas. *In: Fumonisins in Food.* Jackson, L. W., DeVries, J. W. and Bullerman, L. V. (eds). Plenum Press : New York. pp. 193-204.

Voss, K. A., Bacon, C. W., Meredith, F. I. and Norred, W. P. (1996). Comparative subchronic toxicity studies of nixtamilized and water extracted *Fusarium moniliforme* culture material. *Food Chem. Toxicol.* **34**:623-632.

Voss, K. A., Norred, W. P., Plattner, R. D. and Bacon, C. W. (1989). Hepatotoxicity and renal toxicity in rats of corn samples associated with field cases of equine leukoencephalomalacia. *Food Chem. Toxicol.* **27**:89-96.

Wang, D. S., Lang, Y., Chad, N. T., Dien, L. D., Tanka, T. and Ueno, Y. (1995). Natural co-occurrence of *Fusarium* toxins and aflatoxin B_1 in maize for feed in North Vietnam. *Natural Toxin.* **3**:445-449.

Wang, E., Norred, W. P., Bacon, C. W., Riley, R. T. and Merrill, A. H., Jr. (1991). Inhibition of sphingolipid biosynthesis by fumonisins: implications for disease associated with *Fusarium moniliforme*. *J. Biol. Chem.* **266**:14486-14490.

Wotton, H.R. and Strange, R. N. (1985). Circumstantial evidence for phytoalexin involvement in the resistance of peanut to *Aspergillus flavus*. *J. Gen. Microbiol.* **131**:487-494.

Yamashita, A., Yoshizawa, T., Aiura, Y., Sanchez, P.C., Dizon, E. I., Arim, R. H. and Sardjono. (1995). *Fusarium* mycotoxins (fumonisins, nivalenol, and zearelenone) and aflatoxins in maize fron South East Asia. *Biosci. Biotechnol. Biochem.* **59**:1804-1807.

Yoo, H-S., Norred, W. P., Wang, E., Merrill, A. H., Jr. and Riley, R. T. (1992). Fumonisin inhibition of *de novo* sphingolipid biosynthesis and cytotoxicity is correlated in LLC-PK_1 cells. *Toxicol. Appl. Pharmacol.* **114**:9-15.

Yoshizawa, T., Yashimata, A. and Luo, Y. (1994). Fumonisin occurrence in corn from high- and low-risk areas for oesophageal cancer in China. *Appl. Environ. Microbiol.* **60**:1626-1629.

Yousef, A. E. and Marth, E. H. (1984). Kinetics and accumulation of aflatoxin B_1 by *Aspergillus parasiticus* in the presence of butylated hydroxyanisole, isoprothiolane and nystatin. *Biotechnol. Bioeng.* **26**:6-11.

Yousef, A. E., El-Gendy, S. M. and Marth, E. H. (1980). Growth and biosynthesis of aflatoxin by *Aspergillus parasiticus* in cultures containing nisin. *Z. Lebensm, Unters. Forsch.* 171:341-343.

Zaika, L. L. (1988). Spices and herbs: Their antimicrobial activity and its determination. *J. Food Saf.* **9**:97.

Zaika, L. L. and Buchanan, R. L. (1987). Review of compounds affecting biosynthesis or bio-regulation of aflatoxins. *J. Food Prot.* **50**:691-708.

Zaika, L. L. and Kissinger, J. C. (1988). Inhibitory and stimulatory effects of oregano on *Lactobacillus platarum* and *Pediococcus cerevisiae*. *J. Food Sci.* **46**:1205.

APPENDIX ONE

Standard plate count:

Molten Sabouraud agar was maintained at 45 °C. Serial dilutions were carried out on the spore suspension using 9.9 ml dilution blanks (sterile distilled water). Dilutions were prepared from 10^1 to 10^7, and 1 ml of each dilution was dispensed to sterile petri dishes labeled accordingly. The molten agar was poured into the petri dish and swirled to evenly distribute the spore suspension. The plates were incubated at 28 °C for 48 hours.

The number of spores per ml of original cultures was calculated by multiplying the number of spores counted by the dilution factor:

No. of spores per ml = no. of spores x dilution factor

APPENDIX TWO

Media used for identification:

Czapek Yeast Extract Agar (CYA):

K_2HPO_4	1.0 g
Czapek concentrate	10. 0 ml
Powdered Yeast Extract	5.0 g
Sucrose	30.0 g
Agar	15.0 g
Distilled water	1.0 L

Malt Extract Agar (MEA):

Powdered malt extract	20.0 g
Peptone	1.0 g
Glucose	20.0 g
Agar	20.0 g
Distilled water	1.0 L

Czapek Yeast Extract Agar with 20% Sucrose (CY20S):

K_2HPO_4	1.0 g
Czapek concentrate	10.0 ml
Powdered yeast extract	5.0 g
Sucrose	200.0 g
Agar	15.0 g
Distilled water	1.0 L

Medium used for *Fusarium* growth:

Alberts A Broth:

Glucose	90 g
KH_2PO_4	2.0 g
$(NH_4)_2SO_4$	35 g
$MgSO_4$	0.3 g
$CaCl_2$	0.4 g
$MnSO_4$	0.016 g
Distileed water	1.0 L

All chemicals used were of analytical grade (Merck).

The media was sterilized by autoclaving at 121°C for 15 min.

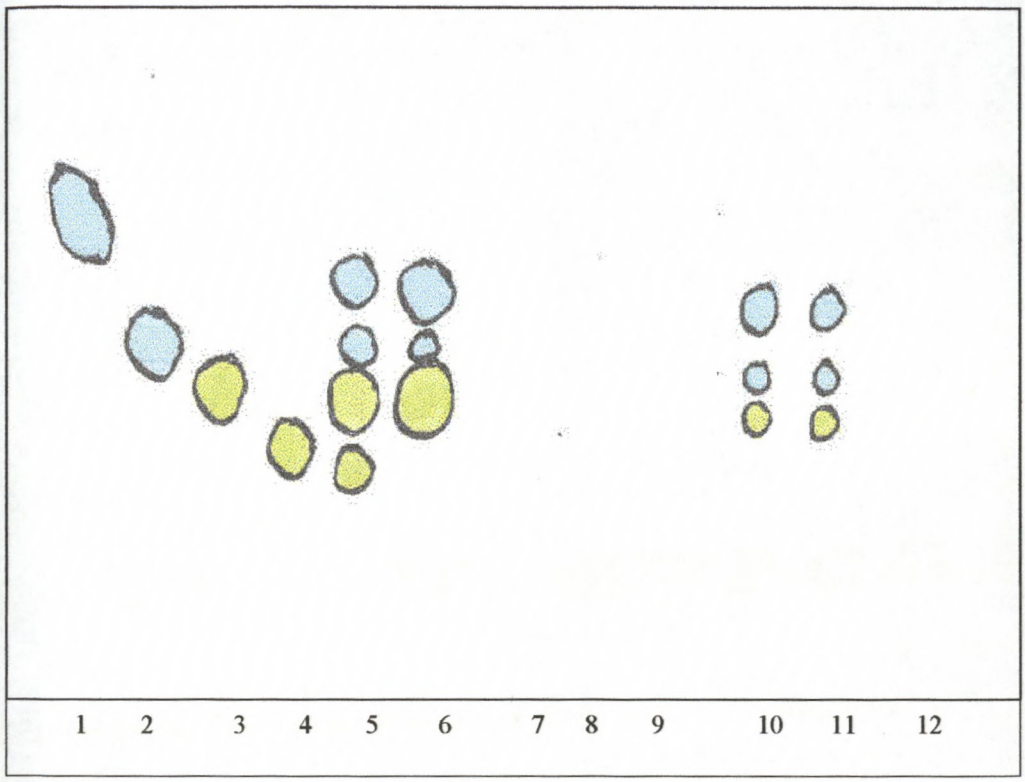
APPENDIX THREE

List of common and botanical names of spices

Common name	Botanical name
Allspice	*Pimenta officinalis*
Parsley	*Petroselium crispum*
Marjorum	*Marjorum hortensis*
Mustard	Blend of *Brassica hirta* and *Sinapis alba*
Garlic	*Allium sativum*
Celery flakes	*Apium graveolens*
Celery seed	*Apium graveolens*
Chives	*Allium schoenporasum*
White pepper	*Piper nigrum* (water soaked)
Black pepper	*Piper nigrum* (dried fruit)
Sweet pepper	*Capiscum annuum*
Paprika	*Capiscum annuum*
Anise	*Pimpinella anisum*
Sage	*Savia officinalis*
Ginger	*Gingiber officinale*
Caraway	*Carum carvi*
Fennel	*Foeniculum vulgare*
Achiote	*Bixa orellano*
Tarragon	*Artemisia dracunulus*
Dill	*Anethum graveolens*
Rosemary	*Rosmarinus officinalis*
Cinnamon	*Cinnamonum zeylanicum*
Cloves	*Eugenia caryophyllata*
Red pepper	*Capsicum frutescens*
Bay leaf	*Laurus nobilis*
Cumin	*Cuminum cyminum*
Oregano	*Lippia graveolens* *Oreganum vulgare*
Tumeric	*Circuma longa*
Onion	*Allium cepa*
Thyme	*Thymus vulgaris*
Nutmeg	*Myristica fragrans* (seed)
Mace	*Myristica fragrans* (external coat)
Coriander seed	*Coriandrum sativum*

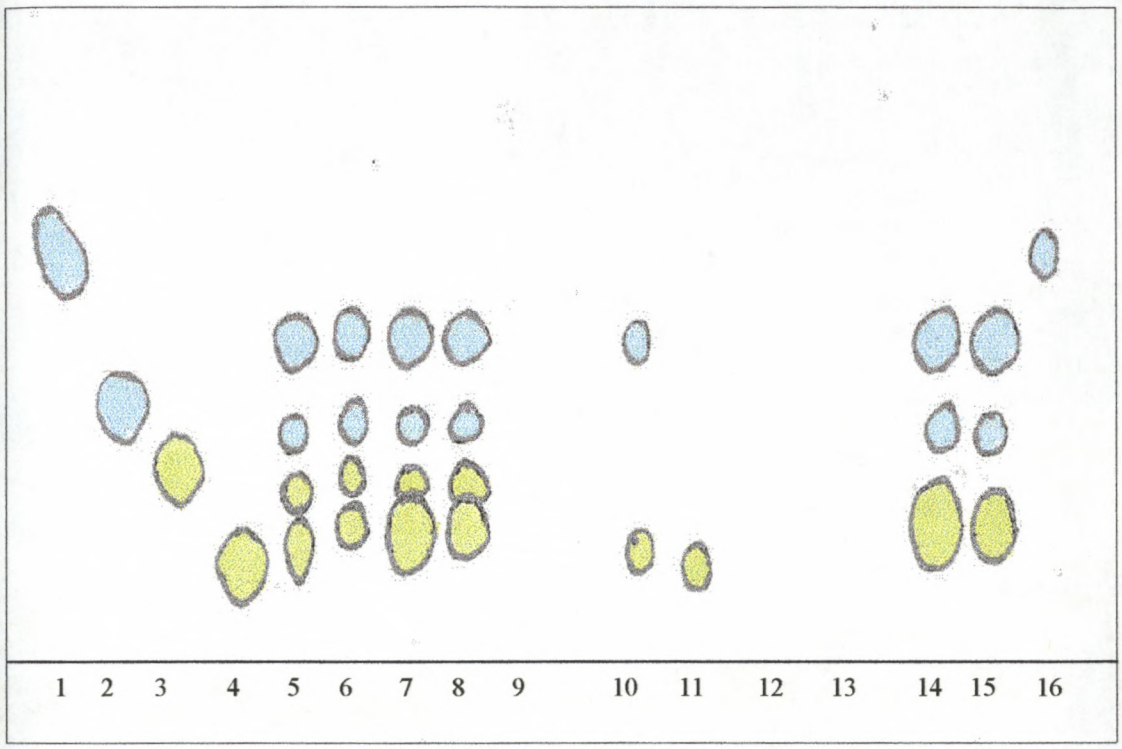
Adapted from Hirasa and Takemasa, 1998

APPENDIX FOUR



1	=	AFB_1	7	=	1.0 ppm nutmeg oil
2	=	AFB_2	8	=	1.5 ppm nutmeg oil
3	=	AFG_1	9	=	2.0 ppm nutmeg oil
4	=	AFG_2	10	=	0.1 ppm mace oil
5	=	control	11	=	0.25 ppm mace oil
6	=	0.5 ppm nutmeg oil	12	=	1.0 ppm mace oil

TLC plate showing aflatoxins produced by *Aspergillus parasiticus* grown in the presence of nutmeg and mace oil in broth culture.



1	=	AFB_1	9	=	2.0 ppm eucalyptus oil
2	=	AFB_2	10	=	0.1 ppm clove oil
3	=	AFG_1	11	=	0.25 ppm clove oil
4	=	AFG_2	12	=	0.5 ppm clove oil
5	=	control	13	=	2.0 ppm clove oil
6	=	0.5 ppm eucalyptus oil	14	=	1.0 ppm tumeric oil
7	=	1.0 ppm eucalyptus oil	15	=	1.5 ppm tumeric oil
8	=	1.5 ppm eucalyptus oil	16	=	2.0 ppm tumeric oil

TLC plate showing aflatoxins produced by *Aspergillus parasiticus* grown in the presence of eucalyptus, clove and tumeric oils in broth culture.