ANTIFUNGAL SECONDARY METABOLITES FROM *Monanthotaxis littoralis* WITH ACTIVITY AGAINST MYCOTOXIGENIC FUNGI FROM MAIZE

CHEPKIRUI CLARA

A Thesis Submitted to the Graduate School in Partial Fulfillment for the Requirement of the Master of Science Degree in Chemistry of Egerton University

EGERTON UNIVERSITY

MAY 2013

DECLARATION AND RECOMMENDATION

DECLARATION

This thesis is my original work and has not been submitted or presented for examination in any institution.

Chepkirui Clara

SM11/2752/10

Signature …… Date ……...

RECOMMENDATION

This thesis has been submitted for examination with our approval as university supervisors.

Prof. J. C. Matasyoh
Egerton University

Signature …… Date ……...

Prof. I. N. Wagara
Egerton University

Signature …… Date ……...

COPYRIGHT

DEDICATION

This work is dedicated to my parents and siblings for their moral, emotional and financial support.

ACKNOWLEDGMENTS

My sincere appreciation goes to my supervisors for their advice, support and guidance throughout my research. I also want to acknowledge the great assistance from the technologists Chepkorir Regina, Langat Ezekiel, Kamau P, Onyego K Sunguti and Anderson Mwangi. A big thank you goes to Egerton University Chemistry Department for allowing me to use their laboratories and equipments during my research. Also a big thank you goes to the Berlin Technical University for assisting in running both the NMR and GS-MS. Finally my dearest thanks to my friend and colleague Cynthia for the support she gave me during this research. Great Thanks to the Almighty God for his love, mercy, blessings and good health throughout my research.

ABSTRACT

This study involved isolation of compounds from the plant *Monanthotaxis littoralis*, screening and evaluating the activity of the compounds against mycotoxigenic fungi isolated from maize collected from LVB and structure elucidation of the bioactive compounds. The non volatile secondary metabolites were extracted from the plant using methanol. The methanol extract was suspended in water and extracted with ethyl acetate. Hydro-distillation was used in the extraction of essential oils. Column chromatography and Preparative Thin Layer Chromatography (PTLC) was used in purification of compounds. The solvent system used for separation in this study was 9:1 chloroform ethyl acetate mixture. The isolated compounds were tested for their anti-mould activity against 31 most common and damaging mycotoxigenic moulds from three genera of fungi (*Aspergillus, Fusarium* and *Penicillium*) using paper disc diffusion inhibition method. The structural elucidation of the bioactive compounds was carried out by a combination of spectroscopic techniques that include 1 and 2 D high field NMR spectroscopy GC-MS spectroscopy and Mass spectrometry. The oil had eight major compounds which include (-)-Beta-necrodol, (+,-)-tetramisole among others while from the crude extract flavonoids 3, 5-dihydroxy-7-methoxy anthocynidines and 3, 7, 5'- trihydroxy anthocynidines were isolated and compound M whose structure could not be elucidated because of the limitation of the purification methods used was also isolated. The highest activity of the oil was against *Penicillium purporogenum*, *Penicillium islandicum* and *Aspergillus niger* with MIC of 26 mg/ml. These species had inhibition zones of 39.17 mm, 29.60 mm and 15.67 mm respectively. The extent of inhibition of fungal growth was dependent on the concentration of the secondary metabolites. The oil MIC generally ranged from 26 mg/ml and 103 mg/ml. The flavonoids mixture had the highest activity against Aspergillus ochraceus where MIC of 1 mg/ml. In general the non volatile compounds had MIC values ranging from 1 to 4 mg/ml against the mycotoxigenic fungi. These results show that the secondary metabolites isolated from *Monathotaxis littoralis* have antifungal activities against mycotoxigenic fungi found in foods. Thus these compounds are potential antifungal that can be used in food preservation systems to inhibit the growth of moulds and retard subsequent mycotoxins production.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

BEN	Balkan Nephropathy
CAST	Council for Agricultural Science and Technology
DMSO	Dimethyl Sulfoxide
FAO	Food and Agricultural Organization
KEPHIS	Kenya Plant Health Inspectorate Service
LVB	Lake Victoria Basin
MIC	Minimum Inhibitory Concentration
SAS	Statistical Analysis System
PTLC	Preparative Thin Layer Chromatography
EFSA	European Food Safety Authority
KEKIM	Kenya Kitale Maize
KEKAM	Kenya Kakamenga Maize
KEKUM	Kenya Kuria Maize
DEPT	Distortionless Enhancement by Polarization Transfer
HSQC	Heteronuclear Single Quantum Correlation
HMBC	Heteronuclear Multiple Bond Correlation
COSY	Correlation Spectroscopy

CHAPTER ONE

INTRODUCTION

1.1 Background information

Moulds are composed of long filaments called hyphae which grow over the surface and inside nearly all substances of plant or animal origin and cause them to decay. They are opportunistic biological agents of ubiquitous nature. They appear as profuse or woolly fungal growth on damp or decaying matter or on surfaces of plant tissues. Moulds are able to colonize diverse substrates including foods. Because of their powerful arsenal of hydrolytic enzymes, these microorganisms can cause a high degree of deterioration when present in foods and are responsible for considerable economic losses (Souza *et al*., 2005). The most common moulds that develop on foods and feeds include *Aspergillus, Penicillium, Fusarium, Rhizopus* and *Mucor*. One of the most destructive activities of moulds in foodstuffs occurs in stored seeds and grains. Some of the moulds such as species of *Eurotium* that attack stored grain commonly appear on the surface of processed foods like jams, jellies and syrups. When moisture conditions are favorable, moulds such as *Rhizopus* and *Mucor* affect fleshy fruits and vegetables after harvest and also stored grains and legumes as well as prepared foods such as bread (Agrios, 1997).

Moulds cause extensive damage on food, feeds and other agricultural commodities in the field, during transportation, storage and processing which actually leads to postharvest losses. According to Agrios (1997) molds are known to destroy 10 to 30% of the total yield of crops and more than 30% for perishable crops by reducing their quality and quantity in developing countries. In addition moulds produce mycotoxins that can cause illness or even death to the consumer. Mycotoxins contaminate 25% of agricultural crops worldwide and are a source of morbidity and mortality throughout Africa, Asia and Latin America (Smith *et al.,* 1994; Azziz-Baumgartener *et al*., 2005). Acute liver damage, liver cirrhosis, induction of tumors and attack on central nervous system, skin disorders and hormonal effects are mostly caused by mycotoxicoses (Ibrahim *et al*., 2000; Oguz *et al*., 2003). Most deterioration of grains and legumes after harvest is caused by several species of *Aspergillus* which are responsible for many cases of feed contamination (Katta *et al*., 1995; Agrios, 1997). Aflatoxins produced by

Aspergillus flavus and *Aspergillus parasiticus* are the most economically important mycotoxins. Acute aflatoxicosis epidemics have been reported in several parts of Africa and Asia leading to the death of several hundreds of people (Varga *et al*., 2009). Over the past years, Kenya has experienced dramatic aflatoxicoses outbreaks resulting in loss of life in some cases. In 2004 an acute aflatoxicosis outbreak occurred in Machakos, Kenya resulting in 317 cases of acute hepatic failure and 125 deaths (Nyikal *et al*., 2005; Lewis *et al.*, 2005). Cases of liver cancer have been associated with high levels of aflatoxins in Ugandan food while in the Lake Victoria basins esophageal cancer is associated with high levels of fumonisins (Kedera *et al.*, 1994).

Aflatoxins are known to be potent hepatocarcinogens in animals and humans. Ochratoxin A, which has been experimentally shown to be teratogenic, a potent renal carcinogenic and immunosuppressive is largely produced by *Aspergillus ochraceus* and less frequently by *Aspergillus niger* (Nielsen *et al*., 2009). As an enzyme inhibitor, ochratoxin A affects lipids peroxidation and have been implicated in Balkan Nephropathy (BEN) in humans (Hohler, 1998). Mycotoxins produced by other *Aspergillus* species include malformins by *Aspegillus niger*, fumitoxins and gliotoxin by *Aspergillus fumigatus* (Hof and Kupfahl, 2009).

The wide and indiscriminate use of chemical preservatives has been cause of appearance of resistant microorganisms, leading to occurrence of emerging food borne diseases (Akinpelu, 2001). As a result of this, there is an increasing interest to obtain alternative antimicrobial agents from natural sources for use in food preservation. Recent research has shown that some plants contain compounds like essential oils mainly geranial, neral and myrecene which are able to inhibit the microbial growth (Matasyoh *et al*., 2006). Systematic screening of interaction between microorganisms and plant products is widely applied in the search for biologically active compounds. The plant used in this study was *Monanthotaxis littoralis* which belongs to the family Annonaceae and the genus *Monanthotaxis*. Numerous diverse compounds and extracts containing activity inhibitory to aflatoxin production have been reported. Most of these inhibitors are phenyl propanoids, terpenoids and alkaloids all of which have been isolated from majority of plants in the genus *Monanthotaxis* to which *M. littoralis* belong (Holmes and Payne, 2008).

1.2 Statement of the problem

Moulds cause extensive damage on food, feeds and other agricultural commodities in the field, during transportation, storage and processing. They are known to destroy 10 to 30% of the total yield of crops and more than 30% for perishable crops in developing countries by reducing their quality and quantity. In addition, moulds produce mycotoxins that can cause illness or even death to the consumers. Mycotoxicoses outbreak has been reported in recent years in Kenya especially in the eastern parts of the country. The LVB experience high temperature and high humidity which favours moulds infestation and mycotoxin production thus, the residents of this area are exposed to mycotoxins contamination before birth and throughout their lives. They suffer the long term chronic effects of mycotoxins accumulation in their bodies. These chronic effects include: liver cancer, respiratory problems, liver cirrhosis, induction of tumors and attack on the central nervous system, skin disorders and hormonal effects. The mycotoxin fumonisins particularly have been associated with high rates of aesophageal cancer in humans around Lake Victoria region. Thus the need to design strategies for the reduction or elimination of mycotoxigenic fungi and prevention of mycotoxin production in grains is an urgent task especially in the LVB where climate conditions naturally favours moulds infestation and mycotoxins production in grains. The use of synthetic chemical preservatives has been ineffective in solving this problem hence the need for alternative methods.

1.3 Objectives

1.3.1 General objective

To evaluate the antifungal activities of secondary metabolites from Monanthotaxis littoralis against mycotoxigenic fungi from maize collected from LVB

1.3.2 Specific objectives

1. To isolate and identify mycotoxigenic moulds associated with maize from the LVB.
2. To extract and isolate secondary metabolites from *Monanthotaxis littoralis.*
3. To screen and carry out bioassay fractionation of crude extracts isolated from *Monanthotaxis littoralis.*

4. To evaluate the antimould activities of the bio-active secondary metabolites isolated from *Monanthotaxis littoralis*.
5. To elucidate the structures of the bio-active secondary metabolites isolated.

1.4 Hypotheses

1. The maize samples collected from LVB are not infested with moulds.
2. The secondary metabolites from *Monanthotaxis littoralis* cannot be isolated.
3. The secondary metabolites from *Monanthotaxis littoralis* do not show antimould activity.
4. The isolated secondary metabolites from *Monanthotaxis littoralis* do not show significant antimould activity.
5. The structure of the bioactive compounds could not be elucidated.

1.5 Justification

Kenya has experienced dramatic aflatoxicoses outbreaks resulting in loss of lives. Cases of liver cancer and aesophageal cancer in LVB have been linked to high levels of aflatoxins and fumonisins. Designing strategies for the reduction or elimination of mycotoxins and knowing their fungal sources is important, hence the need to identify the mycotoxin producing moulds in grains and reliable methods of preventing such moulds' growth in grains is an urgent task. One of the available methods of controlling moulds is the use of synthetic chemical preservatives like sodium nitrate but resistance of micro-organisms to these synthetic chemical preservatives has resulted in emerging food borne diseases and food poisoning. Also, drying maize in the LVB does not help so much because there is too much humidity in the air that even if the maize is dried, it will still be contaminated. Thus, it is important to improve food and health security through postharvest food protection using plant based compounds to replace the ineffective methods being used. One way of achieving this is by screening and evaluating alternative antimicrobial activities of secondary metabolites from plant-based sources to which development of microorganisms' resistance has not been reported. In addition plant compounds do not pose any danger to the environment. It is also quite important to know the composition of the active compounds in plants as this will help in identifying the active ingredient in the extracts and this could appear in food preservation systems as the main antimicrobial agents.

CHAPTER TWO

LITERATURE REVIEW

2.1 Moulds

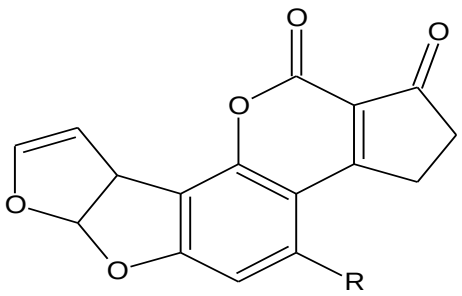
Moulds are composed of long filaments called hyphae which grow over the surface and inside nearly all substances of plant or animal origin and cause them to decay. Moulds not only contaminate our air but also our food. As they grow on food, they produce enzymes that break down the food resulting to spoilage (Kung'u, 2005). Moulds derive energy from the organic matter in which they live. Typically, moulds secrete hydrolytic enzymes, mainly from the hyphal tips. Moulds include all species of microscopic fungi that grow in the form of multicellular filaments, called hyphae. A connected network of these tubular branching hyphae has multiple, genetically identical nuclei and is considered a single organism, referred to as a colony or in more technical terms a mycelium (Ryan and Ray, 2004). Although some moulds cause disease or food spoilage, others are useful for their role in biodegradation or in the production of various foods, beverages, antibiotics and enzymes (Madigan and Martinko, 2005). Moulds cause the loss of millions of dollars to the economy every year and, even worse, may be a menace to human and animal health. According to Agrios (1997), postharvest diseases caused by moulds destroy 10 to 30% of the total yield of crops and in some perishable crops, especially in developing countries they destroy more than 30% of the crop yields.

2.2 Mycotoxins and mycotoxicoses

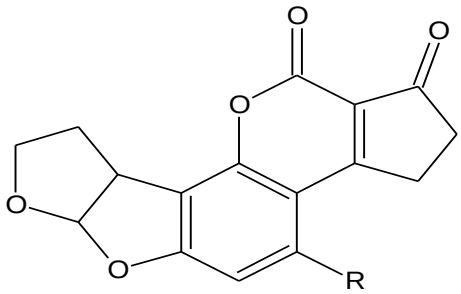
Mycotoxins are toxic secondary metabolites produced by certain fungi in agricultural products that are susceptible to mould infestations (Wagacha and Muthomi, 2008; Moreno *et al*. 2009). Mycotoxins contaminate 25% of agricultural commodities worldwide and are a source of morbidity and mortality throughout the world. Consumption of mycotoxin-contaminated foods has been associated with several cases of human poisoning or mycotoxicoses (Munimbazi and Bullerman, 1996). Most mycotoxins are produced by *Aspergillus*, *Penicillium* and *Fusarium*. Mycotoxins differ in their chemical formula, in the products and conditions under which they are produced, in their effects on various animals and humans, and in their degree of toxicity (Agrios, 1997). The most important ones in terms of economic importance are aflatoxins, deoxynivalenol,

fumonisins, ochratoxins and zearalenones produced by *Aspergillus flavus*, *Aspergillus parasiticus*, *Fusarium moniliforme*, *Aspergillus ochraceus* and *Fusarium graminearum*, respectively (FAO, 1999). Most famous of these are the aflatoxins B_1, B_2, G_1 and G_2 which are produced by some strains of *Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus nomius* with aflatoxin B_1 being the most common. The structure of aflatoxin B_1, B_2, G_1 and G_2 are shown below. These compounds are not only immediately toxic but are also known to be carcinogenic in humans and domestic animals (Ibrahim *et al.,* 2000). These mycotoxins are produced in infected cereal seeds, most legumes especially groundnuts, cotton seed, fishmeal, nuts etc (Kaaya *et al.,* 2006). Aflatoxins exist in a variety of derivatives with varying effects. Some of the toxins e.g. ochratoxins can persist in meat and milk of animals fed on contaminated feed and can be transmitted to humans through the food chain (Lanyasunya *et al*., 2005).

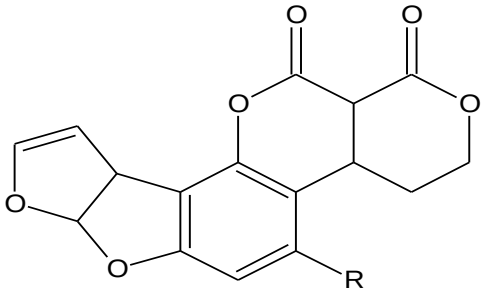
Aflatoxins are a group of polyketides derived from furanocoumarins. They are biosynthesized in two stages from malonyl Co A, first with formation of hexanoyl Co A, followed by formation of decaketide anthraquinone. A series of highly organized oxidation-reduction reactions then allows the formation of aflatoxin (Dutton 1988, Townsend 1997).



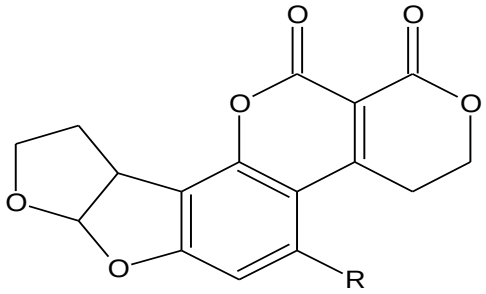
Aflatoxin B_1



Aflatoxin B_2



Aflatoxin G_1



Aflatoxin G_2

R=OCH_3

2.3 Impacts of the mycotoxins and mycotoxicoses

Maize is the most important stable food for the majority of the Kenyan population (Kimanya *et al*., 2008), and it is consumed as a dietary stable at an average intake of 400 gm per person per day (Shephard, 2008). However, the grain is vulnerable to degradation by mycotoxigenic fungi which include *Aspergillus, Fusarium* and *Penicillium.* Maize contaminations by fungi reduce its quality through discoloration as shown in Figure 1 below. In addition, moulds infestations in maize reduce the nutritional value and also lead to mycotoxin production. The mycotoxins are produced by fungal action during production, harvest, transportation, storage and food processing (CAST, 2003; Murphy *et al*., 2006). Mycotoxin attracts worldwide attention because of the significant economic losses associated with their impact on human health, animal productivity and trade (Wagacha and Muthomi, 2008). The Food and Agricultural Organization (FAO), estimates that between 25% and 50% of agricultural crops worldwide is contaminated by mycotoxins (Lewis *et al*., 2005; Wagacha and Muthomi, 2008). The estimated value of maize lost to aflatoxin is $225 million and $ 932 million per year in the United States and Kenya respectively (Betran and Isakeit, 2003).

Figure 1: Maize contaminated with moulds causing discoloration
Source: KEPHIS (2006).

Mycotoxin poisoning in humans and animals occurs through ingestion, inhalation and absorption through the skin (Wagacha and Muthomi, 2008). High-level exposure may cause instant death while long-term chronic effects include cancer, mutagenicity and nervous disorders

(KEPHIS, 2006). At the farm level, the real problem is that contaminated maize may appear just like the normal grain without any outward physical signs of fungal infection. Destruction of aflatoxins by conventional food processing is difficult because they are typically resistant to heat and detection is complicated due to limitations in analytical capacity.

Aflatoxin poisoning has continued to cause disease and death of many people in rural areas of Eastern and Central provinces of Kenya. The first outbreak of aflatoxicosis in Kenya was reported in 1978 and again in 1984-85 when a large number of dogs and poultry died due to aflatoxin poisoning (Manwiller, 1987). Other aflatoxicoses outbreaks have been reported over the years resulting in sickness, death and destruction of contaminated maize. The largest outbreak reported in the world during the last 20 years was in 2004 when 317 cases were reported with 125 deaths in eastern province of Kenya. Most of the aflatoxin poisoning outbreaks occurred in remote villages and, therefore, the number of people affected could have been higher than reported. Also cases of liver cancer have been linked to high levels of aflatoxins in the LVB (Lewis *et al*., 2005). Maize from the affected area contained as much as 4,400ng/g aflatoxin B1, which is 440 times greater than the 10 ng/g tolerance level set by the Kenya Bureau of Standards.

Aflatoxin contamination of agricultural produce has been reported to be a serious problem also in Uganda (Kaaya and Warren, 2005). Maize and groundnuts, which are some of the major staple foods in the country, are reported to be highly contaminated. Since 75% of maize consumed in Uganda originates from the mid-altitude agroecological zones which have high mould and aflatoxin incidences, the consumers in the country are exposed to the danger of aflatoxin poisoning (Kaaya *et al*. 2006). For example, maize samples from Iganga, Kamuri, Mayuge, Masaka and Kapchworwa in the 2003 season had 32, 27, 25, 21 and 32 ppb, respectively, which are higher than the WHO regulatory limit of 20 ppb (Bigirwa *et al*., 2006).

Aflatoxin contamination in maize has been associated with drought combined with high temperature as well as insect injury (Betran and Isakeit, 2003). Optimal conditions for fungal development are 36 to 38°C, with a high humidity of above 85% (Diener *et al.,* 1987). Suitable conditions for the growth of the fungi and aflatoxin production occur in most areas of Africa hence African communities are exposed to aflatoxin and other harmful mycotoxins before birth and throughout their lives with serious impact on their health (Wild and Gong, 2010). Also poor

harvesting practices, improper storage and less than optimal conditions during transport and marketing can also contribute to fungal growth (Figure 2) and proliferation of mycotoxins (Bhat and Vasanthi, 2003; Wagacha and Muthomi, 2008).

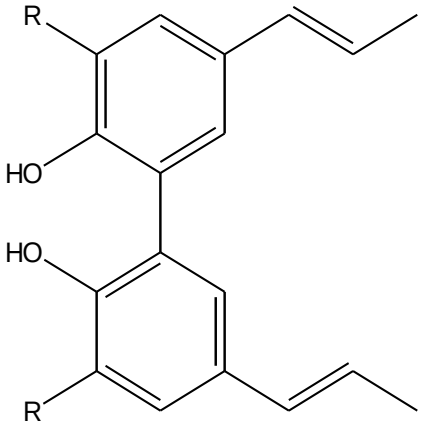
Figure 2: Maize contaminated with moulds after harvest
Source: KEPHIS (2006).

2.4 The non-volatile secondary metabolites

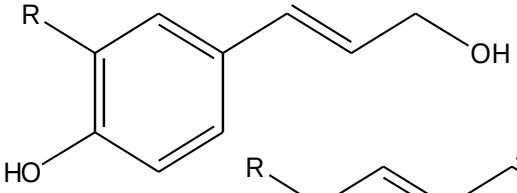
Monanthotaxis littoralis belongs to the family Annonaceae and the genus *Monanthotaxis*. The Annonaceae family includes 130 genera and about 2300 species distributed in tropical and subtropical areas of America, Africa and Asia (Chang *et al.,* 1998). *Monanthotaxis littoralis* grows widely in Mabira equatorial forest in Uganda. The Buganda people call it kasiba mpingu meaning the one that ties itself around others (figure 3). The family Annonaceae is important phytochemically because of the frequent presence of isoquinoline alkaloids and also a very active class of natural products; the acetogenins. These compounds have been shown to have strong antifungal properties (Chang *et al.,* 1998). From the *Monanthotaxis* genus flavonoids, lignoids and terpenoids have been isolated (Moveira *et al.,* 2005). Numerous diverse compounds and extracts containing activity inhibitory to aflatoxin production have been reported. Most of these inhibitors are phenyl propanoids, terpenoids and alkaloids all of which have been isolated from majority of plants in the genus *Monanthotaxis* to which *M. littoralis* belong (Holmes *et al*., 2008). Cyclohexane epoxides containing a benzoyloxymethylene unit have been isolated from Tanzanian *Monanthotaxis* species that are found in coastal forests near Dar es Salaam (Liang *et al*., 1988).

Figure 3: Photograph of *Monanthotaxis littoralis*

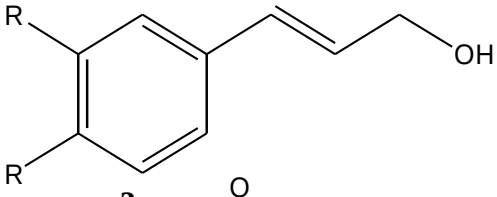
The phenyl propanoids which have been shown to inhibit the accumulation of aflatoxins include: 1,1-di-2-propenyl-4,4-dihydroxy-3,3-dimethoxy-5,5-biphenyl (**1**), trans-3-(4-hydroxy-3-methoxy phenyl)-2-propenol (**2**), trans-3-(3,4-dimethoxy phenyl)-propenol (**3**), trans-3-(4-dimethoxy phenyl)-propenaldehyde (**4**) and trans-3-(4-hydroxy-3-methoxy phenyl)-2-propenaldehyde (**5**). Compound **1** was shown to have the highest activity by suppressing 93% of aflatoxin B_1 at concentration of 0.26µmol/ml. Compound **4** suppressed 70% at concentration of 0.84 mg/ml (Ohat *et al*., 2002). According to Shanmugam *et al.*, (2008) alkaloids isolated from *Xylopia championii* of the Annonaceae family showed strong antifungal activity against the *Aspergillus* fungi. Some of the alkaloids they isolated are: oxopurpureine (**6)**, (+)-laudanidine (**7)**, (−)-discretine (**8)**, nordicentrine (**9),** and dehydrocorytenchine (**10).** Compound **8** and **10** particularly showed the highest inhibition of 57.01% and 55.84% respectively at concentration of 0.5 mg/ml. The flavonoid 3, 5, 7-trihydro-3, 4-dimethoxy flavone (**11**) was also shown to have strong inhibition against aflatoxin B_1 (Lee *et al*., 2001). Structures of these compounds are shown below.



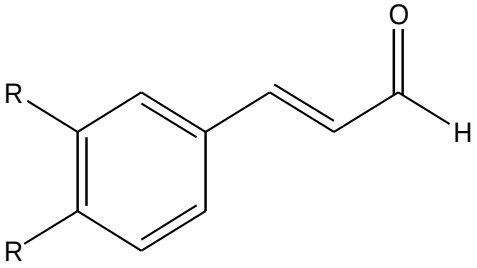
1



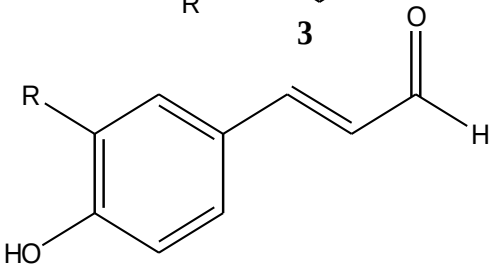
2



3

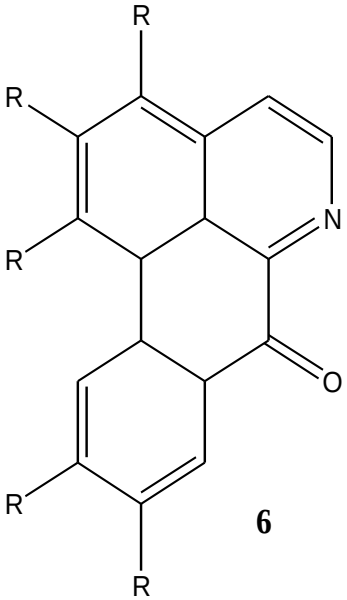


4

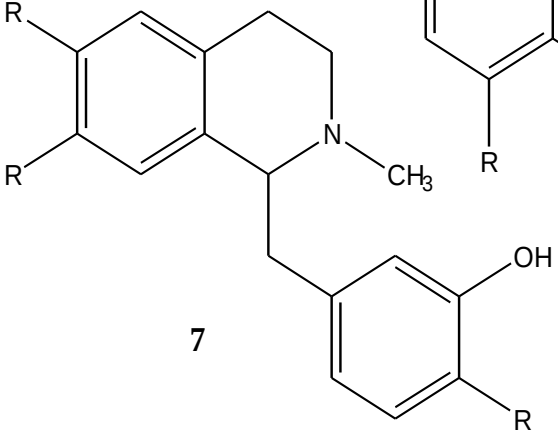


5

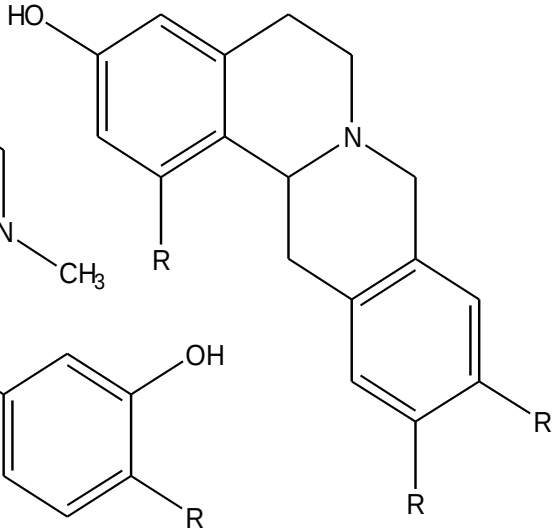
R= OCH_3



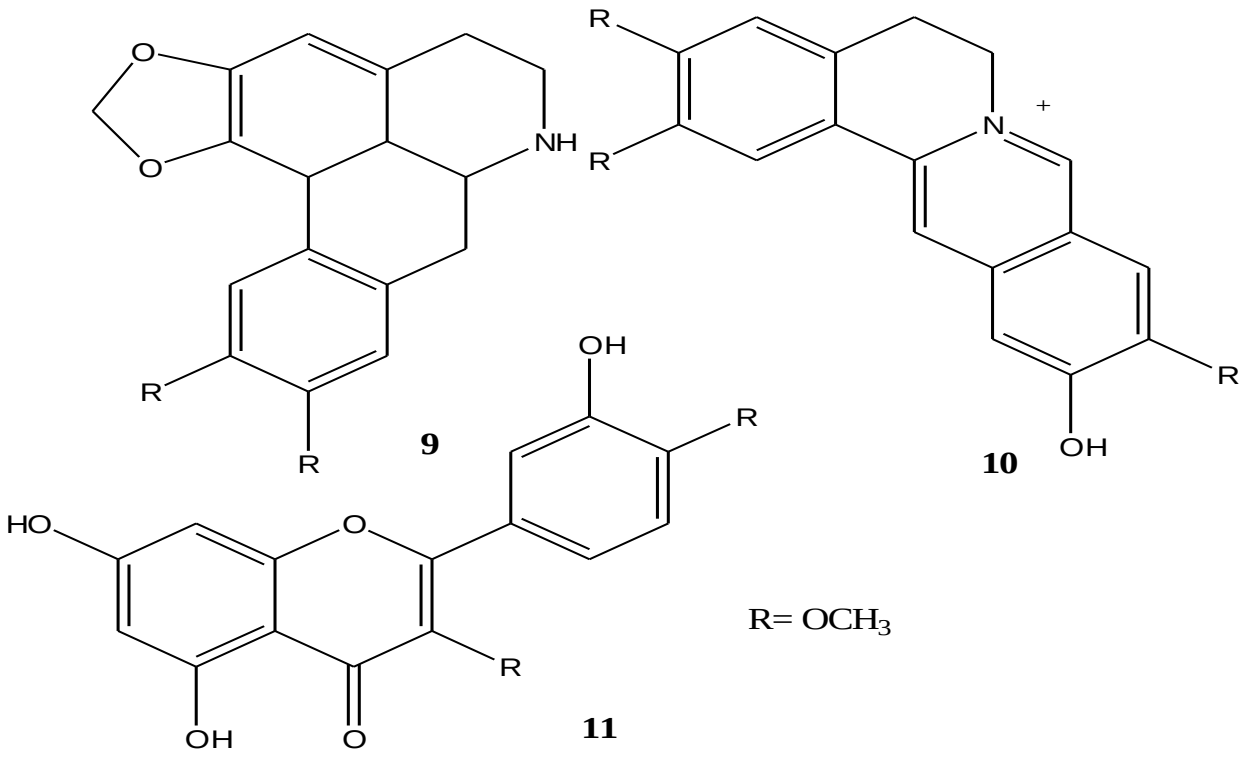
6



7



8



2.5 Flavonoids and their biosynthetic pathway

Flavonoids are a large family of compounds synthesized by plants. The function of flavonoids in flowers is to provide colour attractive to pollinator .In leaves these compounds are increasingly believed to promote physiological survival of the plants protecting it from physiological fungal pathogens (Harbrones and williams, 2000). Increasingly flavonoids are becoming subject of medicinal research. They have been reported to posses many useful properties including anti- inflammatory activity, enzyme inhibitors, antimicrobial activity and antitumor activity (Harbornes and Baxtex, 1999).

Flavonoids are synthesized through the mevalonic pathway. Flavonoids are synthesized by extension of p-hydroxycoularoyl CoA with three molecules of malonyl CoA in head to tail manner forming tetraketide intermediate (Figure 4). This step is catalyzed by the enzyme chalcone synthase. The intermediate then folds and condenses to give a chalcone. This reaction is the first committed step in flavonoids biosynthesis and the reaction is catalyzed by strereospecific intermolecular cyclization of the chalcone isoliquiritigenin and

naringeninchalcone into flavonones (2S) – liquiritigenin and (2S)-navigenin (Stafford, 1991, Winkel-shrley, 1999 Winkel-shrley, 2001).

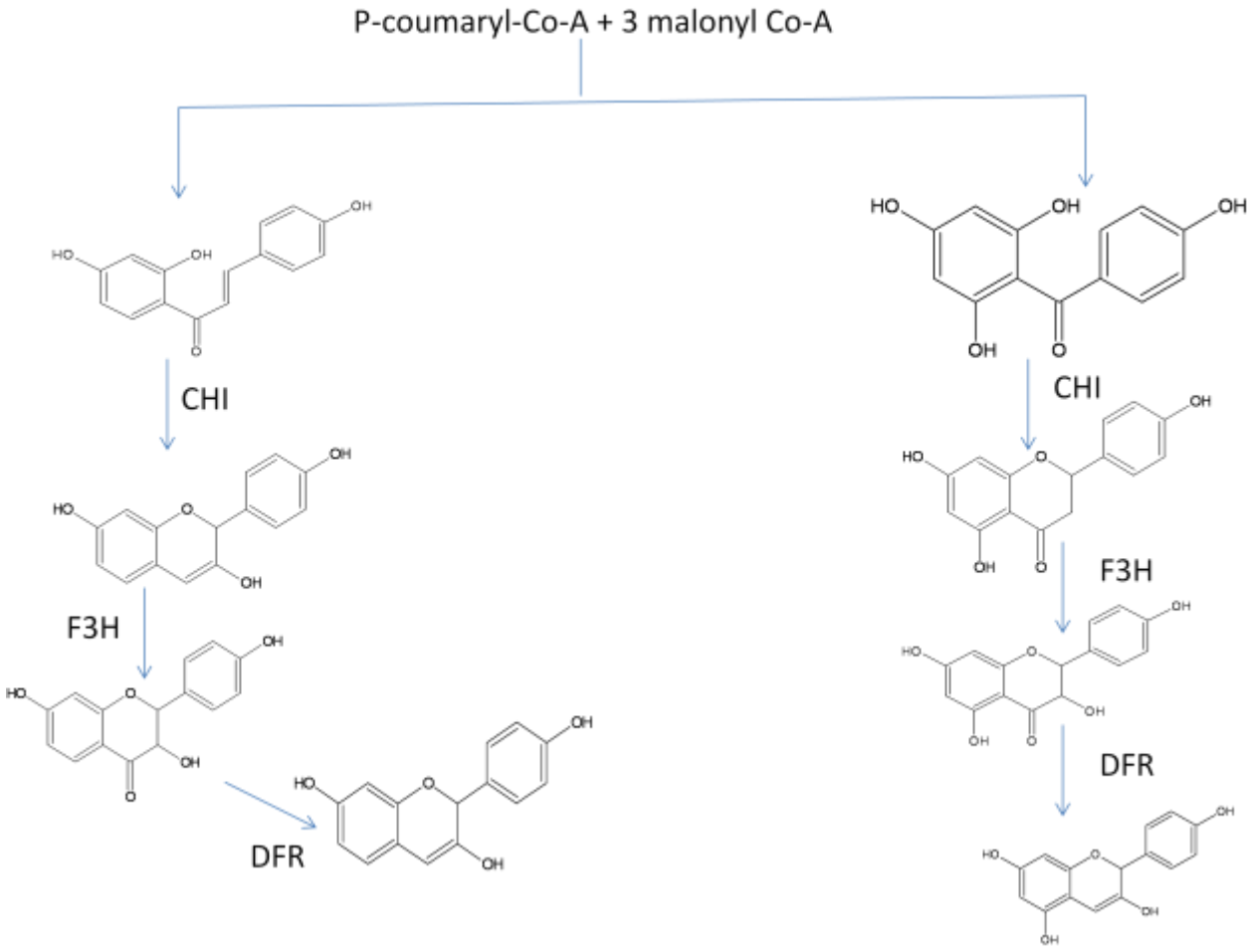


Figure 4: Schematic diagram showing major steps in biosynthesis of flavonoids

2.6 Essential oils

Essential oils are volatile plant oils which are composed chiefly of terpenoids: mono-, sesqui- and di-terpenes plus various alcohols, ketones and aldehydes with frequently occurring aromatic compounds arising from the phenylpropanoid pathway (e.g. eugenol and safrole). In some species alkanes, aliphatic alcohols and ketones may be obtained. They are also considered as a complex mixture of various aroma chemicals. Each of these constituents contributes to the

beneficial or adverse effects of the oil (Buchbuaer, 1993). These volatile oils are more or less modified during the preparation process.

Essential oils are used as raw materials in many fields, including perfumes, cosmetics, aromatherapy, phytotherapy, pharmaceuticals, spices and nutrition (Buchbuaer, 1993). The antimicrobial activities of essential oils are well recognized for many years. Essential oils of the plant *Cymbopogon citratus* have been shown to have strong activity against *Aspergillus* moulds isolated from maize and ground nuts (Matasyoh *et al*., 2010). This activity could act as chemical defense against plant pathogenic diseases. Furthermore, higher and aromatic plants have traditionally been used in folk medicine as well as to extend the shelf life of foods, showing inhibition against bacteria, fungi and yeast (Alves *et al.,* 2000; Sartoratto *et al.,* 2004). Most of their properties are due to essential oils produced by their secondary metabolism (Adams *et al.,* 1995). The essential oils eugenol and mircene isolated from *Annona cherimola* and *Monanthotaxis faulknerae* have been shown to have strong antimicrobial activity. Both of these plants belong to the same family and genus respectively with *Monanthotaxis littoralis*. Essential oils such as anisum, boldus, myrecene, geraniol, neral and geranial isolated from plants of different families including Annonaceae family can stop fungal growth and aflatoxin B_1 accumulation in food (Bluma *et al*., 2007). Thus these oils can be added to grains in store to protect it from fungal infection since they are natural and non toxic to humans and animals.

2.7 Effects of essential oils on microorganisms

Research into the antimicrobial action of the monoterpenes suggests that they diffuse into and damage cell membrane structures (Sikkema *et al*., 1995). Monoterpenes are lipophilic and by definition will preferentially partition from an aqueous phase into membrane structures. This causes expansion of the membrane, increased fluidity or disordering of the membrane structure and inhibition of membrane-embedded enzymes (Sikkema *et al*., 1995). Some authors have attributed this action to the interaction of their functional groups (phenols especially) with the microbial cell envelope (Lahlou and Berrada, 2001). Bammi *et al.,* (1997) demonstrated an action of essential oil of thyme on the cell life cycle. The appearance of profound lesions in different micro-organisms (*Escherichia coli, Bacillus subtilis* and *Saccharomyces cerevisiae*) clearly demonstrated the action of this oil (Bammi *et al*., 1997).

Antimicrobial activity of volatile compounds results from the combined effect of direct vapour absorption on micro-organisms and indirect effect through the medium that absorbed the vapour (Moleyar and Narasimham, 1986). The vapour absorption in micro-organisms is determined by their membrane permeability. In general, the inhibitory action of natural products on mould cells involves cytoplasm granulation, cytoplasmic membrane rupturing and inactivation and synthesis inhibition of intercellular and extracellular enzymes. These actions can occur in isolate or concomitant ways and culminate in inhibition of mycelium germination (Cowan, 1999).

2.8 Control of moulds

One of the available methods of controlling moulds is the use of synthetic chemical preservatives which have been the cause of appearance of resistant microorganisms, leading to occurrence of emerging food borne diseases. Furthermore, other methods such as use of solar driers are expensive for the small scale farmers. It has been established that some plants contain compounds able to inhibit moulds growth (Adams *et al*.; 1995, Matasyoh *et al*, 2006). These compounds can have different structures and different action when compared with conventional antimicrobials used to control microbial growth and survival. The potential antimicrobial properties of plants had been related to their ability to synthesize, by the secondary metabolism, several chemical compounds of relatively complex structures with antimicrobial activity. This activity could act as chemical defense against plant pathogenic diseases. For example, higher and aromatic plants essential oils have traditionally been used in folk medicine as well as to extend the shelf life of foods as they show inhibition against bacteria, fungi and yeast (Alves *et al.*, 2000; Sartoratto *et al.*, 2004). Therefore, use of secondary metabolites with anti-mould activities in food preservation would provide a technology to ensure that foods and feeds are free of moulds and mycotoxins. Such compounds of recognized antimicrobial spectrum could appear in food preservation systems as main antimicrobial compounds or as adjuvants to improve the action of other antimicrobial compounds (Kaur and Arora, 1999).

The appearance of resistant micro-organisms, leading to occurrence of emerging food borne diseases has been attributed to the wide and indiscriminate use of chemical preservatives (Kaur and Arora, 1999). Due to this, there is an increasing interest to obtain alternative antimicrobial agents to use in food conservation systems. One of the main procedures used in the research of biologically active substances is the systematic screening of interaction between

micro-organisms and plant products. This procedure has been useful in identifying agents to control the microbial survival in different microbiology applications. This project aims at identifying secondary metabolites isolated from *Monanthotaxis littoralis* that can control the growth of moulds in maize and therefore inhibit the production of associated mycotoxins in the grains. A protocol for antimould product formulation could be derived from the research findings that can be used by local entrepreneurs to start small-scale industrial initiatives to produce cheap, affordable and environmentally-friendly antimould products for food preservation systems. This would help alleviate great losses of grains encountered due to moulds and prevent mycotoxin poisoning outbreaks.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection of plant material and maize samples

3.1.1 Collection and identification of the plant

The plant was collected from Mabira equatorial forest in Uganda. The plant grows wildly in the mid altitudes of 1500 m to 1700 m, in the tropical rainforest conditions receiving about 2000 mm of rainfall a year. The peak period of this rainy season is in the months of April and November. They experience short dry season between December and March. The average temperatures range is 15- 28^0C throughout. The plant material was identified by a taxonomist as *Monanthotaxis littoralis* and a voucher specimen deposited at the department of Biological Sciences of Egerton University.

3.1.2 Collection of maize samples

A total of 30 samples categorized as good (maize without moulds) and mouldy maize were obtained randomly from rural households and markets in Kitale, Kuria and Kakamega district. In Kitale, samples were collected from Kitale central and Kiminini divisions. In Kakamega, samples were collected from Kakamega municipality division whereas in Kuria district, samples were collected from Kehancha and Masaba divisions. Most of these areas are in mid altitude agroecological zones with warm and humid conditions which favour development of moulds and production of mycotoxins (Kaaya *et al.,* 2006). One major maize producing districts in north, central and south of LVB were chosen. These areas have unpredictable rainfall patterns making it difficult for small scale farmers to efficiently dry their produce. Ten samples (each weighing half a kilogram) were collected from each district. The samples were collected in properly labeled khaki paper bags to minimize saprophytic fungal contamination and transported in a cool box to the laboratory for analysis. The samples were stored at 4^oC until time for further analysis. The map of the study area is shown in figure 5.

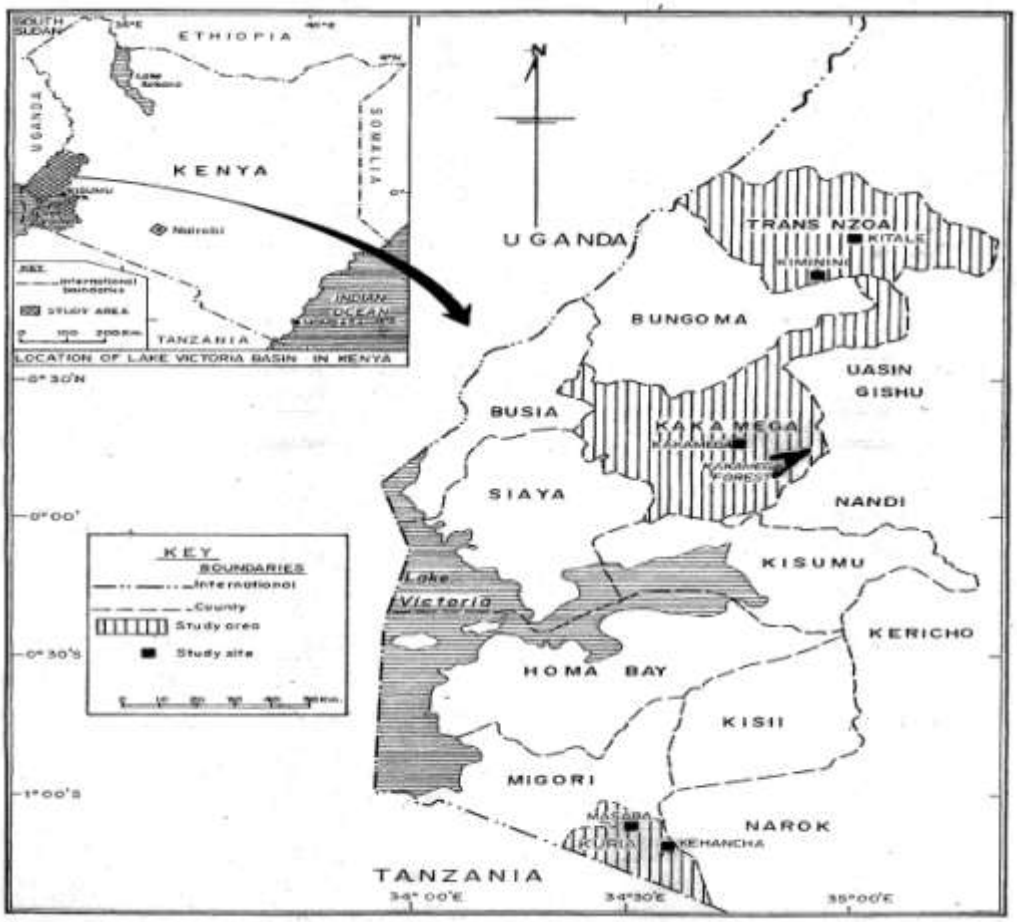


Figure 5: A map of the study area

Source:http://www.vicres.net/?.jc=coverage

3.2 Extraction of non – volatile compounds

The solvents used were bought at Indo laboratories and the purity was GPR and were distilled before being used. The plant leaves were dried under shade to constant weight and ground to a fine powder. One thousand grams of the powders was extracted with 1 L of methanol ten times and after removal of the solvent by rotary evaporation; the extracts were suspended in water and extracted using 100 ml of ethyl acetate eight times. About 50g of activated charcoal was added to the extract, stirred well with magnetic stirrer, filtered and evaporated by rotary evaporation and the extracts were then dried using anhydrous sodium sulphate. The extracts were screened for antimould activities and bioassay guided fractionation. The flow chart in figure 6 summarizes the procedure for the extraction of non- volatile secondary metabolites.

3.3 Extraction of essential oils

Fresh whole plant leaves of *M. littoralis* were subjected to hydro-distillation in a modified Clevenger-type apparatus for at least four hours according to the British pharmacopoeia. The essential oil was obtained in a yield of w/w after drying over anhydrous sodium sulphate. The oil was stored in sealed glass vial (Bijoux bottle) at 4^{o}C.The flow chart for the procedure is shown in figure 7.

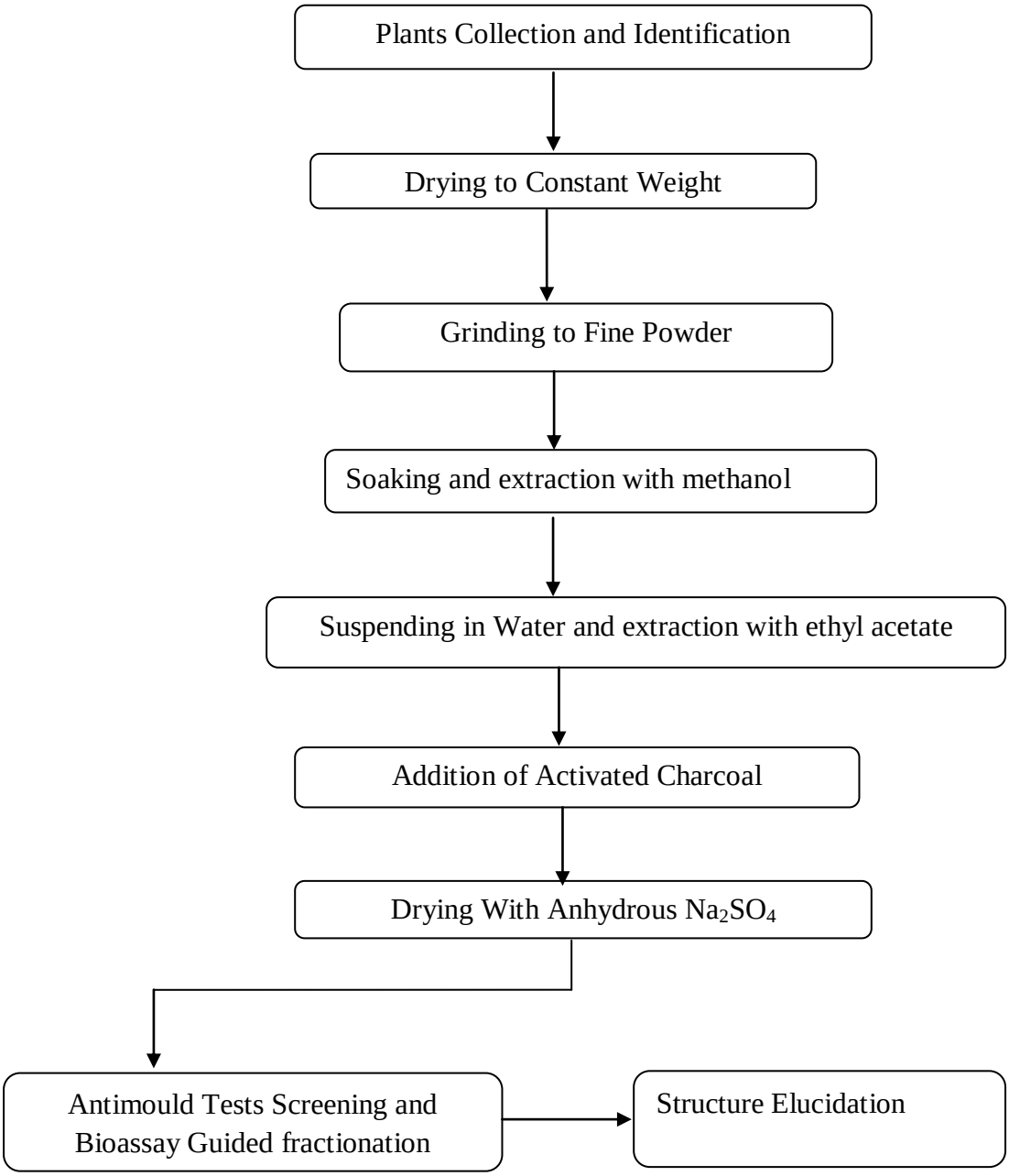


Figure 6: Extraction of non-volotile compounds and antimould tests

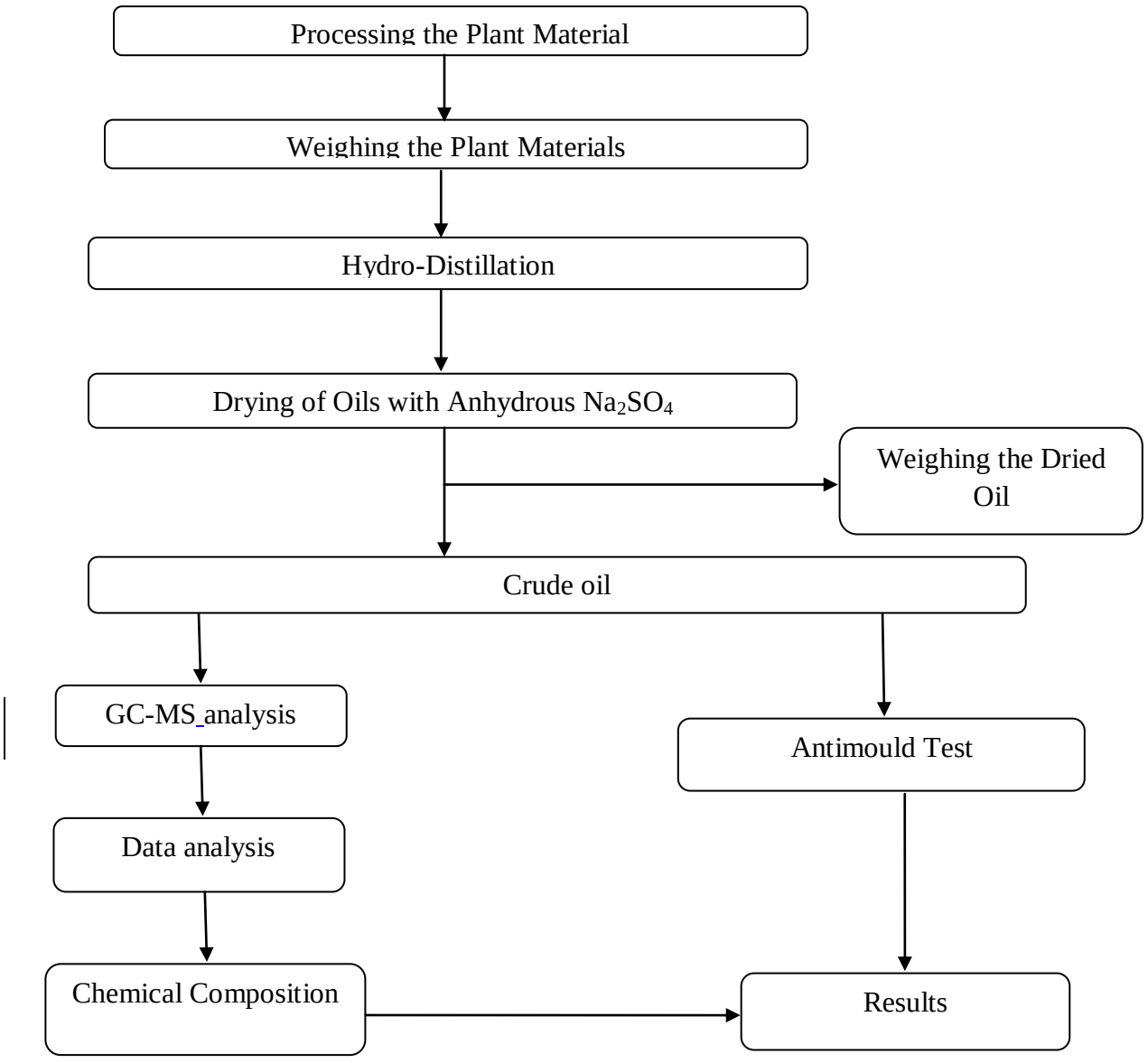


Figure 7: Extraction of volatile compounds and antimould tests

3.4 Isolation and identification of moulds

Moulds were isolated from the maize samples using both direct and dilution techniques. For each sample, 20 seeds were picked randomly and surface sterilized by soaking for one minute in 2.5% sodium hypochlorite, and rinsed three times with sterile distilled water. The samples (5 seeds per plate) were directly plated on Potato Dextrose Agar (PDA) containing 7.5%

sodium chloride and 133 mg of streptomycin sulphate. Addition of streptomycin sulphate is very effective in the inhibition of fast-growing "spreader" moulds such as *Trichoderma* yet, it does not inhibit the growth of other mould species, including the mycotoxin producers (Diba *et al*., 2007). Treatments were replicated four times and the experiments were laid down in a complete randomized design. The resulting cultures were identified based on cultural and microscopic characteristics using taxonomic keys. Morphological features of moulds were studied and the major and remarkable macroscopic features that were looked at were colony diameter, colony color on agar and reverse, exudates and colony texture.

3.5 Fractionation and purification of anti-mould compounds

The ethyl acetate crude extracts were fractionated with silica gel chromatographic column using chloroform ethyl acetate mixtures in the ratio of 9:1 v/v as eluent. The fractions obtained were monitored using thin layer chromatography (TLC), to achieve isolation of compounds. The active extracts were subjected to bioassay-guided fractionation on normal phase column chromatography, using silica gel 70-230 mesh. The compounds isolated were purified using repetitive preparative thin layer chromatography.

3.6 Screening for anti-mould Activity

Paper disc diffusion inhibition test was used to screen for antimicrobial activity of both volatile and non-volatile secondary metabolites as described by Souza *et al.* (2005). One milliliter of mould suspension (approximately 10^6 spores) was uniformly spread on sterile PDA media in petri dishes. Sterile Whatman filter paper discs (No. 1, 6 mm in diameter) were soaked with 10 µl of the secondary metabolites and placed at the center of the inoculated culture plates. The plates were incubated at 25^oC for 7 days. For the non-volatile secondary metabolites the soaked discs were allowed to dry before being placed in the culture plates. This was done to ensure that all the solvent was evaporated completely. The plates were placed in the fridge at 4 ^0C for 48 hours to allow the extracts to diffuse and then incubated for 7 days. After the end of the incubation period, diameter of the inhibition zones were measured. Also the inhibition zones were measured after 14 days.. The most bioactive metabolites were selected based on the antimould spectrum and size of inhibition zones. Nyststin bought from Indol Laboratories Company with concentration of 100 µg was used as the positive control.

3.7 Determination of the Minimum Inhibitory Concentration of the oil

Five fungi from the three genera showing the highest inhibition zones in the screening assay were used to evaluate the bioactive secondary metabolites for their Minimum inhibitory concentration (MIC). One milliliter of mould suspension (approximately 10^6 spores) was uniformly spread on sterile potato dextrose agar media in petri dishes. Serial dilutions of the oil were done using pure dimethyl sulfoxide (DMSO) which was also used as the control. The oil was diluted to the following serial geometric dilutions: 50%, 25%, 12.5%, 6.25%, 3.13%, 1.56% and 0.78%. Sterile Whatman filter paper discs (No. 1, 6 mm in diameter) were soaked with 10 µl of the oil and placed at the center of the inoculated culture plates and then incubated for 7 to 14 days. The experiment was in three replicates. The resultant minimum inhibition zones were used to determine the range for MIC analyses.

3.8 Determination of the Minimum Inhibitory Concentration of the non-volatile compounds

Five fungi from the three genera showing the highest inhibition zones in the screening essay were used to evaluate the bioactive secondary metabolites for their Minimum inhibitory concentration (MIC). One milliliter of mould suspension (approximately 10^6 spores) was uniformly spread on sterile potato dextrose agar media in petri dishes. Sterile Whatman filter paper discs (No. 1, 6 mm in diameter) were soaked with 10 µl of the secondary metabolites. The soaked discs were allowed to dry before being placed in the culture plates. This was done to ensure that all the solvent was evaporated completely. The plates were placed in the fridge at 4 ^{0}C for 48 hours to allow the extracts to diffuse and then incubated for 7 to 14 days at 25 ^{0}C. Serial dilutions of the identified metabolites were done using methanol which was also used as the control. The metabolites were diluted to the following serial geometric dilutions: 8 mg/ml, 6 mg/ml, 4mg/ml, 2 mg/ml and 1 mg/ml. The resultant minimum inhibition zones were used to determine the range for MIC analyses. The experiment was in three replicates.

3.9 Characterization of the bio-active essential oils by GC-MS analysis

Analysis of the essential oils was done by gas chromatography-mass spectrometer (GC - MS). Sample of essential oils were diluted in methyl tert-butylether (MTBE) (1:100) and analyzed on an Agilent GC-MSD apparatus equipped with an Rtx-5SIL MS (‘Restek’) (30m × 0.25 mm i.d., 0.25µm film thickness) fused-silica capillary column. Helium (at 0.8 ml/min) was

used as a carrier gas. Samples were injected in the split mode at a ratio of 1:10 – 1: 100. The injector was kept at 250 ^{0}C and the transfer line at 280 ^{0}C. The column was maintained at 50 ^{0}C for 2 min and then programmed to 260 ^{0}C at 5 ^{0}C /min and held for 10 min at 260 ^{0}C. The MS was operated in the Electron Ionization (EI) mode at 70 eV, in m/z range 42-350. The identification of the compounds was performed by comparing their retention indices and mass spectra with those found in literature (Adams, 1995) and supplemented by Wiley and QuadLib 1607 GC-MS libraries. The relative proportions of the essential oil constituents were expressed as percentages obtained by peak area.

3.10 Characterization and structure elucidation of the bio-active non- volatile compounds

The structural elucidations of the bioactive compounds were carried out by a combination of spectroscopic high field NMR and mass spectroscopy. 1D and 2D high field NMR spectroscopy was used to elucidate the actual structures of the isolated compounds. The ^{1}H NMR was used to determine the protons present in the molecule while ^{13}C NMR was used to determine the number of carbon atoms in the molecule. HSQC was used to allocate the protons to the carbons. COSY was used to determine the adjacent protons while HMBC was used to determine the proton carbon correlation. MS spectroscopy was used to derive the mass of the compound. Known types of compounds were identified by means of databases already developed. The machine was set at 600.10 MHz for ^{1}H NMR and 150.90 MHz for ^{13}C NMR

3.11 Statistical analysis

The most bioactive secondary metabolites were selected based on the antimould spectrum and size of inhibition zones. As for MIC determination, data was analyzed using SPSS 11.5 software to determine the mean and standard deviations and the lowest concentration able to induce inhibition was taken as the MIC. The NMR spectra data was analyzed using ACD 1D and 2 D NMR manager. The GC-MS spectra data were analyzed by GC–MS data base to obtain the chemical composition of the oil.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Moulds isolated

4.1.1 *Aspergillus* species isolated

A total of 10 mycotoxigenic *Aspergillus* species were isolated from the maize collected from the three districts (Table 1). All *Aspergillus* species isolated exhibited conidial heads in shades from yellow–green to brown except for *A. niger* which was black in colour. These fungi were identified according to Kozakiewtez (1989) and Klich (2002).

Table 1: *Aspergillu*s species isolated from the maize and their sources

S.No	Fungi isolated	Source (maize samples)
1	*A. flavus*	KEKUM 45, KEKUM 44, KEKIM 02, KEKIM 06, KEK AM 19, KEKAM 20, KEKAM 21
2	*A. flavipes*	KEKUM 38,
3	*A. parasiticus*	KEKIM 01, KEKUM 38, KEKAM 44, KEKAM 25
4	*A. fumigatus*	KEKAM 21
5	*A. sparsus*	KEKUM38, KEKIM 06, KEKUM 33, KEKUM 35
6	*A. nidulans*	KEKUM 33
7	*A. versicolar*	KEKIM 02, KEKIM 06, KEKIM 07
8	*A. ochraceus*	KEKUM 38,KEKIM 07, KEKUM3, KEKUM 41
9	*A. niger*	KEKIM 03, KEKUM 43, KEKIM 02, KEKIM 05, KEKAM 21
10	*A. tamari*	KEKUM 38, KEKUM 44

Exposure to *Aspergillus* causes diseases called Aspergillosis in humans and the three major ones are allergic bronchopulmonary aspergillosis, acute invasive aspergillosis and disseminated invasive Aspergillosis. Some of the harmful mycotoxins produced by *Aspergillus* include aflatoxins, fumitoxins and gliotoxin.

Aspergillus flavus and *Aspergillus parasiticus* were isolated from maize from the three districts. *A. flavus* grew as a yellow-green mould in culture. The conidia of *A. flavus* appeared yellow green when young and as the fungus matured the spores turn dark green. The underneath colour for this fungus ranged from goldish to red brown. This fungus was isolated from maize from the three districts. *A. parasiticus* which is closely related to *A. flavus* in morphological features was also isolated from maize from the three districts. The difference between the two species is the texture of conidia. *A. parasiticus* produced very rough conidia while *A. flavus* produced smooth and fine conidia. *A. parasiticus* grew as a blue-green mould on the surface when the mould is still young and turns gray as the mould matured. White colour was observed for the reverse. *Aspergillus flavus* and *A. parasiticus* are known to produce the harmful mycotoxins aflatoxin B_1 and B_2, G_1 and G_2 (Schmale and Munkvold, 2009). The primary disease associated with aflatoxin intake is hepatocellular carcinoma (liver cancer). This disease is the third-leading cause of cancer death globally. Also growth and development impairment is associated with ingestion of aflatoxins in small quantities over a long time. Ingestion of large quantities of aflatoxin can lead to death (WHO, 2008).

Aspergillus niger and *A. ochraceus* were isolated from maize collected from Kitale and Kuria. *Aspergillus niger* showed a white colour but produced black spores while the underneath colour was white. *A. ochraceus* grew as yellow-gold mould with light pink underneath. The two molds are known to produce ochratoxin A; a kidney toxin which cause kidney failure. Also ochratoxin A has been reported to be carcinogenic (Schuster *et al.,* 2002; Mantle *et al.,* 2005). *Aspergillus niger* is also known to cause otomycosis, a fungal ear infection and also produces other mycotoxins like oxalic acid and kojic acid abundantly (Schuster *et al.,* 2002). *Aspergillus fumigatus* on the other hand showed a blue-green colour on the surface when the mould is still young and turns gray as the mould aged. White colour was observed for the reverse. *Aspergillus fumigatus* was isolated from maize collected from Kakamega and is known to produce a mycotoxin called gliotoxin which is capable of altering host defenses through immunosuppression (Spikes *et al.,* 2008). *Aspergillus fumigatus* is the most frequent cause of invasive fungal infection in immunosuppressed individuals, which include patients receiving immunosuppressive therapy for autoimmune or neoplastic disease, organ transplant recipients, and AIDs patients. Additionally, *A. fumigatus* can cause chronic pulmonary infections or allergic disease in immunocompetent hosts (Segal, 2009; Ben-Ami *et al.,* 2010).

Aspergillus tamarii was isolated from maize collected from Kuria district. This fungus was yellow with shades of green when young and yellowish brown as it matured. *Aspergillus tamari* is known to produce a mycotoxin called cyclopiazonic acid but its effects on human health is not yet known. The fungus has also been reported to cause Keratitis (Laszlo *et al.*, 2007). *Aspergillus flavipes* and *A. nidulans* were mycotoxigenic moulds that were isolated from maize from Kuria district while *Aspergillus versicolar* was isolated from maize collected from Kitale. *Aspergillus flavipes* fungus grew slowly and its surface appears yellowish when the fungus was still young and it turned brownish as the fungus aged. The reverse of this fungus in the media was faded black. *A. versicolar* showed white colour at the beginning and turned yellow to pale green after maturity. *Aspegillus versicolar* showed white colour at the beginning and turned yellow to pale green after maturity. The underneath was white yellow when the mould was still young and it turns purple red as the mould. *Aspergillus nidulans* was typically green with wooly texture colony. *Aspergillus flavipes, A. nidulans* and *A. versicolar* are mycotoxigenic fungi and are known to produce sterigmatucystin (Lillard-roberts, 2003). A mycotoxigenic fungus *A. sparsus* was isolated from maize collected from Kitale and Kuria.

4.1.2 *Fusarium* species isolated

A total of nine mycotoxygenic *Fusarium* species were isolated and identified from maize collected from the three districts (Table 2). Generally *Fusarium* species colonies showed fast growth, pale or brightly colored (depending on the species) and may or may not have a cottony aerial mycelium. The color of the thallus varies from whitish to yellow, brownish, pink, reddish or lilac shades. Fusarium species were identified according to Nelson *et al*., (1983). *Fusarium* species are known to produce several mycotoxins which include fumonisins and trichothecene which are most strongly associated with chronic and fatal toxic effects in animals and humans and are also known to affect the nervous system and are also carcinogenic (Desjardins *et al.*, 2007).

Table 2: *Fusarium* species isolated from the maize and their sources

S.No	Fungi isolated	Source (maize samples)
1	*F. solani*	KEKIM 08, KEKIM 09, KEKIM 13, KEKIM 01
2	*F. oxysporum*	KEKUM 42, KEKUM 45
3	*F. moniliforme*	KEKUM 42
4	*F. nivale*	KEKUM 33
5	*F. avenaceum*	KEKIM 07, KEKAM 26
6	*F. proliferatum*	KEKAM 33
7	*F. culmorum*	KEKAM 27
8	*F. graminearum*	KEKAM 25
9	*F. subglutinans*	KEKIM 07, KEKAM 25

Fusarium solani was isolated from maize collected from Kitale. This mould produces sparse to abundant, white cream mycelium which starts out as white, cottony colonies. This fungus is known to produce trichothecene mycotoxins like T-2 toxin and neosolaniol. T-2 toxin causes acute and chronic toxicity and induces apoptosis in the immune system and fetal tissues (Lillard-Roberts, 2003). *Fusarium moniliforme* was growing rapidly with colonies initially white becoming tinged with lavender with a colorless to dark purple reverse. *Fusarium culmorum* had fast growing colonies and its surface colour was whitish but becomes brown to dark brown with age *Fusarium moniliforme* and *F. culmorum* were isolated from maize from Kuria district and Kakamega district respectively. The two fungi are the major producers of the mycotoxins like fumonisins B_1, nivalenol, deoxynivalenol among others (Lillard-Roberts, 2003). These mycotoxins are known to have harmful effects in humans and animals. Fumonisins B_1 have been reported to be carcinogenic and have been associated with aesophageal cancer (Wild and Gong 2010). Also this mycotoxin has been reported to affect unborn babies as they cause Neural Tube

Defect (NTD) a defect of the brain and spinal cord in the embryo (Savolainen and Stockmann 2008).

Fusarium nivale and *F. oxysporum* were isolated from maize collected from Kuria district. Based on morphological features *F. nivale* was growing as irregular circular pink to reddish brown patches that merged to cover the whole plate. *Fusarium oxysporum* was growing initially as a white mould but turns yellow as the fungus matured. *Fusarium nivale* is a mycotoxigenic fungus as is it known to produce mycotoxins like fumonisins B_1, nivalenol and deoxynivalenol (Lillard-Roberts, 2003). *Fusarium oxysporum* produces the harmful mycotoxins like scirpentriol, nivalenol among others (CAST, 2003). *Fusarium proliferatum* and *F. graminearum* were isolated from maize collected from Kakamega district. *Fusarium proliferatum* grew rapidly with colonies which were initially white and later become tinged with purple. The reverse was colourless initially but changed to dark purple as the fungus aged. On the other hand *F. graminearum* grew thick pinkish white mycelia with grayish margins and a dark ruby reverse. *Fusarium graminearum* and *F. proliferatum* produces several mycotoxins, including deoxynivalenol (DON) and derivatives, zearalenone, fusarin C, and aurofusarin (CAST, 2003). *Fusarium avenaceum* and *F. subglutinans* were isolated from maize from Kakamega and Kitale. *Fusarium avenaceum* had a light pink surface with reddish brown margines while *F. subglutinans* appeared as a white mould with a violent gray reverse. The two fungi produce mycotoxins which are harmful to human health. The mycotoxins that these fungi produce include fumonisins, deoxynivalenol, fusaproliferin among others (CAST, 2003).

4.1.3 *Penicillium* species isolated

Eleven mycotoxigenic *Penicillium* species were isolated from maize collected from the three districts (Table 3). The *Penicillium* colonies were growing rapidly and in general the fungi were flat, filamentous, velvety, woolly, or cottony in texture (Pitt, 1979). The colonies were initially white and became blue-green, gray-green, olive-gray, yellow or pinkish with time. The plate underneath were pale to yellowish. *Penicillium* species are known to produce mycotoxins which include gliotoxin which has immunosuppressive effects, ochratoxin which is a nephrotoxic mycotoxin, robroskyrin which cause liver damage and patulin which affect the immune system, the nervous system and is carcinogenic among others (Heike *et al.;* 1996, Hopmans, 1997; Satoru *et al*., 1997).

Table 3: *Penicillium* species isolated from the maize and their sources

S.No	Fungi isolated	Source (maize samples)
1	*P. rubrum*	KEKIM 02, KEKIM 07, KEKIM 13, KEKUM 34
2	*P. purporogenum*	KEKAM 47
3	*P. islandicum*	KEKAM 26
4	*P. rugulosum*	KEKAM 14
5	*P .expansum*	KEKUM 45, KEKIM 07, KEKIM 09, KEKIM 11
6	*P. citrinum*	KEKUM 01, KEKIM 13
7	*P. viriidicatum*	KEKIM 08, KEKUM 41
8	*P. claviforme*	KEKAM 17
9	*P. wortmani*	KEKIM 01
10	*P. digitatum*	KEKAM 23
11	*P. cyclopium*	KEKUM 45, KEKIM O7, KEKIM 09

Penicillium rubrum was isolated from maize collected from Kitale and Kuria and was showing rapid growth, initially the colony was light yellow after maturity it had become reddish with some cottony texture and central region was velvety while the back side of the colony was reddish in colour. *Penicillium purporogenum* was isolated from maize obtained from Kakamega distrct while *P. citrinum* was isolated from maize obtained from Kitale. *Penicillium purporogenum* which was isolated from maize obtained from Kakamega showed rapid growth, olive green, yellow margin, velvety colony, with granular appearance and the back side of the colony was yellowish cream. *Penicillium citrinum* showed rapid growth, dark green colour, granular powdery colony and the back side was pale yellow colour. *Penicillium rubrum* and *P. purporogenum* produce the toxic rubratoxin which causes liver damages (Hayes *et al.*, 1970).

On the other hand *P. citrinum* produces citrinin which is a nephrotoxic mycotoxin and is associated with renal damage, vasodilatation and bronchial constriction (EFSA, 2012).

Penicillium rugulosum and *P. islandicum* were isolated from maize collected from Kakamega while *P. expansum* and *P. viridicatum* were isolated from maize from Kitale and Kuria. *Penicillium rugulosum* showed rapid growth, dark green and powdery appearance and back side was yellow in colour while *P. islandicum* was growing rapidly with floccose grayish green surface and brownish orange reverse. *Penicillium expansum* had dark green colour on the surface with velvety texture and the reverse was yellow in colour while *P. viridicatum* grew initially with blue coloured surface which turned grayish bleu as the fungus aged. *Penicillium rugulosum* produce rugulosin and the sterismatocystin which is as harmful as the aflatoxin because it is associated with esophageal, gastric and liver cancers (Sun *et al.,* 2002). *Penicillium islandicum* produces islanditoxin and *P. expansum* produces the carcinogenic palutin (Morales *et al.,* 2007). *Penicillium viridicatum* produce several mycotoxins including ochratoxin, rubrosulphin among others (Lillard-Roberts, 2003). Other *Penicillium* species isolated included *P. cyclopium* which was isolated from maize collected from Kuria and *P. claviforme* isolated from maize obtained from Kakamega. *Penicillium cyclopium* had velutious texture, blue green colour on the surface and cream yellow reverse. *Penicillium claviforme* grew initially with white colour but turned green on maturity. These two fungi produce the toxic palutin. *Penicillium digitatum* was isolated from maize obtained from Kakamega.This fungus showed fast growth, velutious texture, large olive-green colonies and yellowish reverse. A fast growing fungus identified as *P. wortmanni* was isolated from maize collected from Kitale. This fungus showed fast growth, gray-blue colour and orange reverse. Figure 8 are pictures showing the various moulds growing after maize incubation.

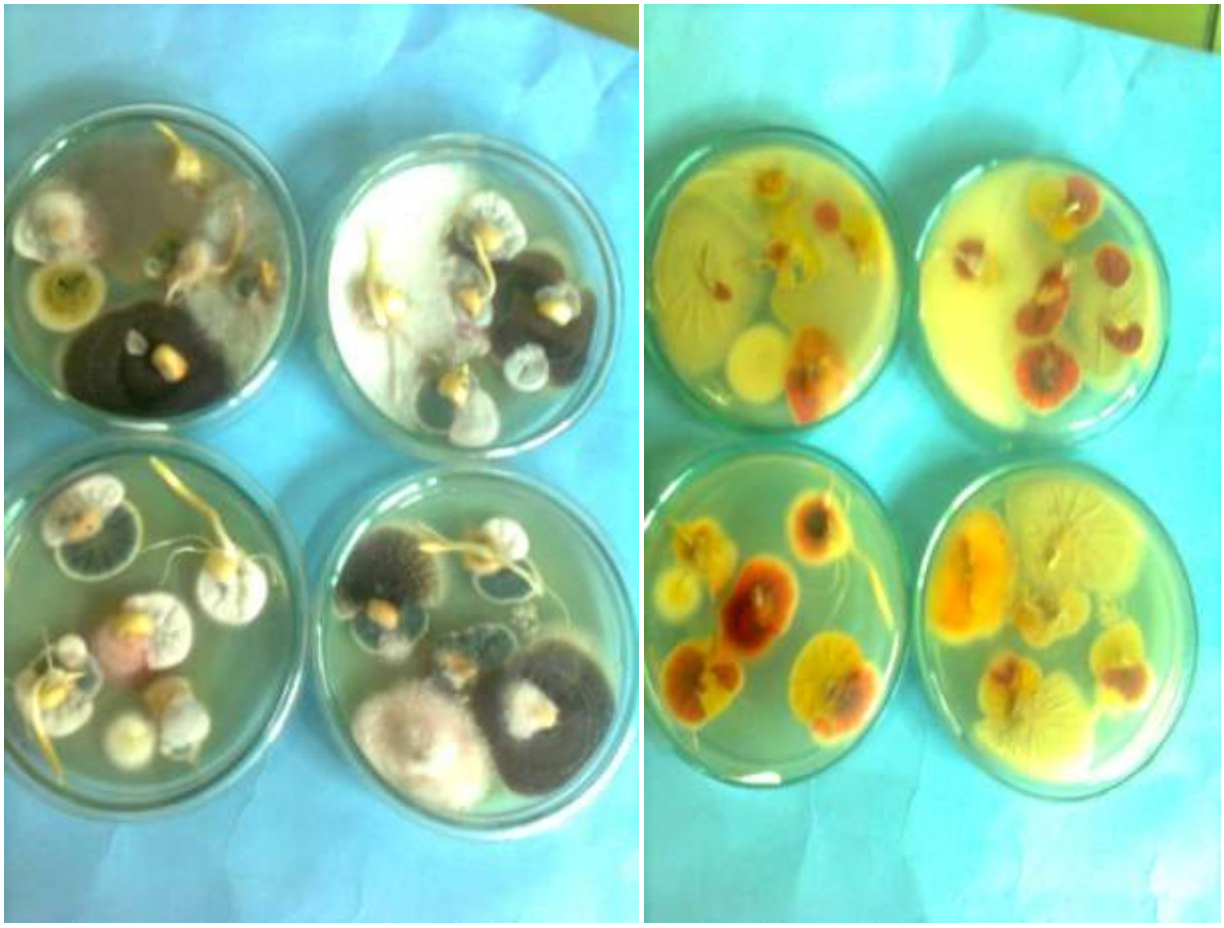


Figure 8: Various moulds growing from maize after incubation

4.2 Extractions of volatile and non volatile secondary metabolites

About 10 kg of fresh *Monanthotaxis littoralis* leaves was hydro-distillated and oil collected. The oil was dried with anhydrous sodium sulphate and the total oil collected was 5.20 ml. To determine the oil density the weight of 1ml of the oil was taken and the value obtained was used to calculate the density of the oil.

Weight of 1ml of oil = 0.82g

Density = mass / volume

=0.82g / 1 ml

=0.82g/ml

For the non volatile compounds *Monanthotaxis littoralis* leaves were dried to constant weight, ground to fine powder before extraction to increase the surface area. One kg of the plant material was soaked in methanol for 24 hours and extracted several times. The dry methanol crude extract weighed 105.30g. The methanol crude extract was suspended in water to remove all water soluble compounds like glycosides. Ethyl acetate was used to extract the crude extract from water to obtain ethyl acetate crude extract which weighed 94.74g. Thus the percentage yield was 9.47%. Figure 9 summarizes this process.

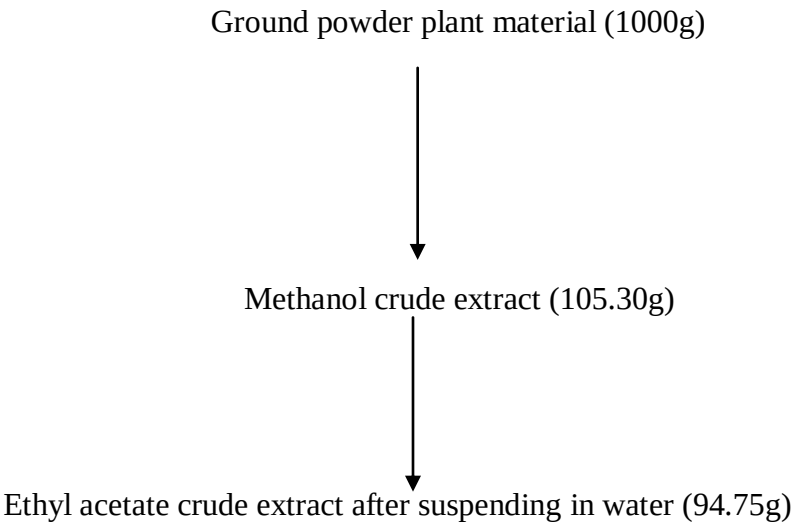


Figure 9: Extraction of non volatile compounds

4.3 Screening of the oil for antifungal activity

4.3.1 Screening of oil activity against *Aspergillus* species

The screening results of the oil against the *Aspergillus* species is shown in table 4. The oil was found to be active against all *Aspergillus* species tested accept for *A. parasiticus*. The oil was very active against *A. flavipes* and *A. flavus* with inhibition zones of 17.00 mm and 17.50 mm respectively. The oil activity against *A. flavus* was quite high compared to standard nystatin which was used as the positive control i.e. nysatin had inhibition zones of 12.50 mm. The nystatin concentration used was 100µg. The nystatin activity against *A. flavipes* was higher than that of the oil extract though the difference was not significant. This is because when the oil was used against this species inhibition zone of 17.00 mm was obtained while an inhibition zone of

20.67 mm was recorded when nystatin was used. The oil also showed high activity against *A. ochraceus* and *A. niger*. This is exhibited by their inhibition zones of 15.25 mm and 14.50 mm respectively. The inhibition zones of the oil against *A. ochraceus* was comparable to that of the nystatin since inhibition zones of 16.00 mm was observed while for *A. niger* the value was less than that of nystatin since 19.67 mm were observed.

Further, inhibition activity of the oil was observed against *A. fumigatus, A. nidulans* and *A. tamari*i where the inhibition zones of13.00 mm, 12.00 mm and 12.25 mm were recorded for the three species respectively. The same inhibition zone of 20.67 mm and 20.33 mm was observed when nystatin was used against *A. nidulans* and *A. fumigatus* respectively. Nystatin activity against *A. nidulans* and *A. fumigatus* was therefore quite high compared to the oil activity against these two species. On the other hand the oil and nystatin activity against *A. tamarii* were almost the same. This is illustrated by the inhibition zones of 12.25 mm and 13.17 mm obtained when the oil and nystatin were used against this species respectively. The highest resistance to the oil was exhibited by *A. versicolar* and *A. sparsus.* This is indicated by the low value of their inhibition zones. Inhibition zone of 20.33 mm was recorded for *A .versicolar* when nystatin was used implying that nystatin activity against this species was more than double that of the oil. Inhibition zone of 12.00 mm was recorded when nystatin was used against *A. sparsus* implying that this species showed high resistance to nystatin.

Table 4: Inhibition zones for *Aspergillus* species

Fungi	*Monanthotaxis littoralis* oil Inhibition zones (mm		Nystatin inhibition zones (mm)	
	7th day	14th day	7th day	14th day
A. niger	14.50 ± 0.50	12.50 ± 1.50	19.67 ± 0.47	19.50 ± 0.48
A. parasiticus	0.00 ± 0.00	0.00 ± 0.00	14.50 ± 0.41	14.00± 0.50
A. fumigatus	13.00 ± 1.00	11.85 ± 0.15	20.33 ± 0.47	20.00 ± 0.96
A. sparsus	7.50 ± 1.50	7.30 ± 0.10	12.00 ± 0.00	11.00 ± 0.50
A. nidulans	12.00 ± 2.00	10.05 ± 0.15	20.67 ± 0.47	20.00 ± 0.50
A. versicolar	7.50 ± 0.50	6.00 ± 0.00	20.33 ± 0.47	19.50 ± 1.31
A. ochraceus	15.25 ± 0.75	14.65 ± 0.35	16.00 ± 0.82	15.00 ± 0.25
A. flavipes	17.00 ± 0.50	16.25 ± 0.25	20.67 ± 0.41	20.50 ± 0.41
A. flavus	17.50 ± 0.50	16.50 ± 0.50	13.00 ± 2.16	12.50 ± 0.45
A. tamarii	12.25 ± 0.75	10.50 ± 0.50	13.17 ± 0.85	12.50 ± 1.25

The activity of the oil against all *Aspergillus* species on the 14th day was lower compared to the activities recorded on the 7th day showing a reduction in the oil strength. The reduction in oil strength is indicated by the reduction in the inhibition zones. The growth of the fungi and inhibition zones were monitored and the best time of making the readings was the 7th day. This is because by the 3rd day the fungi are still growing, on the 7th day the oil activity is at its highest and on the 14th day the oil activity against the fungi is already decreasing. Also this is indicated

by the t value of the paired means which was 5.041 at 95% confidence limit which is out of the expected interval of 0.5798 – 1.4785 (Appendix 1). Thus the means of the inhibition zones on the 7th day and 14th day are significantly different and the 7th day readings are more representative.

4.3.2 Screening of oil activity against *Fusarium* species

The oil was active against all *Fusarium* species screened. The oil inhibited the growth of *Fusarium* species with the highest activity of the oil being observed against *F. proliferatum* with inhibition zone of 20.17 mm (Table 5). The oil activity against this species was double that of nystatin standard since inhibition zones of 10.63 mm was observed when nystatin was used against this species. High activity of the oil was also observed against *F. subglutinans, F. solani* and *F. moniliforme* with inhibition zones of 15.67 mm, 15.83 mm and 15.67 mm respectively. The inhibition zones observed for these three species when nystatin was used were 19.33 mm, 28.33 mm and 18.67 mm respectively. Comparing the activity of nystatin and the oil, their activity was almost comparable for *F. subglutinans* and *F. moniliforme*. On the other hand the nystatin activity against *F. solani* was almost twice that of the oil. Also the oil inhibited the growth of *F. avenaceum, F. culmorum* and *F.graminearum* where inhibition zones of 12.50 mm, 12.33 mm and 11.33 mm were obtained. Inhibition zone of 33.67 mm was recorded when nystatin was used against *F.graminearum* and therefore was three times that of the oil. *Fusarium avenaceum* and *F. culmorum* growth was also inhibited by nystatin and inhibition zones of 12.33 mm and 12.50 mm were observed for the two species respectively. Thus nystatin activity against *F. avenaceum* and *F. culmorum* was close to that of the oil. The lowest activity of the oil was against *F. nivale* and *F. oxysporum* with inhibition zones of 9.67 mm and 7.83 mm respectively. The nystatin inhibition activity against the two fungi species was also low at 10.90 mm and 11.33 mm for the two species respectively.

The inhibition zones recorded on the 14th day for all the *Fusarium* species screened were lower than those recorded on the 7th day. This implies that the oil strength decreases with time. Also the paired means of the 14th day and the 7th day were significantly different at 95% confidence level. This is because the t value of 7.087 at 95% confidence limit was out of the expected interval of 1.5516 – 3.0484 (Appendix 2). Therefore the best time to make the readings is the 7th day when the oil activity is high.

Table 5: Inhibition zones for *Fusarium* species

Fungi	*Monanthotaxis littoralis* oil inhibition zones (mm)		Nystatin inhibition zones (mm)	
	7^{th} day	14^{th} day	7^{th} day	14^{th} day
F. solani	15.83 ± 1.03	13.33 ± 0.94	28.33 ± 1.70	27.00 ± 2.16
F. oxysporum	7.83 ± 0.85	6.33 ± 0.24	11.33 ± 0.47	10.50 ± 0.41
F. moniliforme	15.67 ± 0.47	11.33 ± 1.25	18.67 ± 1.25	17.00 ± 1.41
F. nivale	9.67 ± 0.470	9.33 ± 0.47	10.90 ± 0.70	8.67 ± 1.25
F. avenaceum	12.50 ± 0.41	10.43 ± 0.42	12.33 ± 0.47	11.17 ± 0.62
F. proliferatum	20.17 ± 0.85	17.67 ± 1.25	10.63 ± 0.45	8.50 ± 0.41
F. culmorum	12.33 ± 0.94	8.53 ± 0.42	12.50 ± 0.41	11.27 ± 0.38
F. graminearum	11.33 ± 2.62	9.67 ± 0.94	33.67 ± 0.94	33.00 ± 0.82
F. subglutinans	15.67 ± 0.47	12.67 ± 1.70	19.33 ± 0.47	18 .00 ± 0.82

4.3.3 Screening the oil activity against *Penicillium* species

The oil inhibited the growth of all *Penicillium* species screened as shown in table 6. *Penicillium purpurogenum* and *P. islandicum* exhibited the highest activities with inhibition zones of 39.00 mm and 29.00 mm respectively. The oil showed the highest activity against the

two species compared to all the fungi screened in the three fungal genera. Inhibition zones of 16.97 mm and 15.33 mm were recorded for the two species respectively when nystatin was used. This implies that the oil extract inhibition activity against these two species was double that exhibited by nystatin. The oil was also quite active against *P. rugulosum* and *P. wortmanni* where inhibition zones of 20.17 mm and 17.67 mm were recorded for the two species respectively. The oil and nystatin activity against *P. wortmanni* was comparable since inhibition zones of 17.83 mm which is close to that of the oil was observed. Inhibition zones slightly higher than 10 mm were recorded for *P. cyclopium, P. citrinum* and *P. viridicatum* when oil was used. Inhibition activity of 17.67 mm was obtained when nystatin was used against *P. cyclopium* while the oil activity against this species was 11.00 mm. Inhibition zones of 11.00 mm and 10.67 mm were observed for *P. citrinum* and *P. viriidicatum* respectively when the oil extract was used but inhibition zones of 17.67 mm and 16.67 mm were obtained for the two species respectively when nystatin was used. Thus standard activity against the three species was higher than that of the oil extract. The lowest activity of the oil was that against *P. expansum, P. rubrum, P. claviforme* and *P. digitatum* as evidenced by their inhibition zones which were 7.50 mm, 8.17 mm, 9.33 mm and 7.17 mm respectively. The inhibition activity of nystatin against these four species was twice higher than that of the oil at 17.17 mm, 18.33 mm, 16.67mm and 18.33 mm respectively.

Just like for *Aspergillus* and *Fusarium* species the activity of the oil against all *Penicillium* species on the 14^{th} day was lower compared to the activity observed on the 7^{th} day showing a reduction in the oil strength. The t value of the paired means which was 9.595 at 95% confidence limit was out of the expected interval of 0.8402– 1.3284 (Appendix 3). This implies that the two means of the 7^{th} day and 14^{th} day are significantly different.

Table 6: Inhibition zone for *Penicillium* species

Species	*Monanthotaxis littoralis* oil inhibition zones (mm)		Nystatin inhibition zones (mm) (+ control)	
	7th day	14th day	7th day	14th day
P. rubrum	8.17 ± 0.24	6.50 ± 0.42	18.33 ± 0.47	18.00 ± 0.82
P. cyclopium	11.00 ± 0.82	10.00 ± 0.82	17.67 ± 1.25	17.33 ± 1.25
P. islandicum	29.00 ± 0.82	28.83 ± 0.62	15.33 ± 0.47	14.67 ± 0.47
P. rugulosum	20.17 ± 0.62	18.67 ± 1.25	15.50 ± 0.41	15.30 ± 0.5
P .expansum	7.50 ± 1.08	6.33 ± 0.47	17.17 ± 0.62	17.00 ± 0.82
P. citrinum	11.00 ± 0.82	10.17 ± 0.62	17.67 ± 0.62	15.83 ± 0.45
P. viridicatum	10.67 ± 0.47	9.33 ± 0.47	16.67 ± 0.47	16.67 ± 1.25
P. claviforme	9.33 ± 0.47	8.17 ± 0.24	16.67 ± 0.94	16.67 ± 1.25
P. wortmanni	17.67 ± 0.47	16.33 ± 0.94	17.83 ± 0.24	17.17 ± 0.62
P. digitatum	7.17 ± 0.62	6.33 ± 0.47	18.33 ± 0.47	17.67 ± 1.25
P. purpurogenum	39.00 ± 0.81	37.67 ± 0.47	16.97 ± 0.94	16.33 ± 0.86

4.4 Determination of MIC of the oil

4.4.1 Determination of MIC of the oil against *Aspergillus* species

The antifungal activity of the oil varied with its concentration indicating that the oil activity was proportional to its concentration. Also the activity of the oil as exhibited by the inhibition zones varied from one fungal species to another (Table 7).

Table 7: Inhibition zones of *Aspergillus* species at different oil concentrations

Fungi	Essential oil concentration (mg/ml) $*10^2$								Control	MIC (mg/ml)
	8.20	4.10	2.05	1.03	0.51	0.26	0.13	0.06		
A. niger	15.67 ± 1.25	13.33 ± 0.47	11.00 ± 0.82	8.33 ± 0.47	6.17 ± 0.24	0.00	0.00	0.00	0.00	26.00
A. fumigatus	13.33 ± 0.47	10.00 ± 0.81	6.33 ± 0.24	0.00	0.00	0.00	0.00	0.00	0.00	103.00
A .ochraceous	15.33 ± 0.47	12.00 ± 0.81	7.50 ± 0.41	6.17 ± 0.24	0.00	0.00	0.00	0.00	0.00	51.00
A. flavus	18.33 ± 0.47	12.33 ± 0.47	7.17 ± 0.62	0.00	0.00	0.00	0.00	0.00	0.00	103.00
A. flavipes	17.50 ± 0.50	11.33 ± 0.47	6.95 ± 0.24	0.00	0.00	0.00	0.00	0.00	0.00	103.00

The MIC of the oil ranged from 26.00 mg/ml to 103 mg/ml. The highest inhibition zones recorded were those of *A. flavus* and *A. flavipes* where inhibition zones of 18.33 mm and 17.55 mm were recorded respectively. The oil MIC obtained against the two species was 103.00 mg/ml. *A. ochraceus* growth was inhibited by the oil showing an inhibition zone of 15.33 mm for the highest oil concentration and MIC of 51.00 mg/ml. Although the oil had lower inhibition activity against *A. niger* and *A. ochraceus* as compared to *A. flavus* and *A. flavipes*, their MIC values of 26.00 mg/ml, 51 mg/ml and 103 mg/ml respectively recorded implied that the oil was more active against these species. The least antifungal activity of the oil was observed against *A. fumigatus* with inhibition zone of 13.33 mm and an MIC of 103.00 mg/ml. Although, the oil had lower inhibition zones with *A.fumigatus* compared to *A. flavus* and *A. flavipes*, similar MIC values of 103 mg/ml was observed for the three species.

4.4.2 Determination of the MIC of the oil against *Fusarium* species

The activity of the oil against the *Fusarium* species varied from one species to another. Also the activity of the oil was dependant on the concentration as exhibited by the inhibition zones. As the concentration of the oil was decreased the activity of the oil against all the *Fusarium* species tested was also decreasing. The inhibition activity of the oil at different concentrations against *Fusarium* species is shown in table 8. The highest activity of the oil was observed for *F. proliferatum, F. moniliforme* and *F. subglutinans* with inhibition zones of 19.33 mm, 15.33 mm and 15.67 mm respectively for oil with a concentration of 820 mg/ml. The high activity of the oil against the three species was further indicated by their MIC of 51 mg/ml which was the lowest. Though an inhibition zone of 14.67 mm was obtained when oil with concentration of 820 mg/ml was used against *F. solani*, the oil activity against this species was low since its MIC was 103mg/ml. The lowest activity of the oil was on *F. avenaceum* and *F. culmorum* where inhibition zones of 12.33 mm and 12.67 mm respectively were recorded. Also the resistance of these species to the oil was indicated by their MIC value of 103 mg/ml which was the highest.

Table 8: Inhibition zones of *Fusarium* species at different concentrations of the oil

Fungi	Essential oil concentration (mg/ml) $*10^2$								Control	MIC (mg/ml)
	8.20	4.10	2.05	1.03	0.51	0.26	0.13	0.06		
F. moniliforme	15.33 ± 1.11	13.33 ± 0.44	10.00 ± 0.67	6.83 ± 0.67	0.00	0.00	0.00	0.00	0.00	51.00
F. proliferatum	19.33 ± 0.87	15.00 ± 0.67	11.00 ± 0.56	6.50 ± 0.33	0.00	0.00	0.00	0.00	0.00	51.00
F. solani	14.67 ± 1.11	11.33 ± 0.89	6.5 ± 0.33	0.00	0.00	0.00	0.00	0.00	0.00	103.00
F. subglutinans	15.67 ± 0.89	11.67 ± 1.11	8.87 ± 0.47	6.33 ± 0.22	0.00	0.00	0.00	0.00	0.00	51.00
F. culmorum	12.67 ± 0.94	8.63 ± 0.45	6.23 ± 0.33	0.00	0.00	0.00	0.00	0.00	0.00	103.00
F. avenaceum	12.33 ± 0.44	8.00 ± 0.67	6.20 ± 0.27	0.00	0.00	0.00	0.00	0.00	0.00	103.00

4.4.3: Determination of the MIC of the oil against *Penicillium* species

The growth of *Penicillium* species just like *Aspergillus* species and *Fusarium* species were inhibited by the oil. The inhibition activity of the oil was also directly proportional to the concentration of the oil. The different *Penicillium* species tested showed different inhibition zones implying that the oil's activities against these fungi varied from one species to another (Table 9).

Table 9: Inhibition zones of *Penicillium* species at different concentrations of the oil

Fungi	Essential oil concentration (mg/ml) $*10^2$								Control	MIC (mg/ml)
	8.20	4.10	2.05	1.03	0.51	0.26	0.13	0.06		
P. purpurogenum	39.17 ± 0.53	22.00 ± 0.29	10.33 ± 0.81	7.00 ± 0.33	6.00 ± 0.0	0.00	0.00	0.00	0.00	26.00
P. islandicum	29.60 ± 0.47	18.17 ± 0.81	10.33 ± 0.58	6.96 ± 0.58	6.07 ± 0.12	0.00	0.00	0.00	0.00	26.00
P. rugulosum	20.20 ± 0.26	9.97 ± 0.58	7.87 ± 0.81	0.00	0.00	0.00	0.00	0.00	0.00	103.00
P.wortmanni	17.33 ± 0.57	11.17 ± 0.76	6.67 ± 0.58	6.00 ± 0.00	0.00	0.00	0.00	0.00	0.00	51.00
P. viriidicatum	10.33± 0.58	7.40 ± 0.96	6.17 ± 0.29	0.00	0.00	0.00	0.00	0.00	0.00	103.00

The highest activity of the oil was against *P. purpurogenium* with inhibition zone of 39.17 mm and MIC of 26 mg/ml (Figure 10). This was also the highest activity of the oil among all the fungi from the three moulds genera tested. The oil also showed high activity against *P. islandicum* and *P. rugulosum* with inhibition zones of 29.60 mm and 20.20 mm and MIC of 26 mg/ml and 103 mg/ml respectively. The oil activity against *P. wortmanni* was also high with an inhibition zone of 17.33 mm at 820 mg/ml of the oil and MIC of 51 mg/ml. Even though a high inhibition zone for *P. rugulosum* was observed the oil was more active against *P. wortmanni* as indicated by its lower MIC. The highest resistance to the oil was observed for *P. viridicatum* which had an inhibition of 10.33 mm and MIC of 103 mg/ml. Though *P. viridicatum* had the lowest inhibition zone, an MIC of 103 mg/ml which is the same as that for *P. rugulosum* was observed.

Figure 10: Inhibition zones of *Penicillium purpurogenium* at different oil concentrations

4.5 Determination of the chemical composition of the oil extract

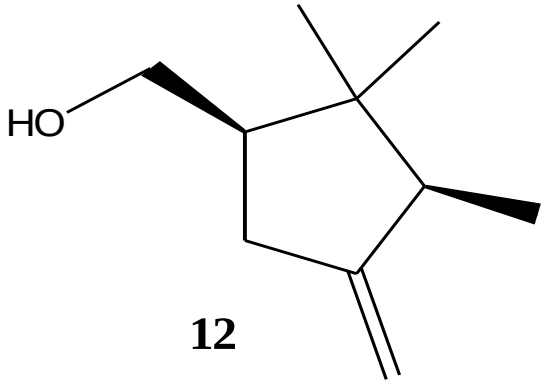
The chemical composition of the essential oil extracted from *Monanthotaxis littoralis* was determined by the GC-MS. The identification of the compounds in the oil was determined by comparing the electron impact mass spectrum of the compounds in the oil and those in the computer library (Wiley7N.l, FLAVORS.L, HPCH1607.L) databases. Though the oil contained 96 compounds only eight compounds were completely identified and are shown in table10 below.

Table 10: The major compounds in the M. littoralis oil extract

Compound Number	R.T (min)	Compound Name	%Concentration
12	12.96	(-)-Beta-necrodol	0.38
13	14.25	(1.α.(s*),5-β.)-1-(phenyl sulfinyl)-bicyclo-[3.3.1]-nona-3-one	4.59
14	15.22	(2R,3R)-spiro-2,3-dimethyl-1,4-dioxolane-5,1',3'-cyanocyclopentane	0.40
15	17.005	1-Adamantyl methyl ketone	1.25
16	20.17	3,5-dimethoxybenzoic acid	0.90
17	22.12	(+,-)-tetramisole	5.22
18	25.65	Cis,trans-2,3-dimethylthiochroman-4-carbonitrile	1.92
19	38.55	1,14-dibromotetradecane	0.02

The compound **(12**) occurring at retention time 12.96 was identified as (-)-beta-Necrodol with molecular mass of 154 and a 0.38% concentration of the total oil. The GC-MS spectrums of the oil extract showed two major peaks m/z 139 and m/z 121 which were also present in the GC-MS of the compound in the database library (figure 11). The molecular ion peak m/z 154

was observed in both spectra though the peaks were very small. The peak m/z 139 belonged to the fragment $[C_9H_{15}O]^+$ after the loss of a methyl group. The peak m/z 122 could be attributed to the fragment C_9H_{14} after the loss of CH_3OH while the peak m/z 121 belonged to the same fragment but after losing a proton. The structure and the mass spectra for (-)-beta. Necrodol are shown below.



12

(-)-Beta-necrodol

Beta-Necrodol is classified as a monoterpene since it has ten carbon atoms. Terpenes and specifically monoterpenes have been reported to have antifungal activities. Research reports have shown that monoterpenes like limonene, geranial, citral and neral isolated from various plants have antifungal activities against mycotoxigenic fungi and other fungi affecting plants (Matasyoh *et al.,* 2010; Saddig and Khayyat, 2010; Adejumo and Langenkamper, 2012). Though necrodol and some necrodol derivatives have been isolated from plants like *Lavandula luisieri* (Vallejo *et al.,* 1994) no antifungal activity of this compound have been reported. Necrodane compounds are also the major constituents of the defensive spray from rectal glands of a carrion beetle from Surinam but the reason for their presence in plants is not known (Baldovini *et al.,* 2005). Thus even though compound **(12)** is a monoterpene the antifungal activity of the oil extract may not be attributed to the presence of this compound in oil.

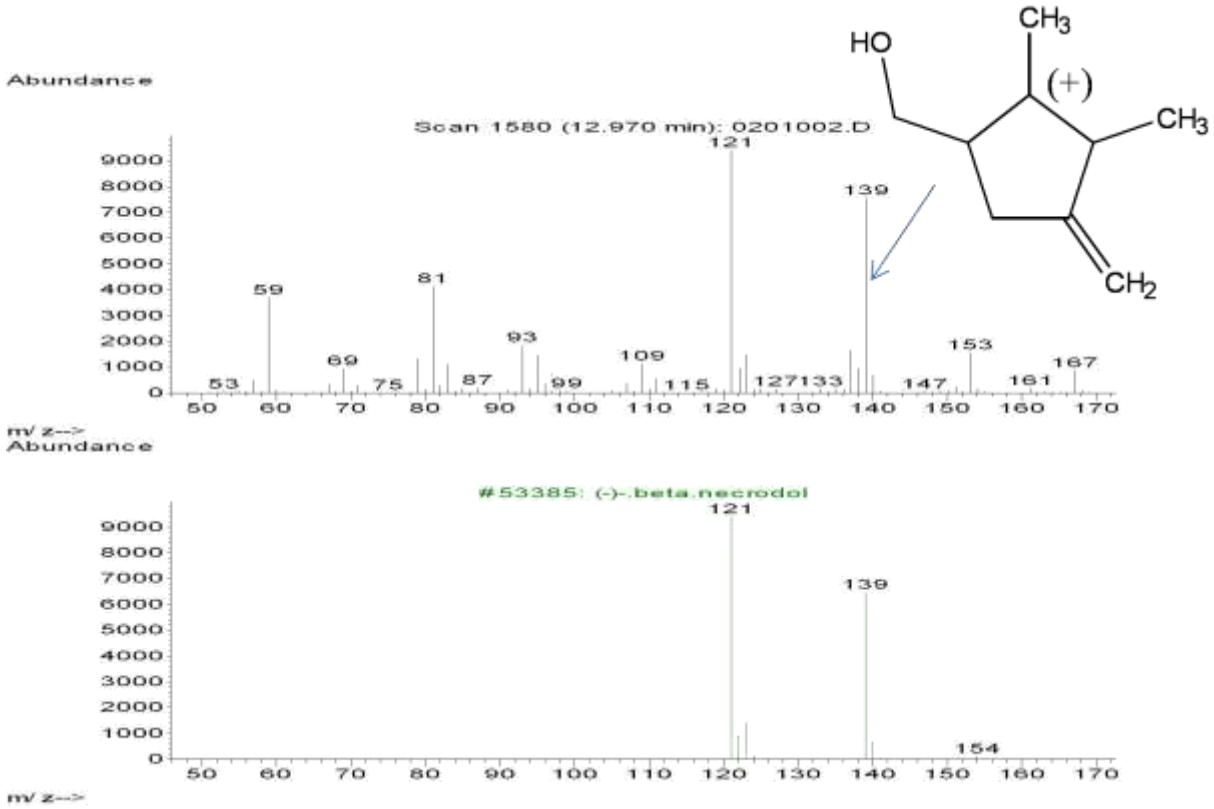
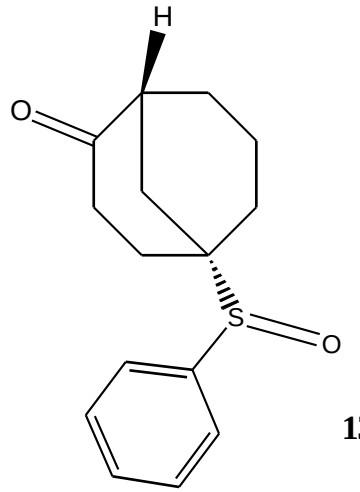


Figure 11: Mass spectra of beta- Necrodol from the oil spectra and the computer database

The compound **(13)** occurring at retention time14.25 was identified as [1.α.(s*),5-β.]-1-(phenyl sulfinyl)-bicyclo-[3.3.1]-nona-3-one with molecular weight of 262. Compound **(13)** had a concentration of 4.95% of the total oil. The spectra of the oil extract and that of the compound in the computer database (Figure 12) showed a peak at m/z 138 which could be attributed to the loss of the phenyl sulfinyl group and the peak m/z 137 could belong to $[C_9H_{13}O]^+$. Another peak was observed at m/z 81 corresponding to the fragment $[C_5H_5O]^+$ while the peak m/z 82 was attributed to the fragment C_5H_6O. The peak m/z 109 belonged to the fragment $[C_7H_9O]^+$. The antifungal activity of this compound could be attributed to the components making up the molecule. Compounds containing sulfinyl group have been reported to have antifungal activity against fungi like *F. oxysporum* (Weiming *et al*., 2011). Further, recent research has shown that imidazoles containing sulfinyl group have increased antifungal activity against *Candidas albicans* and *A. niger* (Prasanthy *et al*., 2011). Thus the presence of sulphenyl containing compound and imidazole in the oil could have contributed to the antifungal activity of the oil.



13

[1.alpha.(s*),5-beta.]-1-(phenyl sulfinyl)bicyclo[3.3.1]nona-3-one

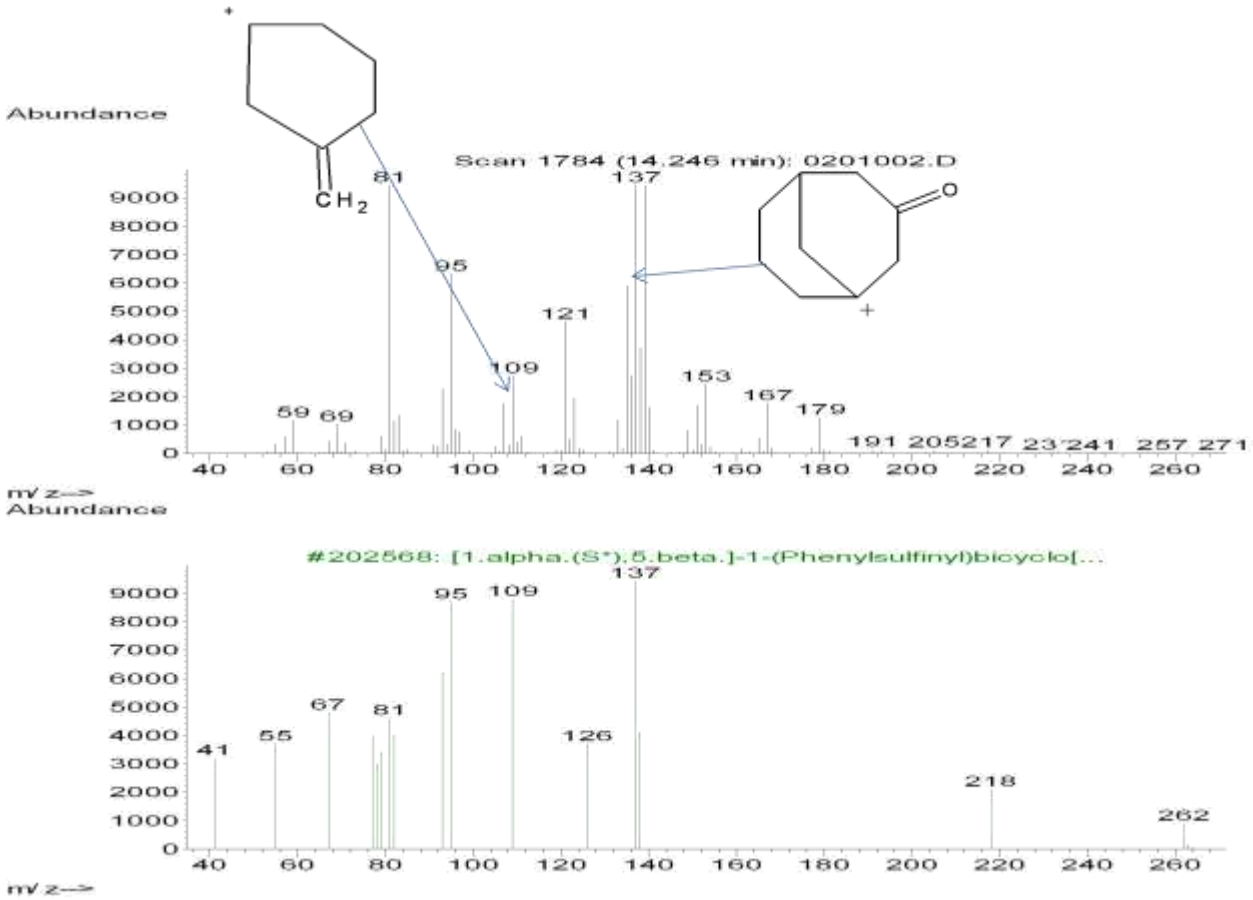
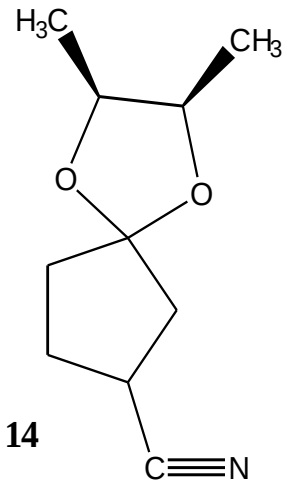


Figure 12: Mass spectra of [1.α.(s*),5-β.]-1-(phenyl-sulfinyl)bicycle [3.3.1]nona-3-one from the oil spectra and the computer database

The peak occurring at 15.22 was assigned to compound **(14)** and was identified as (2R, 3R)-Spiro-2,3-dimethyl-1,4-dioxolane-5,1',3'-cyanocyclopentane. Compound **(14)** had a concentration of 0.4% and molecular mass of 181. Both the spectra of this compound in the oil extract spectra and the computer database spectra (Figure 13) gave a molecular ion peak at m/z 181 and M+1 peak just next to it. The peak m/z 153 occurred after the loss of the two methyl groups in the dioxolane ring. Also the peak m/z 154 was attributed to the loss of the cyanide ion and a proton. The antifungal activity of the oil extract against the various fungal species could have resulted from the fact that the oil contains the dioxolane ring. This is because similar results have been reported in the past. According to Adel *et al.,* (2005) compounds which were isolated from actinomycetes species exhibit high antifungal activity against plant pathogens *F. oxysporum* and *P. digitatum*. Furthermore, oil extracted from leaves and stem of *Dieffenbachia picta* contained some dioxolane compounds which were active against the clinical *Candida albicans* and *F. oxsyporum* (Ganiyat *et al.,* 2011).



14

(2R, 3R)-spiro[2,3-dimethyl-1,4-dioxolane-5,1',3'-cyanocyclopentane]

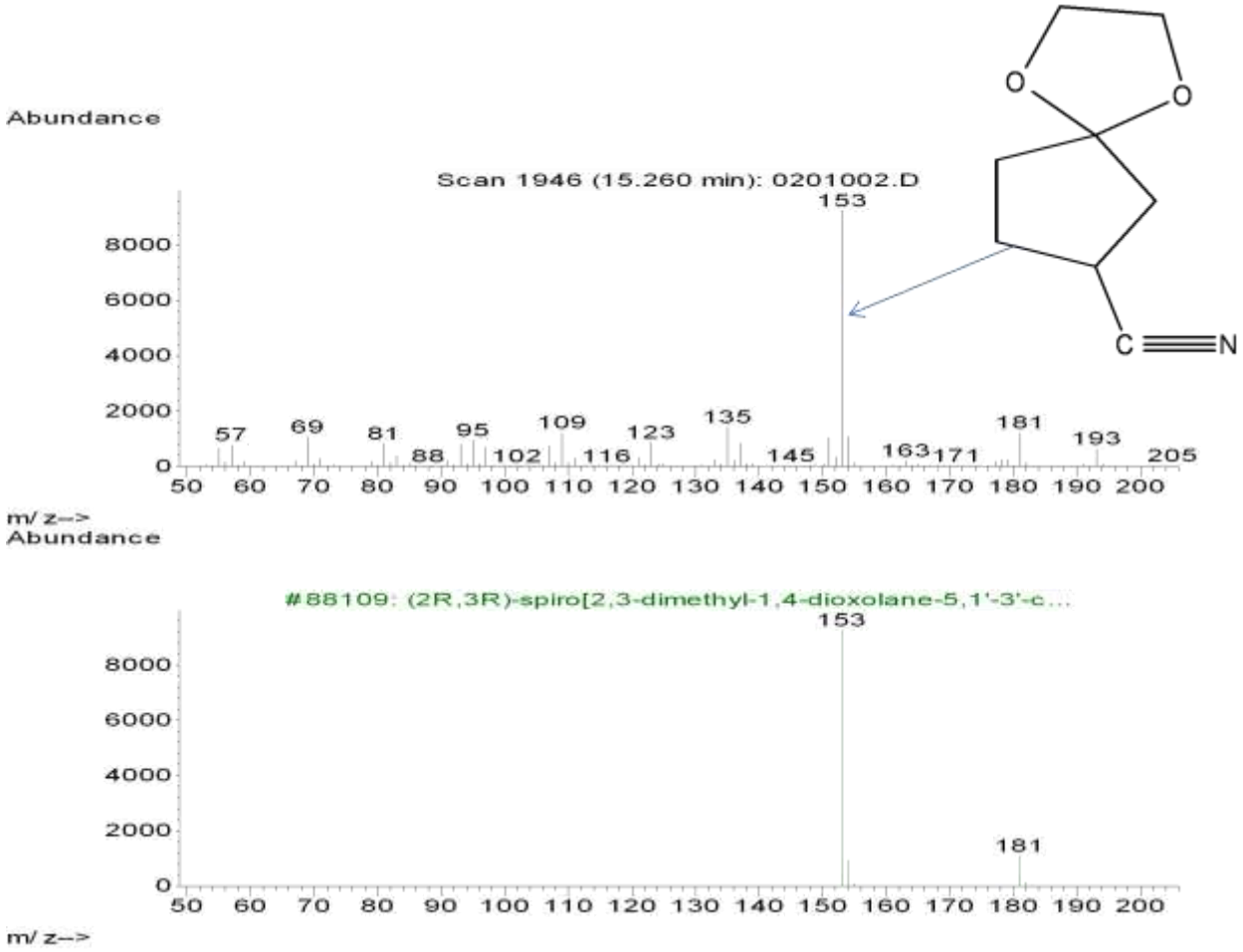
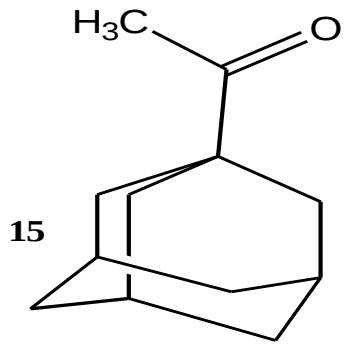


Figure 13: Mass spectra of (2R, 3R)-Spiro-2,3-dimethyl-1,4-dioxolane-5,1',3'-cyanocyclopentane from the oil spectra and the computer database

The compound (**15**) occurring at retention time 17.005 was identified as 1-Adamantyl methyl ketone. Compound (**15**) accounted for 1.25% of the total oil and molecular mass of 178. The oil spectra and the spectra of the compound in the database showed three major peaks (Figure 14). The peak occurring at m/z 135 resulted from the detachment of the isopropyl unit leaving the fragment $[C_{10}H_{15}]^+$ while the peak m/z 136 could have been obtained after the m/z 135 fragment gained a proton. Adamantane derivatives have long been known to possess antibacterial and antifungal activities. Its use in containing influenza started as early as 1946 (Krimmel, 1968). To date several adantane derivatives have been isolated from natural sources or synthesized. Potent antifungal activity has been reported for 2-(1-adamanty)-5-amino-1, 3, 4 -

diathiazole and is considered a typical fungal disinfectant (Papadaki- Valiraki *et al.,* 1993). From the plants oil extracted from the leaves of *Blumea balsamitera* was found to contain various adamantane derivatives (Nazrul *et al.,* 2009). According to recent research Adamantane compounds containing nitrogen and amino groups have been reported to inhibit *A. fumigatus* and *Candida glabrate* (Hemant *et al.,* 2011). Based on this past research the presence of this compound may have contributed to the fungal inhibition activity of the oil extract.



1-Adamantyl methyl ketone

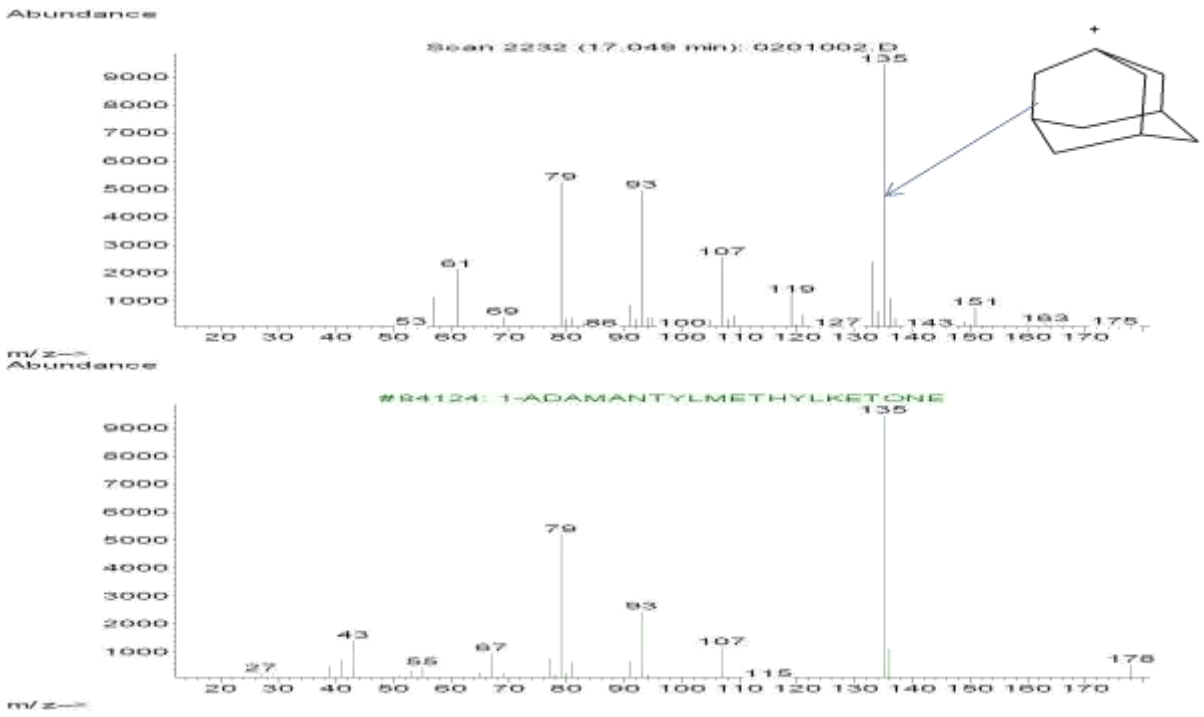
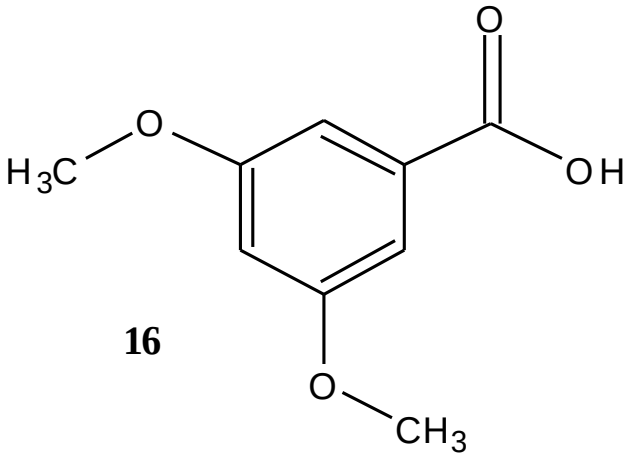


Figure 14: Mass spectra of 1-Adamantyl methyl ketone from the oil spectra and the computer database

The peak occurring at retention time 20.17 was assigned to compound **(16)** and was identified as 3, 5-dimethoxybenzoic acid with molecular mass of 182. Its spectra showed a molecular ion peak m/z 182 and M+1 peak just next to it and similar peaks were observed in the spectra of the compound in the computer library (Figure 15). The peak m/z 152 was observed and could be attributed to the loss of one methoxy while the peak m/z 122 could have resulted from the loss of the two methoxy groups. The peak observed at m/z 165 could have resulted from the loss of hydroxy group. The concentration of this compound in the oil was 0.90%.

It has been shown that natural compounds such as derivatives of benzoic acid can serve as potential alternative conventional antimicrobial agents (Beekrum *et al*., 2003). Benzoic acid has been shown to inhibit the growth of A. *flavus, A. fumigatus and A. terreus* and the activity of the acid increases with introduction of substituent like methoxy group (Jong *et al*., 2010). Further, 3, 4-dibenzoic acid is reported to have antifungal activity against *Botrytis cinerea* (a brown mold fungus) (Sofia-Vio *et al.*, 2012). According to Jong *et al*. (2010) the activity of the benzoic acid derivatives depends on the position of the substituent i.e. the further the substituent is from the carboxyl group the higher the activity of the compound. Therefore the antifungal activity of compound **(16)** could be because of the presence of the benzoic acid moiety in the molecule and the positioning of the substituents in the compound. Thus the inhibition activity of the oil against the various moulds species could be attributed to the presence of compound **(16)** in the oil.



16

3, 5-methoxybenzoic acid

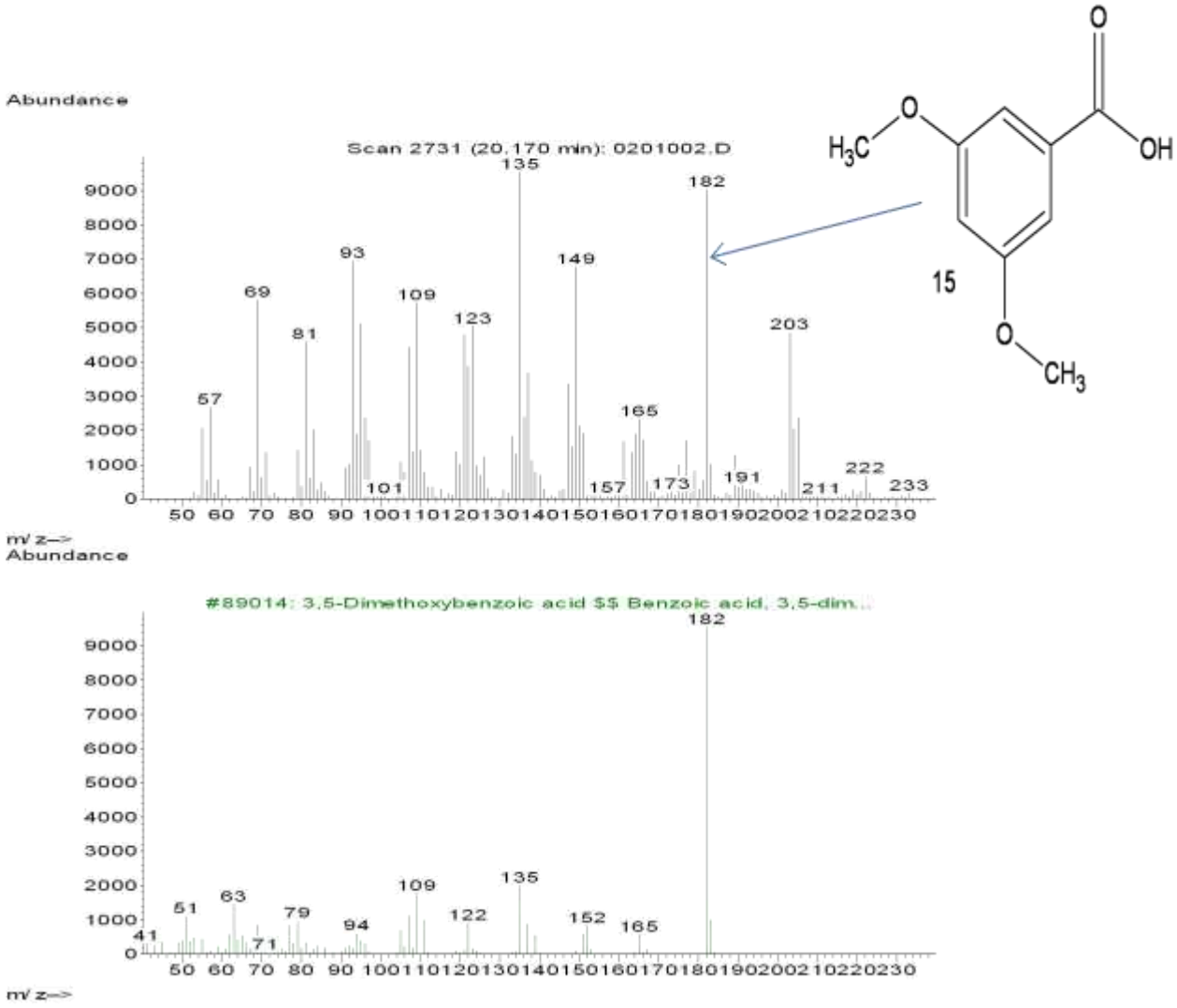
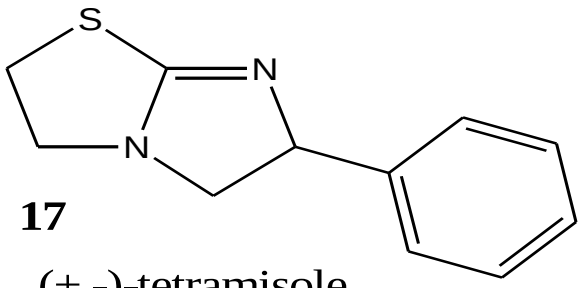


Figure 15: Mass spectra of 3, 5-dimethoxybenzoic acid from the oil spectra and the computer database

The peak occurring at 22.12 was assigned to compound **(17)** which had the highest concentration of 5.22% of the total oil. Compound **(17)** had molecular formula $C_{11}H_{12}N_2S$ and molecular mass of 204 and was identified as (+,-) - tetramisole. Some of the peaks which were observed in both the spectra of the compound in the oil and that of the compound in the computer library include: m/z 204, 203, 205 and 148 (Figure 16). The molecular ion peak m/z 204 was observed in the two spectra and the M+1 peak at m/z 205 was also observed. The peak m/z 203 belong to the fragment $[C_{11}H_{11}N_2S]^+$ after the loss of a proton. The fragment $[C_9H_{12}N_2]^+$showed a peak at m/z 148.

Imidazole is a planar five member hetero aromatic molecule with pyrrole type and pyridine type annular. Imidazole derivatives are widely used in antifungal chemotherapy because of their broad spectrum and availability (Zahra *et al*., 2011). It has been recognized that imidazole antifungals acts with at least two distinct mechanisms. One is the inhibition of ergosterol biosynthesis at low concentration which is responsible for fungistatic action. The other is physicochemical cell membrane damage exerted at high concentration which causes fungicidal effects (Beggs and Hughes, 1987). According to Zahra *et al*. (2011), 1, 2, 4-triazole and imidazole derivatives showed activity against the fungi *Candida albicans, Candida tropicalis* and *Candida parapeilosis* which infect humans. Also these compounds showed activity against pathogenic fungi *A. flavus* and *A. fumigatus.* Furthermore, compounds containing imidazo [2, 1-b] thiozole have been shown to have antifungal activities against *Candida tropicals* (Juspin *et al*., 2010). Imidazo [2, 1-b] thiazole derivatives have also been reported to inhibit *A. niger* (Amandeep *et al*., 2012). Apart from antifungal activities imidazoles have been shown to have anticancer, antiemetic activities (Huaiwei *et al*., 2012). Therefore the antifungal activity of the oil against the different mould species could be because of the presence of tetramizole in the oil.



17

(+,-)-tetramisole

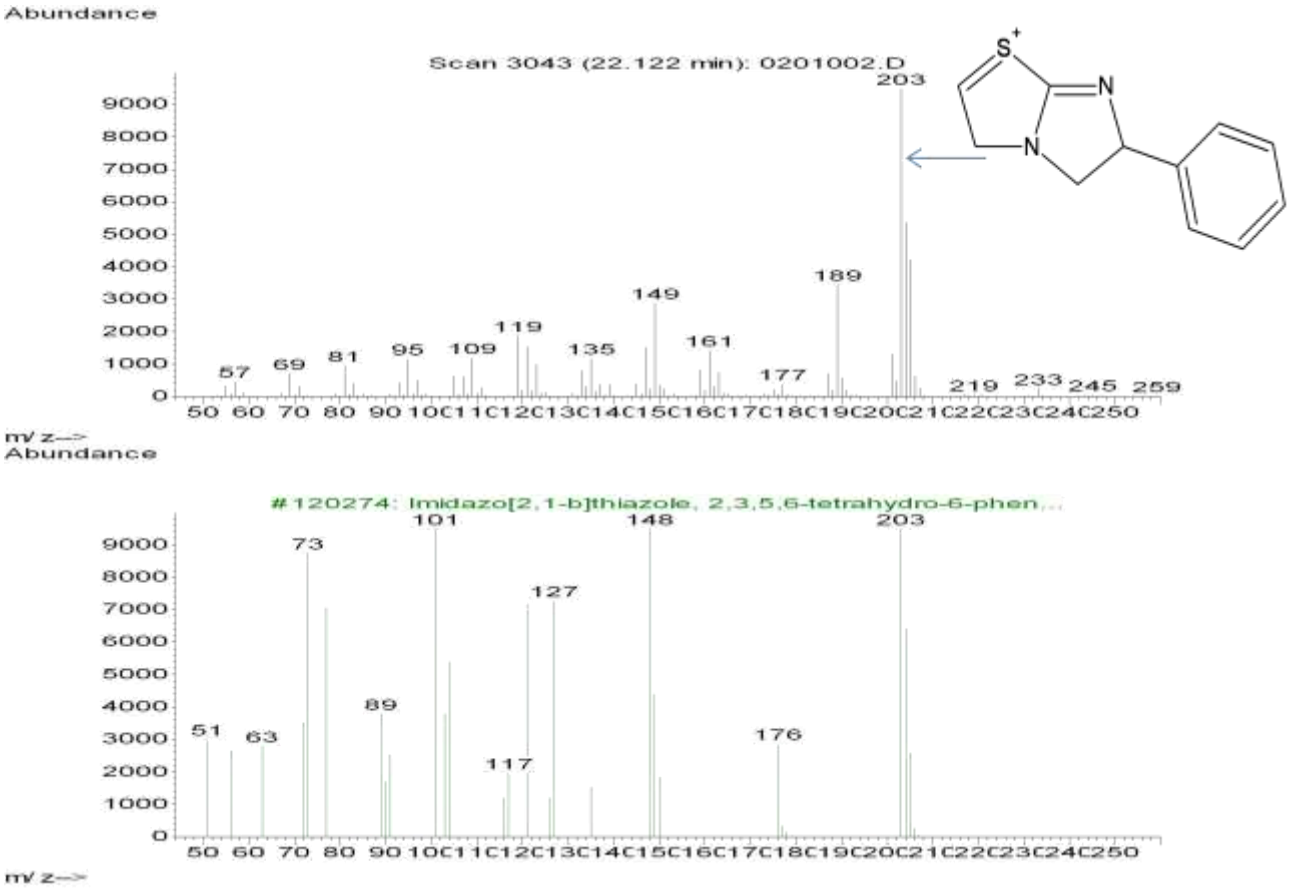
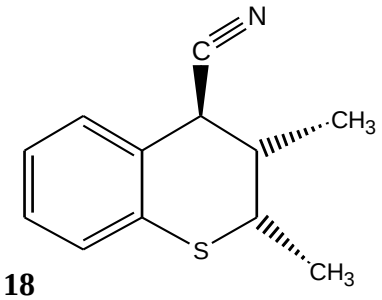


Figure 16: Mass spectra of (+,-)-tetramisole from the oil spectra and the computer database

The compound **(18)** which had a concentration of 1.92% of the total oil and a peak at retention time 25.65 was identified as Cis, trans-2, 3-dimethylthiochroman-4-carbonitrile. This compound has a molecular weight of 203. The computer library compound spectra showed five major peaks which were also observed in the spectra of compound **(18)** (Figure 17). The molecular mass ion peak m/z 203 was observed and the M+1 peak was also observed next to the molecular ion peak. The peak at m/z 173 was observed due to the loss of the cyanide ion. The peak m/z 147 could be attributed to the fragment $[C_{11}H_{15}]^{+\cdot}$

The presence of this compound in the oil could have contributed to the antifungal activity of the oil. This is because compounds containing carbonitriles have been reported to have antifungal activity. According to Wilamowski *et al*., (2001) compounds containing 2-carbonitriles showed activity against two plant pathogens *F.culmorum* and *P. expansum*. Further, high activity was recorded for carbonitriles with hydrogen atoms and short alkyl groups like methyl group at position 3 or 4. Carbonitriles with a methyl group in position 2 have also been reported to have

antifungal activity against the two pathogens above (Bartomiej *et al*., 2006). Therefore the presence of the carbonitrile moiety with a methyl group attached to it in the compound **(18)** which have been shown to have antifungal activity could have contributed to the activity of this particular compound and hence the oil extract. In addition to that carbonitrile fused with imidazoles have been reported to inhibit *F. expansum* (Madkour *et al*., 2011). The presence of compound **(18)** and imidazole in the oil extract could have contributed extensively to the antifungal activity of the oil.



18

Cis, tras-2, 3-dimethylthiochroman-4-carbonitrile

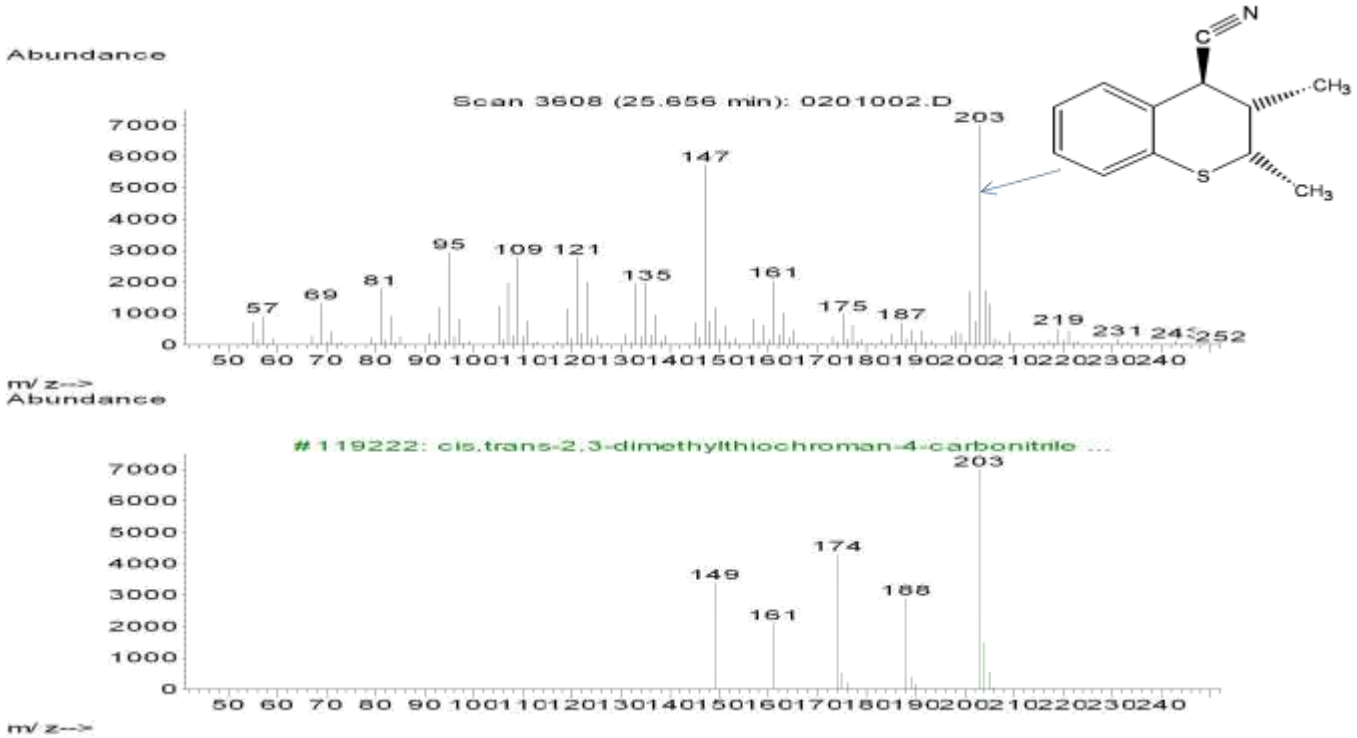
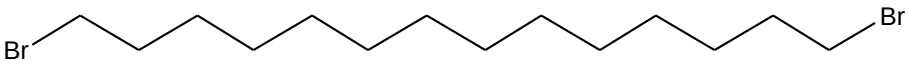


Figure 17: Mass spectra of Cis, tras-2, 3-dimethylthiochroman-4-carbonitrile from the oil spectra and the computer database

The compound **(19)** occurring at retention time 38.55 with molecular formula $C_{14}H_{28}Br_2$ and molecular mass of 356 was identified as 1, 14- dibromotetradecane. This compound had the least concentration of 0.02% of the total oil. The spectra of compound (18) in the oil and that in the library data base showed the molecular ion peak and the M+1 peak m/z 356 and 357 respectively (Figure 17). The peak at m/z 135 was attributed to the fragment C_4H_7Br. Compound **(19)** and in general alkyl halide are not known to show any antifungal activity and therefore the presence of this compound may not have contributed to the antifungal activity of the oil.



19

1, 14-Dibromotetradecane

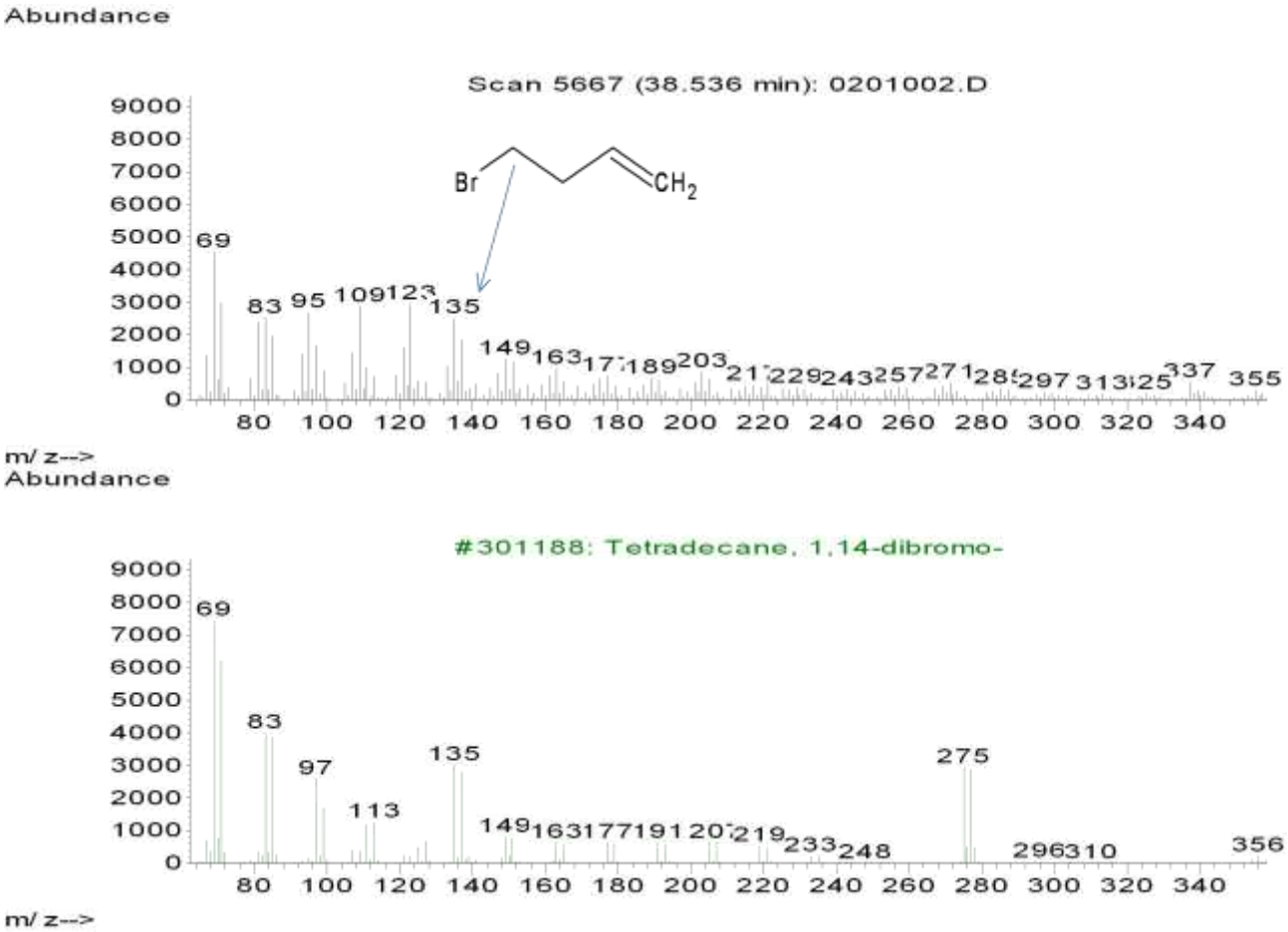


Figure 18 : Mass spectra of 1, 14- dibromotetradecane from the oil spectra and the computer database

Most of the compounds making up the oil contain sulphur atom in their structures. These compounds include compounds **13, 17** and **18**. Natural sulphur containing compounds are known to inhibit fungal growth. This is because sulphur containing natural compounds can be redox-active and inhibit the microbial growth by interfering with cellular redox homeostasis. Thus the presence of these sulphur containing compounds in the oil could have contributed to the antifungal activity of the oil observed against various fungal species (Jacob, 2006).

4.6 Screening of non volatile compounds and bioassay fractionation

The crude extract was fractionated by column chromatography using silica gel 70-200 mesh as the stationary phase and the 9:1 chloroform ethyl acetate mixture as the mobile phase. Fractions collected were sported on TLC plates and fractions with the same compounds were pooled together. Five major fractions were obtained which are S_1, S_2, S_3, S_4 and S_5 (Figure 18).

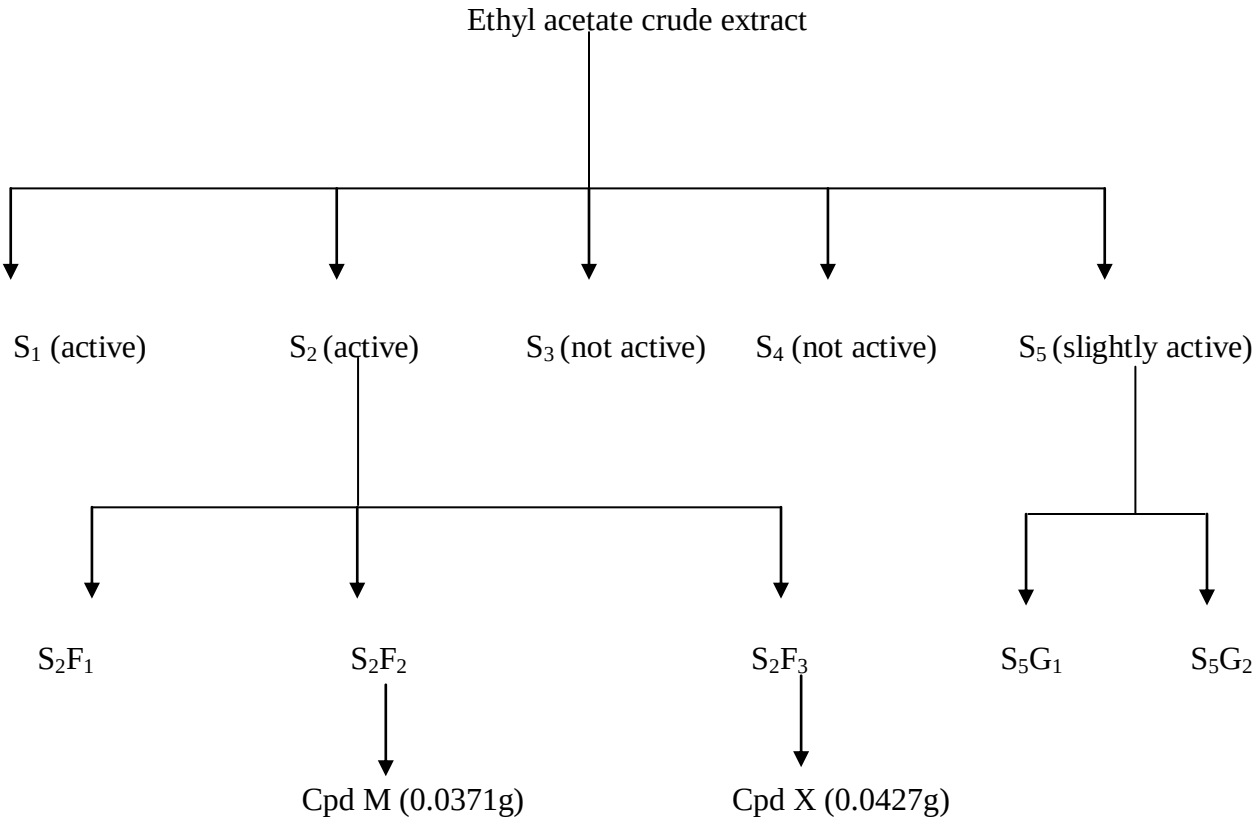


Figure 19: Schematic diagram showing fractionation and purification of various fractions

The crude extract (methanol extract, ethyl acetate) extract and all the fractions were screened for their antimicrobial activities against the *Aspergillus* species, *Fusarium* species and

Penicillium species. For all the fractions the samples concentration used was 2 mg/ml. Both methanol and ethyl acetate extracts were active against all fungi screened except *F. proliferatum* and *P. rugulosum*. Though fraction S_1 was found to be active against most of the fungi species tested, it was not purified further because it consisted mainly of non polar oily compounds which could have been captured in the non volatile compounds. This fraction was weighing 20.06g. Fractions S_3 and S_4 which were weighing 15.25g and 8.05g respectively were left out because they were not active against most of the fungi species tested.

Fraction S_2 which was weighing 27.40g was quite active against all of the fungi apart from *F. proliferatum* was further purified by repetitive column chromatography using the 9:1 solvent system. The fractions were also sported on TLC plates and fractions with similar compounds were pooled together and three fractions were obtained i.e. S_2F_1, S_2F_2 and S_2F_3. From the TLC fraction S_2F_1 was found to be similar to fraction S_1 and was combined with it. Fraction S_2F_2 and S_2F_3 were all active against most of the fungi screened. Fractions S_2F_2 and S_2F_3 were weighing 2.5 g and 3.5 g respectively. Even after passing through the column several times the two fractions were not pure and PTLC were used to further purify these fractions. These fractions were applied on the PTLC plates and developed using the 9:1 chloroform ethyl acetate solvent mixtures. After the solvent front was reached the plates were removed and allowed to dry. The compounds were then scrubbed from the plates based on the separation patterns and the compounds extracted from the silica gel using ethyl acetate. The compound from fraction S_2F_2 which was weighing 0.078g was labeled as compound M. The compound labeled X which had a yield of 0.083g was obtained from fraction S_2F_3. Both compounds were absorbing at 254 nm and 365 nm in the U.V region where purple sports were observed on the TLC plates. Compound X was showing under the U.V two overlapping purple sports. Based on the methods used the two compounds in X could not be separated completely. Fraction S_5 was also purified by repetitive column chromatography and two major fractions were obtained which are S_5G_1 and S_5G_2.These fractions did not undergo further purification due to the complexities of the TLC pattern.

4.7 Screening of crude extracts and fractions for antifungal activity

4.7.1 Screening of crude extracts and fractions against *Aspergillus* species

The crude extracts were active against all the *Aspergillus* species tested. The methanol fraction was more active against *A. niger, A. ochraceus, A. fumigatus* and *A. flavus* with inhibition zones of 11.33 mm, 11.67 10.67 mm and 10.00 mm respectively (Table 11). Ethyl acetate fraction was more active against the four species above with inhibition zones 11.67 mm, 11.00 mm, 11.83 mm and 11.67 mm being recorded. The lowest activity of the fractions was that against *A. flavipes* where inhibition zones of 9.33 mm was observed for the two fractions. Fraction S_1 was active against all the *Aspergillus* species screened. The highest activity of this fraction was that against *A. flavipes* with inhibition zone of 14.00 mm. *Aspergillus niger* and *A. fumigatus* were showing inhibition zone of 10.67 mm when this fraction was used. The highest resistance to S_1 was exhibited by *A. flavus* with inhibition zone of 9.30 mm. Fraction S_2 was also active against all the Aspergillus species screened. *Aspegillus niger* and *A. ochraceus* had inhibition zones of 14. 33 mm and 12.33 mm respectively being recorded. Fraction S_2 also inhibited the growth of *A. fumigatus*, *A. flavipes* and *A. flavus* where inhibition zones of 10.00 mm, 11. 00 mm and 10.50 mm respectively were obtained. Fraction S_3 and S_4 were not active against all the Aspergillus species tested. Fraction S_5 was showing high activity against *A. niger* and *A. flavipes* with inhibition zones of 11.37 mm and 10.00 mm respectively. Low activity was observed against *A. ochraceus*, *A.fumigatus* and *A. flavus* with inhibition zones less than 10.00 mm.

4.7.2 Screening of crude extracts and fractions against Fusarium species

The extracts were active against all the *Fusarium* species screened accept F. proliferatum. The highest activity of this extracts was exhibited by *F. avenacium* and *F. moniliforme* with inhibition zones 14.50 mm, 14.00 mm, 15.33 mm and 15.00 mm (Table 12). *Fusarium subglutinans* and *F. culmorum* had inhibition zones greater than 10.00 mm. Fraction S1 was active against not active against *F. proliferatum* and *F. moniliforme*. The highest activity of S_1 was observed against *F. culmorum* with inhibition zone of 14.33 mm. *Fusarium avenacium* was showing inhibition zone of 10.10 mm. The highest resistance to fraction S_1 was exhibited by *F. subglutinans* with inhibition zone of 9.93 mm which was the lowest.

Table 11: Screening results of crude extract and major fractions against *Aspergillus* species

Fungi /fraction	**Methanol extract**	**Ethyl acetate extract**	**S_1**	**S_2**	**S_3**	**S_4**	**S_5**
A .niger	11.33 ± 1.15	11.67 ± 1.53	10.67 ± 0.58	14.33 ± 0.58	0.00 ± 0.00	0.00 ±0.00	11.37 ± 0.67
A .ochraceus	11.67 ± 1.53	11.00 ± 1.00	6.17 ± 0.29	12.33 ± 1.53	0.00 ±0.00	0.00 ±0.00	9.67 ± 0.58
A .fumigatus	10.67 ± 0.58	11.83 ± 1.26	10.67 ± 0.58	10.00 ± 0.00	0.00 ±0.00	0.00 ±0.00	7.33 ± 0. 1.57
A .flavipes	9.33 ± 0.58	9.93 ± 0.12	14.00 ± 1.00	11.00 ± 0.00	0.00 ±0.00	0.00 ±0.00	10.00 ± 0.00
A .flavus	10.00 ± 0.00	11.67 ± 1.53	9.30 ± 0.52	10.50 ± 0.44	0.00 ±0.00	0.00 ±0.00	7.50 ± 0.87

Table 12: Screening results of crude extract and major fractions against *Fusarium* species

Fungi /fraction	**Methanol extract**	**Ethyl acetate extract**	**S_1**	**S_2**	**S_3**	**S_4**	**S_5**
F. avenacium	14.50 ± 0.50	14.00 ± 1.00	10.10 ± 0.85	12.83 ± 0.76	0.00 ± 0.00	11.67 ± 0.58	12.57 ±0.51
F. culmorum	11.37 ± 0.64	11.67 ± 0.58	14.83 ± 0.29	12.63 ± 0.55	0.00 ± 0.00	11.67 ± 0.58	12.83 ± 0.76
F. subglutinans	11.67 ± 1.53	12.50 ± 0.50	9.93 ± 0.12	14.00 ± 1.00	0.00 ± 0.00	9.50 ± 0.50	12.33± 1.15
F. proliferatum	0.00 ±0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
F. moniliforme	15.33± 0.58	15.00 ± 1.00	0.00 ± 0.00	8.83 ± 1.29	13.00 ± 1.00	15.67 ± 1.15	12.53 ± 0.50

Fraction S_2 was showing the lowest activity against *F. subglutinans* with inhibition zone of 14.00 mm. *Fusarium avenacium* and *F. culmorum* had inhibition zones of 12.83 mm and 12.63 mm respectively. Lowest activity was observed against F. moniliforme with inhibition zone of 8.83 mm. Fraction S_3 inhibited only *F. moniliforme* with inhibition zone of 13.00 mm being recorded. Fraction S_4 was active against all the *Fusarium* species tested except *F. proliferatum*. The highest activity of this fraction was shown by *F. moniliforme* with inhibition zone of 15.67 mm. *Fusarium avenacium* and *F. culmorum* had inhibition zone of 11.67 while *F. subglutinans* had inhibition zone of 9.50 mm. Fraction S5 was also active against all the *Fusarium* species tested except *F. proliferatum*. Inhibition zones of 12.57 mm, 12.83 mm, 12.33 mm and 12.53 mm were exhibited by *F. avenacium*, *F. culmorum*, *F.subglutinans* and *F. moniliforme* respectively when S_5 was used.

4.7.3 Screening of crude extracts and fractions against *Penicillium* species

The crude extracts and S_1 were active against all *Penicillium* species screened except *P. rugulosum*. The highest activities of the crude extracts were exhibited by *P. islandicum* and *P. purporogenum* with inhibition zones of 12.67 mm, 11.33 mm, 12.00 mm and 11.67 mm. (Table 13). The lowest activity was observed against *P. viridicatum* and *P. wortmani* with inhibition zones of 9.50 mm and 6.17 mm respectively. For S_1, high activity was observed against *P. islandicum* and *P. purporogenum* with inhibition zones of 10.83 mm and 10.67 mm respectively. Low activity was observed against *P. viridicatum*, *P. rugulosum* and *P. wortmani* with inhibition zones of 8.67mm, 7.47 mm and 6.83 mm being recorded.

Fraction S_2 was also active against all the *Penicillium* species tested. The lowest resistance was exhibited by *P. islandicum* and *P. purporogenum* with inhibition zones of 13.67 mm and 14.33 mm respectively. *Penicillium viridicatum* and *P. rugulosum* had inhibition zones of 11.00 mm and 10.00 mm respectively. Lowest activity was observed against *P. wortmani* with inhibition zone of 7.83 mm. Fraction S3 was active against only *P. islandicum* and *P. purporogenum* where inhibition zones of 12.33 mm and 12.00 mm were observed. Fraction S4 was not active against all the Penicillium species screened. Fraction S5 was exhibiting high activity against *P. islandicum* and *P. purporogenum* with inhibition zones of 11.00 mm and 11. 50 mm respectively. Low activity of this fraction was observed against *P. viridicatum*, *P. rugulosum* and *P. wortmani* with inhibition zones less than 10.00 mm being observed.

Table 13: Screening results of crude extract and major fractions against *Penicillium* species

Fungi /fraction	**Methanol extract**	**Ethyl acetate extract**	**S_1**	**S_2**	**S_3**	**S_4**	**S_5**
P. islandicum	12.67 ± 0.58	12.00 ± 1.00	10.83 ± 1.26	13.17 ± 1.04	12.33 ± 1.53	0.00 ± 0.00	11.0 ±1.00
P. purpurogenium	11.33 ± 1.15	11.67 ± 1.53	10.67 ± 0.58	14.33 ± 0.58	12.00 ± 1.73	0.00 ± 0.00	11.50 ± 0.50
P. viridicatum	9.50 ± 0.87	10.50 ± 0.44	8.67 ± 0.58	11.00 ± 1.00	0.00 ± 0.00	0.00 ± 0.00	6.30 ± 0.52
P. rugulosum	0.00 ±0.00	0.00 ± 0.00	7.47 ± 0.9	10.00 ± 1.00	0.00 ± 0.00	0.00 ± 0.00	6.17 ± 0.29
P. wortmani	6.17 ± 0.29	6.4 ± 0.26	6.83 ± 0.29	7.83 ± 1.26	0.00 ± 0.00	0.00 ± 0.00	6.33 ± 0.55

4.8 Screening of the purified compounds for antifungal activity

Table 14: Screening for antifungal activity of non volatile purified compounds

SPECIES/SAMPLE	**Compound X**		**Compound M**	
(a). Aspergillus species	**Inhibition zones (mm)**	**Inference**	**Inhibition zones (mm)**	**Inference**
A. niger	15.33 ± 0.58	Active	0.00 ± 0.00	Inactive
A. ochraceus	20.00 ± 1.00	Active	10.00 ± 0.50	Active
A. fumigators	14.33 ± 0.58	Active	0.00 ± 0.00	Inactive
A. flavipes	11.50 ± 0.50	Active	0.00 ± 0.00	Inactive
A. flavus	12.67 ± 1.50	Active	13.67 ± 0.58	Active
(b*). Fusarium species*				
F. moniliforme	0.00 ± 0.00	Inactive	0.00 ± 0.00	Inactive
F. culmorum	10.17 ±0.29	Active	0.00 ± 0.00	Inactive
F. avenaceum	13.50 ± 0.50	Active	13.67 ± 0.58	Active
F. proliferatum	14.17 ± 0.76	Active	0.00 ± 0.00	Inactive
F. subglutinans	12.33 ± 0.58	Active	0.00 ± 0.00	Inactive
(c). *Penicillium species*				
P. purporogenum	13.67 ± 0.58	Active	11.00 ± 1.00	Active
P. islandicum	12.50 ± 0.50	Active	0.00 ± 0.00	Inactive
P. rugulosum	0.00 ± 0.00	Inactive	10.33 ± 0.58	Active
P.wortmanni	0.00 ± 0.00	Inactive	0.00 ± 0.00	Inactive
P. viriidicatum	0.00 ± 0.00	Inactive	0.00 ± 0.00	Inactive

Compound X was the most active against all the fungal species screened. The sample concentration used for this screenings was 8g/ml. This sample was active against all *Aspergillus* species tested. The highest activity of this compound was that against *A. ochraceus* where an inhibition zone of 20.00 mm was recorded. Inhibition zones of 15.33 mm and 14.33 mm were observed for *A. niger* and *A. fumigatus* respectively implying that compound X was quite active against these species (Table 11). The Compound showed lower activity against *A. flavus* and *A. flavipes* where inhibition zones of 12.67 mm and 11.50 mm were observed for the two species respectively.

Compound X was active against all the *Fusarium* species screened except for *F. moniliforme.* As indicated by the inhibition zones this sample was not as active against the *Fusarium* species as compared to *Aspergillus* species. The highest activity of compound X against the *Fusarium* species was that against *F. proliferatum* where an inhibition zone of 14.17 mm was observed. *F. avenaceum* and *F. subglutinans* were inhibited by this sample and the inhibition zones recorded were 13.50 mm and 12.33 mm respectively. The highest resistance to compound X by the *Fusarium* species was that observed for *F. culmorum* where an inhibition zone of 10.17 mm was observed. Lowest activity of this compound was recorded against the *Penicillium* species where only two species were inhibited by the sample. The two species inhibited were *P. purpurogenum* and *P. islandicum* with inhibition zones of 13.70 mm and 12.5mm respectively.

Compound M on the other hand was not very active against most of the fungi screened. For the *Aspegillus* species Compound M was active against only *A. flavus* and *A. ochraceus* where inhibition zones of 13.67 and 10.00 mm were observed respectively. The highest resistance to this compound was observed among the *Fusarium* species as only *F. avenaceum* was inhibited by this compound with an inhibition zone of 13.67 mm. *Penicillium purpurogenium* and *P. rugulosum* were the only species inhibited in the *Penicillium* genus with inhibition zones of 11.00 mm and 10.33 mm respectively. The low values of inhibition zones for *P. purpurogenium* and *P. rugulosum* indicated the low activity of compounds M.

4.9 Determination of MIC for compound X

Table 15: Inhibition zones of *Aspergillus, Fusarium* and *Penicillium* species at different concentrations of compound X

Fungi	**Concentration of compound X (mg/ml)**					**(-)control**	**MIC (mg/ml)**
	8.00	6.00	4.00	2.00	1.00		
A .niger	15.00 ± 0.58	12.00 ± 1.00	8.33 ± 0.58	0.00 ± 0.00	0.00 ± 0.00	0.00	2.00
A .ochraceus	20.17 ± 0.76	14.33 ± 0.58	10.67 ±0.58	7.00 ± 1.00	0.00 ± 0.00	0.00	1.00
A .fumigators	14.33 ± 0.58	11.00 ± 1.00	7.50 ± 0.50	0.00 ± 0.00	0.00 ± 0.00	0.00	2.00
A .flavipes	11.17 ± 0.29	9.67 ± 0.58	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00	4.00
A .flavus	13.50 ± 0.50	9.17 ± 0.76	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00	4.00
F. culmorum	10.17 ± 0.29	6.770 ± 0. 93	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00	4.00
F. avenaceum	13.50 ± 0.50	7.45 ± 0.50	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00	4.00
F. proliferatum	13.83 ± 1.04	9.6 ± 0.53	6.33 ± 0.57	0.00 ± 0.00	0.00 ± 0.00	0.00	2.00
F. subglutinans	12.33 ± 0.58	7.50 ± 0.50	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00	4.00
P. purporogenum	13.17 ± 0.76	9.00 ± 1.00	6.17 ± 0.29	0.00 ± 0.00	0.00 ± 0.00	0.00	2.00
P. islandicum	12.33 ± 0.58	9.83 ± 0.76	6.33 ± 0.29	0.00 ± 0.00	0.00 ± 0.00	0.00	2.00

The dilutions for MIC was done using the formula $M_1V_1 = M_2V_2$. The stock solution used for compound X was 8 mg/ml. Methanol was used as the control since it was used to make the dilutions. Compound X was the most active among the three compounds because it inhibited the growth of most of the fungi tested. The highest activity of the compound X was that against *Aspergillus* species. The highest oil activity was observed against *A. ochraceus*, with inhibition zone of 20.17 mm when 8 mg/ml of the compound was used and the lowest MIC of 1 mg/ml (Table 12). Also among the *Aspergillus* species high activity of this compound was that against *A. niger* and *A. fumigatus* where an MIC of 2 mg/ml was obtained for the two species even though they had different inhibition zones of 15.00 mm and 14.33 mm respectively. The highest resistance to compound X was shown by *A. flavus* and *A. flavipes* where an MIC of 4 mg/ml was recorded. The two species showed inhibition zones of 13.50 mm and 11.17 mm respectively. Activity of compound X was proportional to the concentration since the compound's activity against all the fungi tested was decreasing with decrease in concentration. Also since lower MIC implies higher activity of the compound, compound X was more active against the *Aspergillus* species compared to *Fusarium* and *Penicillium* species.

For the *Fusarium* species the highest activity of compound X was recorded for *F. proliferatum* with inhibition zone of 13.83 mm and an MIC of 2 mg/ml. Though *F. avenacium, F. culmorum* and *F. subglutinans* had the same MIC of 4 mg /ml the three had different inhibition zones which were 13.50 mm, 10.17 mm and 12.73 mm respectively when sample with concentration of 8 mg/ml was used. Though *F. avenacium, F. culmorum* and *F. subglutinans* had almost the same inhibition zones as that of *F. proliferatum* their MIC of 4 mg/ml showed a decrease in the compound activity against the three species. The lowest activity of compound X was against *Penicillium* species since it was active against only two species. However, the compound was quite active against the *P. purpurogenum* and *P. islandicum* where an MIC of 2 mg/ml was recorded. Thus the activity of the compound X against these two species was higher than those of *Aspergillus* and *Fusarium* species with MIC of 4 mg/ml.

4.10 Determination of MIC for compound M

Compound M was active against only five fungal species. The *Aspergillus* species inhibited by this compound were *A. flavus* and *A. ochraceus* where inhibition zones of 10.17 mm and 13.67 mm were observed for the two species respectively. The compound M was more

active against *A. flavus* since the lowest MIC of 2 mg/ml was recorded (Table 13). MIC of 4 mg/ml was obtained for compound M against *A. ochraceus. Fusarium avenaceum* was the only *Fusarium* species inhibited by compound M and this species showed an inhibition zone of 12.33 mm and MIC of 4 mg/ml. Compound M had activity against *P. purporogenum* and *P. rugulosum* where inhibition zones of 11.00 mm and 10.33 mm respectively were recorded. For both species an MIC of 4 mg/ml was recorded. Compound M was less active compared to compound X. Since most fungi species which were inhibited by compound X were not inhibited by compound M. Also for the species which were inhibited by the two compound higher inhibition zones were observed for compound X compared to M. As an example the inhibition zone of 20.17 mm was recorded for *A. ochraceus* when 8 mg/ml of Compound X was used but when the same concentration of Compound M was used the inhibition zone recorded was 10.17 mm. *Penicillium rugulosum* however, was inhibited by compound M and no activity was observed when compound X was used.

Table 16: Inhibition zones of *Aspergillus, Fusarium* and *Penicillium* species at different concentrations of compound M

Fungi	**Concentrations of compound M (mg/ml)**					**(-) control**	**MIC (mg/ml)**
	8.00	6.00	4.00	2.00	1.00		
A. ochraceus	10.17 ± 0.29	6.33 ± 0.58	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00	4.00
A. flavus	13.67 ± 0.58	11.50 ± 0.50	6.33 ± 0.58	0.00 ± 0.00	0.00 ± 0.00	0.00	2.00
F. avenaceum	12.33 ± 0.0.57	7.50 ± 0.50	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00	4.00
P. purporogenum	11.00 ± 1.00	7.17 ± 0.76	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00	4.00
P. rugulosum	10.33 ± 0.58	6.83 ± 0.76	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00	4.00

4.11 Structure elucidation for compound X

Analysis of the NMR spectra of compound X indicated that it consist of two closely related compounds designated as X_1 (**20**) and X_2 (**21**). When the sample containing these compounds was spotted on TLC two purple spots were overlapping suggesting the presence of two closely related compounds. The ^{13} C NMR spectrum of the same sample showed a lot of peak splitting and overlapping peaks. From the ^{1}H NMR spectrum there were 14 aromatic protons and one methoxy group. This implies that the difference between the two compounds was a methoxy group.

Compound (**20**) had 16 carbons, 14 hydrogen and 4 oxygen and therefore molecular formula $C_{16}H_{14}O_4$. The NMR data for this particular compound is shown in table 14.

Table 17: NMR information of compound (**20**)

Position	**δ^{13} C (ppm)**	**DEPT**	**δ^{1}H/HSQC (ppm)**	**J (Hz)**	**HMBC**	**COSY**
2	141.70	Q				
3	144.50	Q				
4	37.53	CH_2	2.92 (s)		3, 2	
5	106.11	CH	6.65 (d)	9.79	8, 6, 9, 7	6
6	104.71	CH	6.66 (d)	9.79	8, 5, 7	5
7	161.08	Q				
8	101.11	CH	6.38 (s)		5, 6, 9, 7	
9	157.21	Q				
10	-	Q				
1’	137.12	Q				
2’	126.61	CH	7.52 (d)	7.53	1’, 3’, 4’, 6’	3’
3’	128.70	CH	7.38 (t)	7.53	2’, 6’, 4’, 1’	2’
4’	129.31	CH	7.06 (d d)	16.32,16.30	2’, 3’, 1’, 5’	
5’	139.61	Q				
6’	127.76	CH	7.29 (s)		2’,3’, 1’, 2	
OCH_3	55.25	CH_3	3.82 (s)		7	

The ^{13}C NMR spectrum of **20** showed 16 signals. Seven aromatic CH signals at δ 101.11, δ 104.71, δ 106.11, δ 126.61, δ 127.76, δ128.70 and δ 129.31 were observed. A total of seven quaternary carbons at δ 137.12, δ 139.61, δ 141.70, δ 144.50, δ 157.21 and 161.08 were obtained. One signal for sp^3 carbon was observed at δ 55.25 and a signal also for a sp^2 carbon at δ 37.53. From the ^1H NMR seven signals for aromatic protons were observed. Two singlet's at δ 6.38 and δ 7.29 and three doublets at δ 6.66, δ 6.65 and δ 7.52, also a triplet was observed at δ 7.38. Also a doublet of doublets signal was observed at δ 7.06. Two more singlets were observed at δ 3.82 and δ 2.92 for methoxy group and methylene group respectively.

Carbon atoms absorbing at δ 141.70 and 144.70 were assigned to C-2 and C-3 respectively and C-3 was hydroxylated. Flavonoids hydroxylated at C-3 have been isolated from different plants (Ponce *et al*., 2004; Malgorzata and Irena, 2005). C-4 was assigned the carbon atom absorbing at δ 37.53. The carbon atoms absorbing at δ 106.11, 104.71, 161.08, 101.11 and 157.21 were allocated to the carbons in ring A. Carbon atoms δ 106.11 and δ 104.71 were assigned to C-5 and C-6 respectively. The quaternary carbon absorbing at δ 161.08 was allocated to C-7 with a methoxy group attached to it. The methoxy group carbon was absorbing at δ 55.25. C-8 and C-9 were assigned the carbon atoms 101.11 and 157.21 respectively. Closely related compound to this have been isolated from *Dracaena cochinchinensis* and *Salix matsudana* (Zheng *et al.,* 2006; Xiang *et al.,* 2008). Also ^{13}C NMR values for C-6 to C-9 which were almost similar to the one obtained in this research have been reported (Zheng *et al.,* 2006; Bennardi *et al*., 2008). Two quaternary carbon and four CH carbons were allocated to the ring B carbon atoms. C-1' and 5' were assigned the quaternary carbons absorbing at δ 137.12 and δ 139.61 respectively. C-2', C-3', C-4' and C-6' were allocated the carbon atoms absorbing at δ 126.61, 128.70, 129.31 and 127.76 respectively. Signal for C-10 was not observed. This peak is normally very small and may not be observed in the ^{13} C NMR.

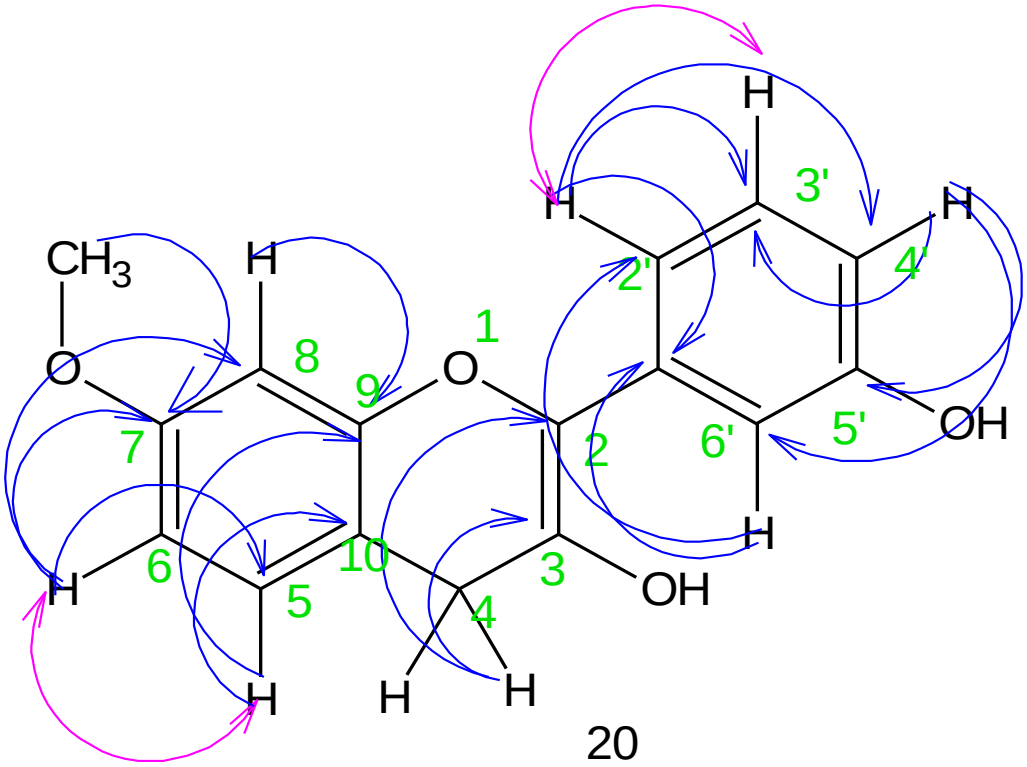
The HSQC spectrum gave clear correlations of the carbon atoms with the protons directly attached to them. From the spectrum there was a correlation between C-4 and the proton δ 2.92, proton δ 3.82 was correlating with the methoxy group carbon. C-5, C-6 and C-8 were correlating with protons δ 6.65, δ 6.66 and δ 6.38 respectively. Further, correlation was observed between C-2', C-3' and C-4' and the protons δ 7.52, 7.38 and 7.06 respectively. The singlet at δ 7.29 was correlating with C-6'. The carbon/proton HMBC correlations were also observed. HMBC

spectrum shows protons correlation with carbon atoms which are two or three bonds away. This helps to know the carbons atoms which are next to each other and those which are two bonds away from each other.

From the HMBC spectrum the proton H-4 absorbing at δ 2.92 showed correlation with carbons C-2 and C-3 which were quaternary carbons. Proton H-5 absorbing at δ 6.65 showed HMBC correlations to carbon C-6 which was one bond away. Also the same proton correlates with carbons C-7 absorbing at δ 161.08 and C-9 absorbing at δ 157.21 both of which were three bonds away. Further, Proton H-5 showed para correlation to C-8 absorbing at δ 101.11. Proton H-6 showed correlations to three carbons i.e. C-5, C-7 and C-8. Further HMBC correlation was observed between the proton H-8 absorbing at δ 6.38 and the carbons C-6, C-7 and C-9 absorbing at δ 104.71, 161.08 and 157.21 respectively. Para correlation was also observed between H-8 and C-5. In the ring B four signals for four protons were observed. HMBC correlation between protons and carbons in this ring was observed. Correlation between Proton δ H-2' and carbons C-1', C-3', C-4' and C-6' was observed. Furthermore, proton H-3', absorbing at δ 7.38 was correlating with the carbons C-2', C-4', C-6' and C-1'. Also this proton showed para correlation to carbon C-6' absorbing at δ 127.76. Carbons C-2', C-3' and C-5' were correlating to proton H-4' absorbing at δ 7.06. Para correlation was observed between proton H-4' and carbon C-1' absorbing at δ 137.12. HMBC correlation between proton H-6' and C-1', C-2'and C-3' was also observed. The position of the methoxy group at position 7 was confirmed by the correlation of methoxy protons at δ 3.82 with carbon C-7.

The proton /proton COSY correlation was also determined for compound (**20**). COSY spectrum gives information on protons which are attached to adjacent carbons. In ring A COSY correlation was observed between the protons H-5 and H-6 absorbing at δ 6.65 and 6.66. Proton H-2' and δH-3' were showing COSY correlating in ring B. COSY correlation is also supported by the coupling constants. Coupling constants for protons showing COSY correlation are equal. The coupling constant for the peaks absorbing at δ 7.52 and 7.38 was 7.53 Hz while the coupling constant for the peaks absorbing at δ 6.65 and 6.66 was 9.79 Hz. The two peaks for H-5 and H-6 were overlapping as shown by the integration factor. The integration factor was 0.02 implying that one proton was equal to 0.02. The integration of doublet at δ 6.65 or 6.66 gave a value of

0.04 implying that there are two protons for that peak (Figure 20). The proposed structure for compound (**20**) based on the 1D and 2D NMR information is shown below.



20

3, 5-dihydroxy -7- methoxy anthocynidines

HMBC correlation

COSY correlation

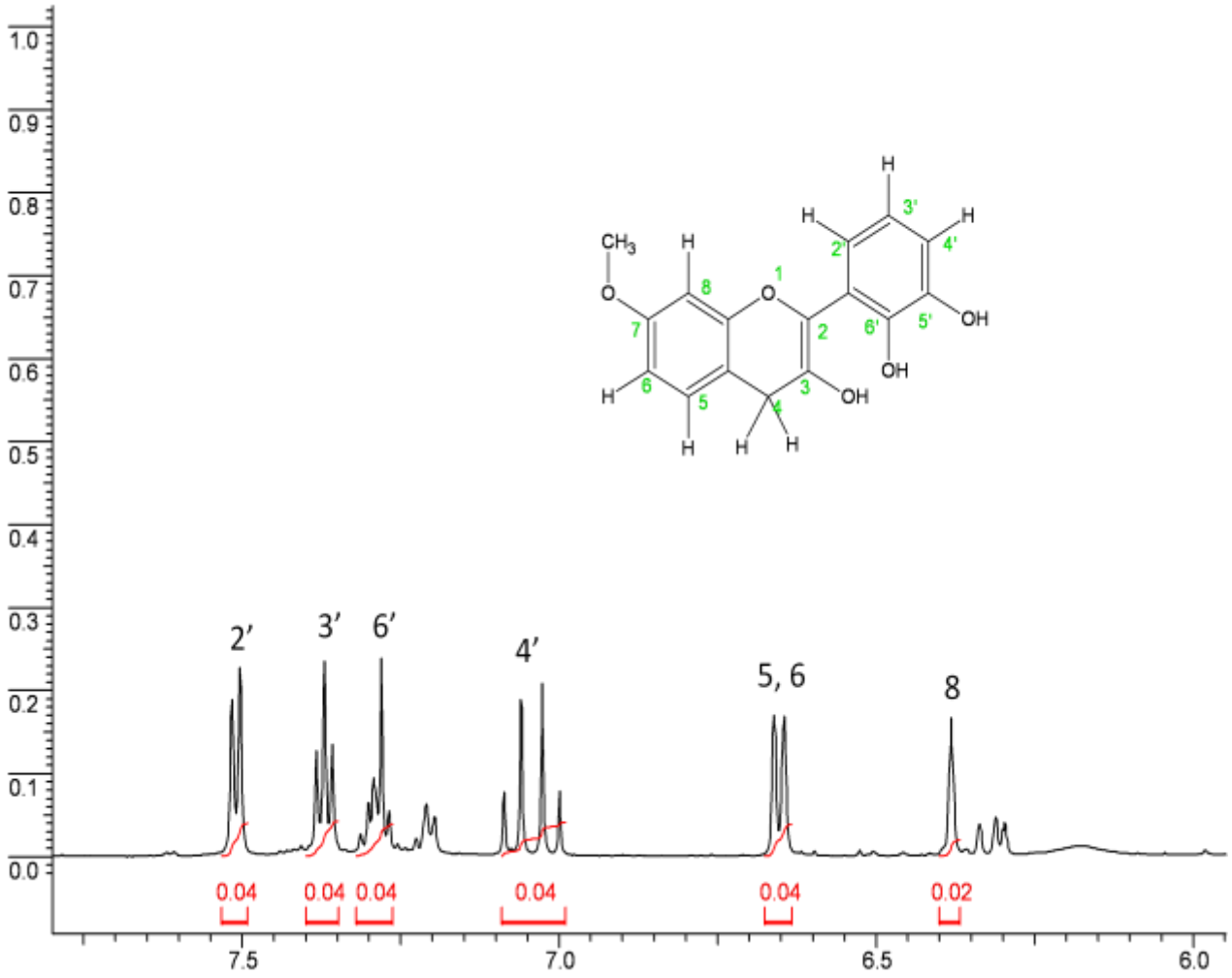
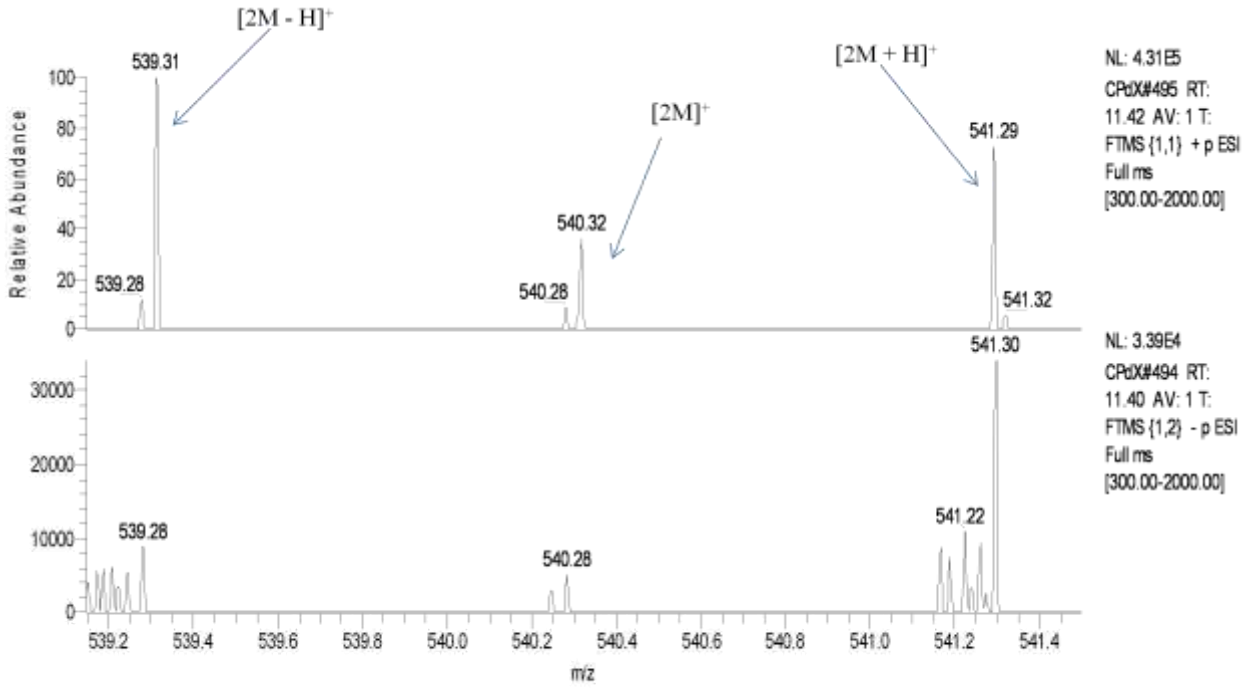


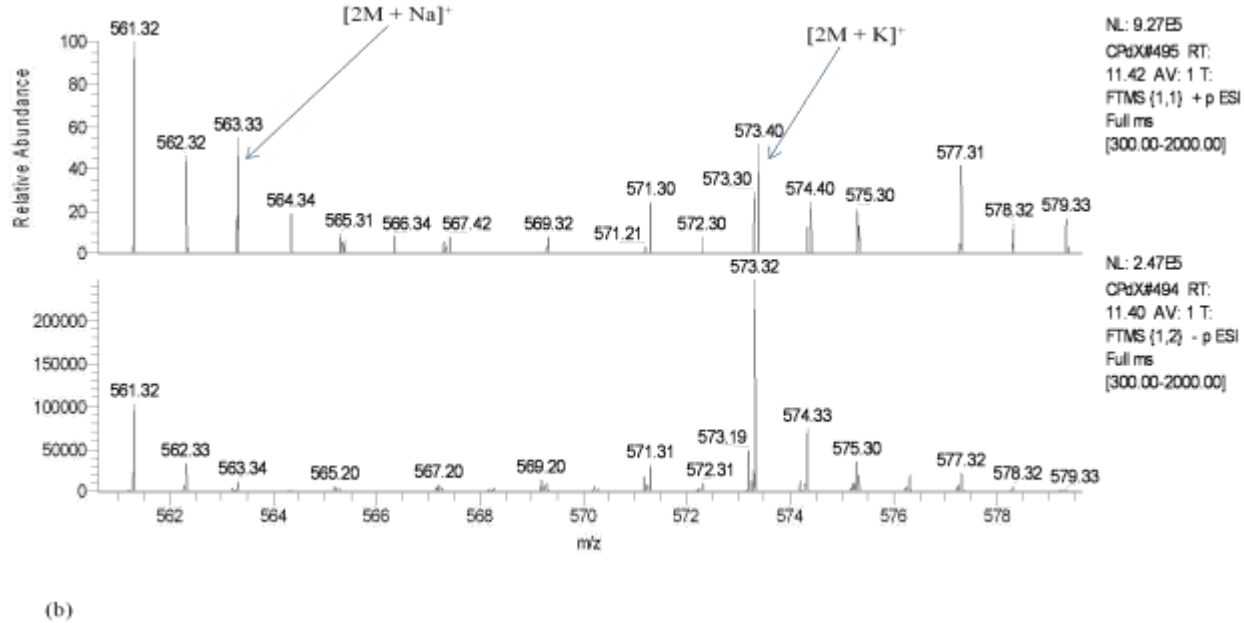
Figure 20: ^{1}H NMR showing aromatic protons in compound (20) and their integration factors

Compound (**20**) was identified as 3, 5-dihydroxy-7-methoxy anthocynidines with molecular ion peak m/z 270.280 calculated for $C_{16}H_{14}O_4$ (m/z 270) as revealed by the EIMS spectrum. The sample was analyzed at a wavelength range of 300-2000 thus only the 2M peaks were recorded. The EIMS spectrum of compound (**20**) revealed $[2M-H]^+$ ion peak m/z 539.21, and $[2M+H]^+$ ion peak m/z 541.29. Further the peaks m/z 563.30, and 579.30 were observed for peaks $[2M + Na]^+$ and $[2M + K]^+$ respectively. The peak m/z 309.21 was attributed to the peak $[M + K]^+$. The mass spectra for compound (**20**) are in Figures 21 (a), 21 (b) and 21 (c).



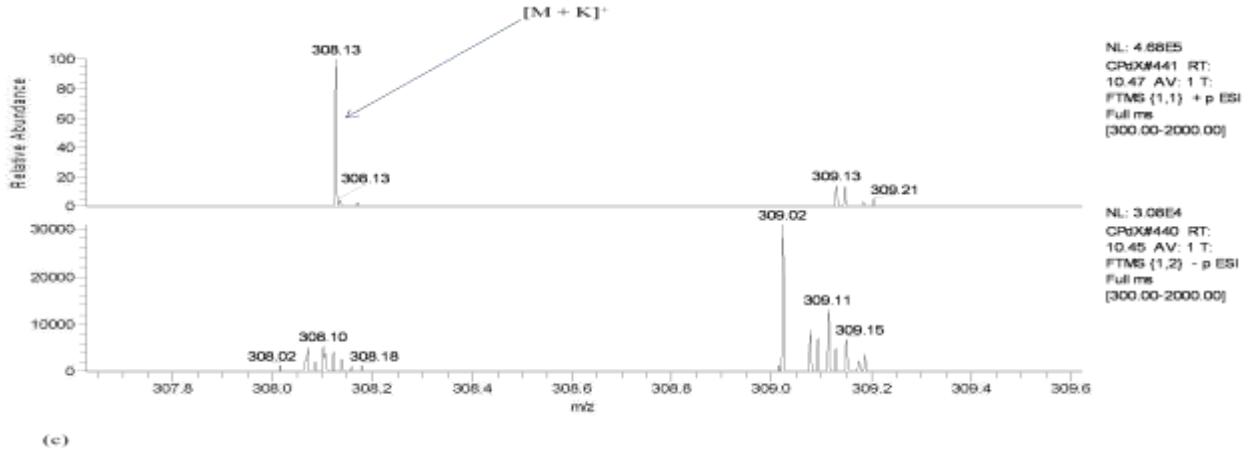
(a)

Figure 21: Mass spectra for compound **(20)**



(b)

Figure 22: Mass spectra of compound **(20)**



(c)

Figure 23: Mass spectra of compound **(20)**

Compound (**21**) had 15 carbons, 12 hydrogen and 5 oxygen and therefore molecular formula $C_{15}H_{12}O_5$. The NMR data for this particular compound is in table 15 below:

Table 18: NMR information for compound (**21**)

Position	**δ^{13}C (ppm)**	**DEPT**	**δ^{1}H/HSQC (ppm)**	**J (Hz)**	**HMBC**	**COSY**
2	141.70	Q				
3	144.50	Q				
4	37.93	CH_2	2.92 (s)		5, 2, 3	
5	106.56	CH	6.33 (d)	15.06	4, 8, 6, 7, 9	6
6	108.06	CH	6.31 (d)	14.29	8, 5, 7	5
7	161.06	Q				
8	99.11	CH	6.29 (s)		5,6, 9, 7	
9	156.87	Q				
10	-	Q				
1’	137.13	Q				
2’	126.61	CH	7.52 (d)	7.53	1’, 3’, 4’, 6’	3’
3’	128.76	CH	7.38 (t)	7.53	2’, 4’, 1’ 6’	2'
4’	128.45	CH	7.04 (d d)	20.23, 15.81	6’, 2’, 3’, 1’, 5’	
5’	139.65	Q				
6’	127.76	CH	7.29 (s)		2’,3’, 1’, 2, 4’	

Fifteen peaks were observed in the ^{13}C spectrum i.e. 7 quaternary carbons, 7 tertiary carbons and 1 secondary carbon. The quaternary carbons were absorbing at δ 141.70, δ 144.50, δ 156.87, δ 137.13 and δ 139.65. The CH carbons peaks appeared at δ 106.56, δ 108.06, δ 99.11, δ 126.61, δ 127.76, δ 128.76 and δ 128.45 in the ^{13}C spectrum. The only CH_2 in the molecule was absorbing at δ 37. 93. The ^{1}H spectrum showed 7 aromatic protons. Two singlets at δ 6.29 and δ 7.29 and three doublets at δ 6.33, δ 6.31 and δ 7.52, also a triplet was observed at δ 7.38. A doublet of doublets signal was observed at δ 7.04. One more singlet was observed at δ 2.92 for the methylene group.

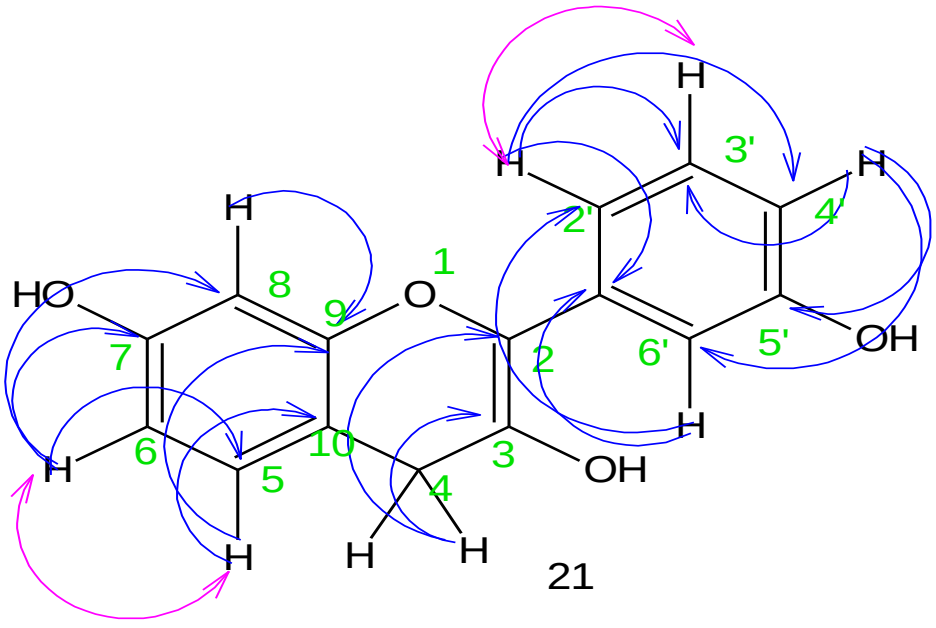
Carbon atoms absorbing at δ 141.70 and 144.50 were assigned to C-2 and C-3 respectively. C-4 was assigned the carbon atom absorbing at δ 37.93. The carbon atoms absorbing at δ 106.56, 108.06, 161.06, 99.11 and 156.87 were allocated to the carbons in ring A. Carbon atoms δ 106.56 and δ 108.06 were assigned to C-5 and C-6 respectively. The quaternary carbon absorbing at δ 161.06 was allocated to C-7 with hydroxyl group attached to it. C-8 and C-9 were assigned the carbon atoms 99.11 and 156.87 respectively. Like compound X_1 C-10 signal was not observed in the ^{13} C NMR. Two quaternary carbon and four CH carbons were allocated to the ring B carbon atoms. C-1' and 5' were assigned the quaternary carbons absorbing at δ 137.13 and δ 139.65 respectively. C-2', C-3', C-4' and C-6' were allocated the carbon atoms absorbing at δ 126.61, 128.76, 128.45 and 127.76 respectively.

The carbons correlation with protons directly attached to them was obtained from the HSQC spectrum. The proton absorbing at δ 2.92 was correlating with carbon C-4 while protons absorbing at δ 6.33 and δ 6.31 were correlating with the carbons C-5 and C-6 respectively. HSQC correlation was further observed between proton δ 6.29 and C -8. Ring B had 4 protons absorbing at δ 7.52, δ 7.38, δ 7.04 and δ7.29 which were correlating with carbons C-2', C-3', C-4' and C-6' respectively. The correlation information between protons and carbons which are two to three bonds away from them was obtained from the HMBC spectrum. The proton H-4 showed correlation to carbons C-2, C-3 and C-5. Correlation between proton H-5 with carbons C-4, C-6, C-7 and C-9 was also observed in the HMBC spectrum. Further, para correlation between H-5 and C-8 which was absorbing at δ 99.11 was observed in the HMBC spectrum.

HMBC correlation was recorded between proton H-6 and the carbons C-8 absorbing at δ 99.11, C-5 absorbing at δ 106.56 and C-7 absorbing at δ 161.06. Proton H-8 was correlating to

carbons C-7 and C-9 both of which were two bonds away and the same proton showed HMBC correlation to carbon C-6 which was three bonds away from it. In ring B there were four protons and all showed HMBC correlation to the carbons in the ring. Proton H-2' was correlating with carbons C-1' C-3', C-4'and C-6' in the HMBC spectrum. HMBC correlation also existed between proton H-3'and the carbons C-1', C-2'and C-4'. Further correlation between proton H-4an d carbons C-2', C-3', C-5' and C-6' was observed. The same proton showed para correlation to carbon C-1' absorbing at δ 137.13. Proton H-6' absorbing at δ 7.29 showed HMBC correlation to the carbons: C-1', C-2' and C-2. Para correlation was also observed between proton H-6' and C-3' absorbed.

The proton /proton COSY correlation was also determined for compound (**21**). COSY spectrum gives information on protons which are attached to carbons adjacent to each other. Proton H-5 and H-6 were showing COSY correlation in ring A while proton H-2'and H-3' were showing COSY correlation in ring B. Based on the 1 D, 2 D NMR and EIMS information described above the proposed structure of compound (**21**) is as given below.



3, 7, 5'- trihydroxy anthocynidines

HMBC correlation

COSY correlation

Compound (**21**) was identified as 3, 7, 5'- trihydroxy anthocynidines with molecular mass of 256.253 calculated for $C_{15}H_{12}O_4$. The sample was analyzed at wavelength ranges of 300-2000 and therefore only the 2M peaks were recorded. The EIMS spectrum of compound **(21)** revealed $[2M-H]^+$ ion peak m/z 511.18. Further the peaks m/z 513.33, 535.17 and 551.30 for peaks $[2M + H]^+$, $[2M + Na]^+$ and $[2M + K]^+$ respectively were observed. The mass spectra for compound (**21**) are in figures 22 (a), 22 (b) and 22 (c).

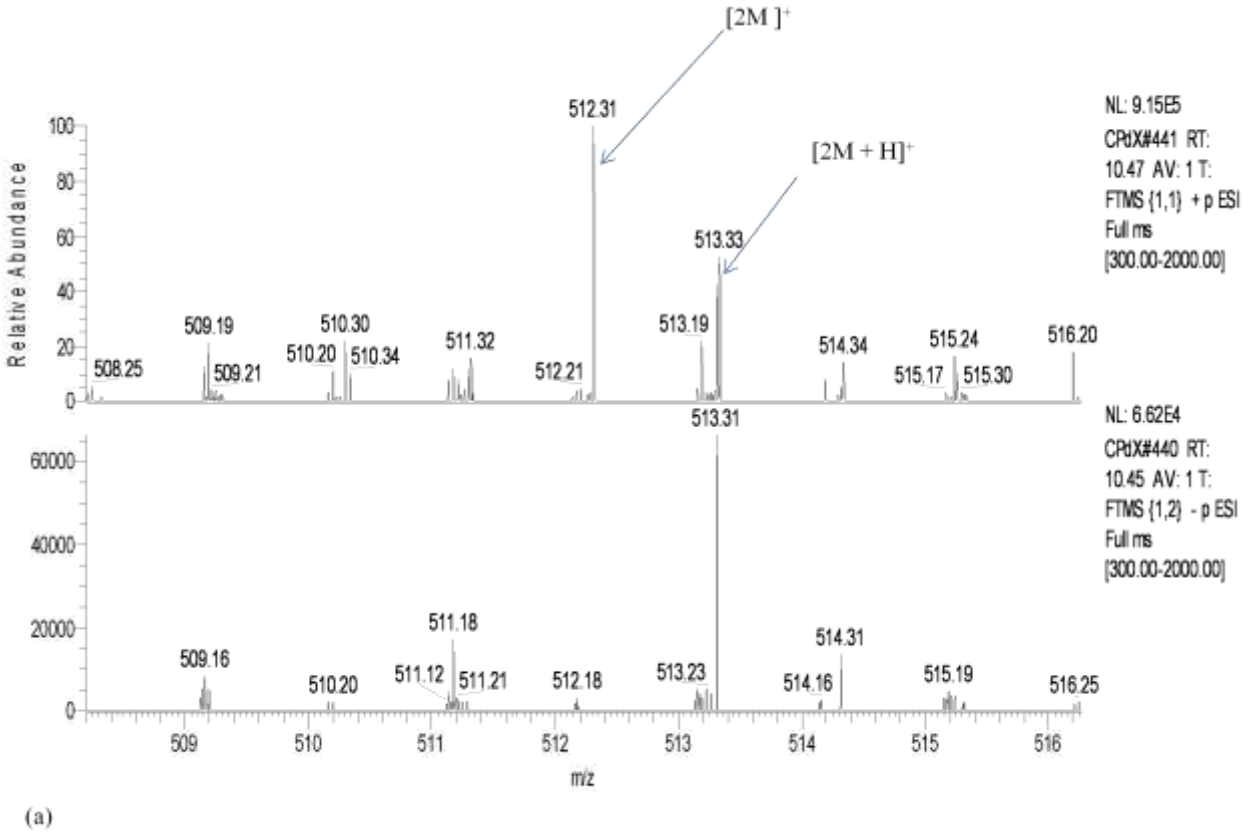
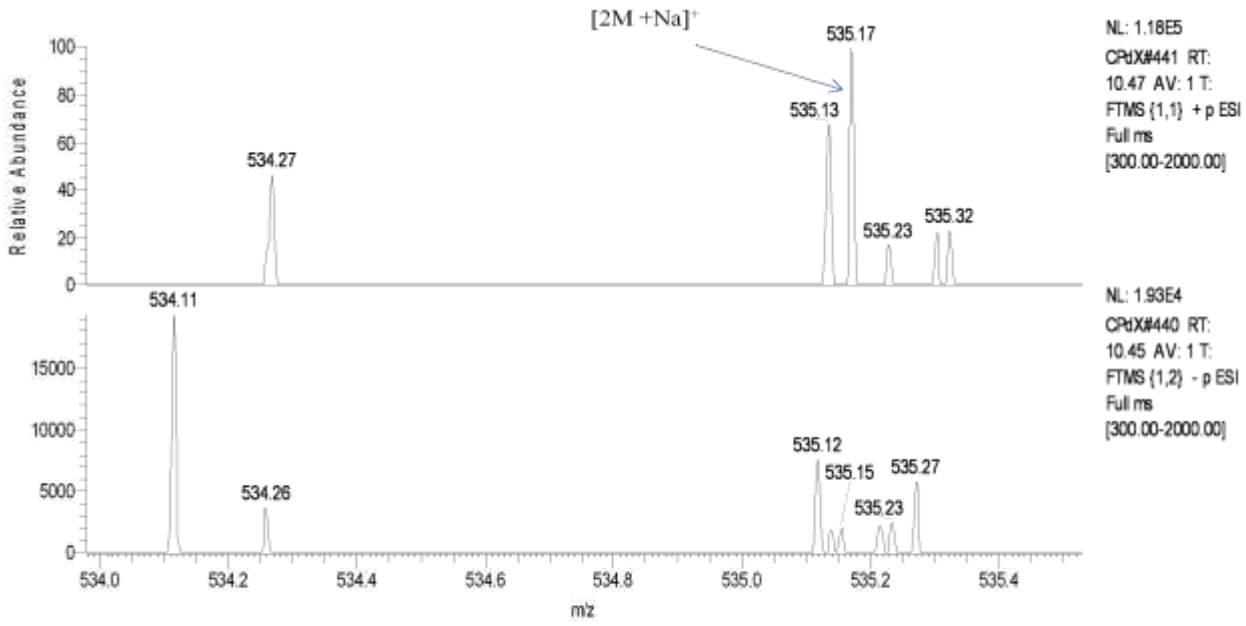
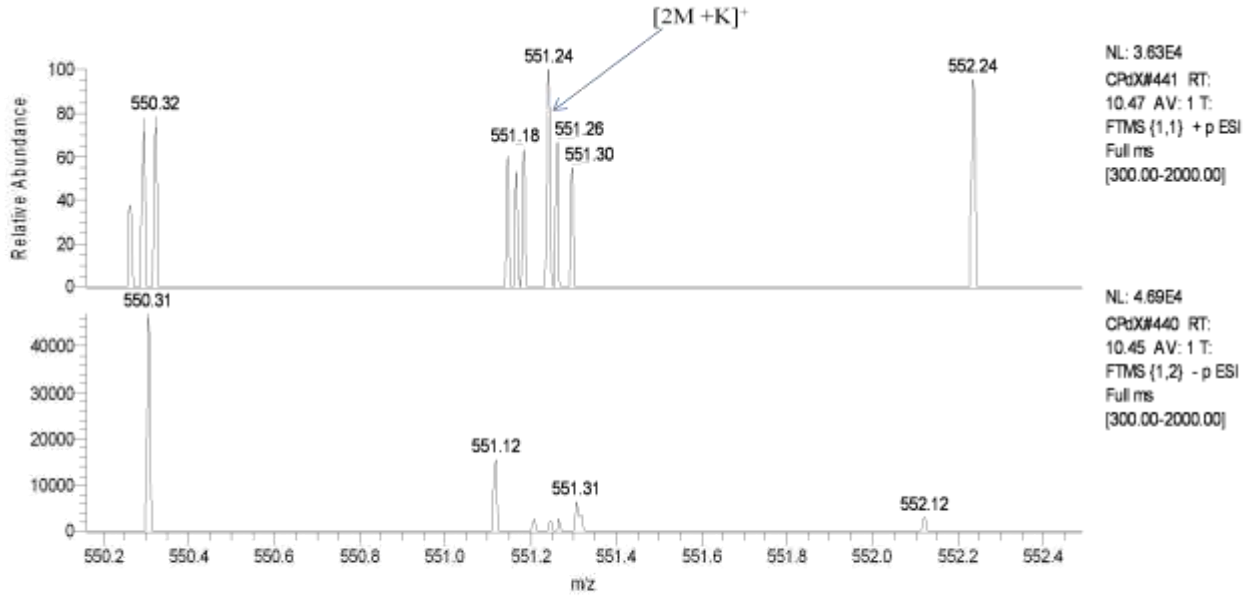


Figure 24: Mass spectra of compound **(21)**



(b)

Figure 25: Mass spectra of compound **(21)**



(c)

Figure 26:Mass spectra of compound **(21)**

The structure of compound M was not elucidated because the spectra obtained were very poor as a result of many impurities. The methods used for purification i.e. column chromatography and PTLC could not remove these impurities.

The flavonoids are biosynthesized through the mevalonic pathway and the isolated flavonoids pass through the (2S) – Liquiriteginin. The activity of the compound containing the mixture of the two flavonoids could be attributed to the two flavonoids. This is because different researchers have reported the ability of flavonoids to inhibit fungal growth (Pandey *et al.,* 2002; Irawan *et al* 2005; Kanwal *et al.,* 2011). Flavonoids are known to be synthesized by plants in response to microbial infection. Their activity is probably due to their ability to complex with extracellular and soluble proteins (Dhiman *et al.,* 2011). Generally flavonoids in plants leaves are believed to promote physiological survival of the plant, protecting it from physiological fungal pathogens and U.V radiation (Harborne and Williams *et al.,* 2000). Several flavonoids with antifungal activity have been isolated from different plants. Liquintigenin and isoliquintigenin isolated from Amboyna wood was found to be active against *F. oxsysporum, A. niger* and *P. italicum* (Irawan *et al.,* 2005). Mishra *et al.,* (2007) reported that the chalcone 2-hydroxy-4, 4',6'- trimethoxy chalcone could inhibit the spores germination of *A. flavus*. Further eicatechin isolated from *Azadirachta indica* leaves was found to inhibit the growth of *A. niger, A. fumigatus* and *P. citrii* (Kanwal *et al.,* 2011).

Anthocyanidines have also been shown to have antifungal activity. Anthocyanidine isolated from *Bryophyllum pinnatum* was found to be active against the plant pathogen *A. niger* and the clinical fungus *Candida albicans* (Ebere and Uchenna, 2011). The Compound X consisted of a mixture of two compounds compound **(20)** and **(21)**. The activity of this particular compound could be attributed to the two compounds. The high antifungal activity of this mixture of compounds could therefore have resulted from their synergic effect. Enhanced antimicrobial activity of flavonoids as a result of synergistic effects have been reported where the mixture of the different flavonoids had high antimicrobial activity than the activity of the individual flavonoids (Pankaj *et al.,* 2010).

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

This study has shown that maize samples collected from LVB were infested with mycotoxigenic fungi from three genera which are *Aspergillus, Penicillium* and *Fusarium*. This study has also shown that essential oils of *Monathotaxis littoralis* have antifungal activity against the mycotoxigenic fungi isolated from maize from LVB. The activity of the oil against these fungi had MIC values ranging from 26 to 103 mg/ml. The flavonoids mixture of 3, 5-dihydroxy-7-methoxy anthocynidines and 3, 7, 5'- trihydroxy anthocynidines isolated from this plant also showed remarkable antifungal activity against the fungi from the three genera with MIC values ranging from 1 to 4 mg/ml being observed. From these results the plant derivative products from *M. littoralis* could be used as alternatives to control microbial contaminants of foods such as moulds and can become useful tools for application in foods preservation systems. Thus, the results obtained justify future researches emphasizing the antimicrobial properties of plant products and their possible use as viable alternatives to control the microbial growth in food.

RECOMMENDATIONS

1. More advanced methods of compounds purification like HPLC should be used to purify compound M and separate the flavonoids mixture X_1 and X_2 so that their structure can be elucidated and tested for antifungal activity.

2. Fractions S_5G_1 and S_5G_2 should be separated and screened for antifungal activity.

3. The unknown compounds in the oil should be separated and their structures elucidated

4. The safety of the extracts should be determined before the products can be recommended for use by the farmers in preserving their grains.

REFERENCES

Adams, R. P. (1995). Identification of Essential Oil Components by Gas Chromatography/ Mass spectroscopy. Carol Stream, USA. Allured Publishing Cooperation. p 30-45.

Adejumo, T. O. and Langenkämper, G. (2012). Evaluation of botanicals as biopesticides on the growth of *Fusarium verticillioides* causing rot diseases and fumonisin production of maize. *Journal of Microbiology and Antimicrobials.* **4**: 23-31.

Adel, A. E., Abd-allah, N.A., Mohamed, R.M and Abu-shady, M. R. (2005). Actinomycetes antagonizing plant and human pathogenic Fungi. II. Factors affecting antifungal production and chemical characterization of the active components. *International Journal of Agriculture & Biology.* **2**: 188-196.

Agrios, G.N. (1997). Plant Pathology, 4th edition. Academic Press, New York. p 635.

Akinpelu, D. A. (2001). Antimicrobial activity of *Anacardium occidentale* Bark. Fitoterapia., **72**: 286-287.

Alves, T. M. A., Silva, A. F., Brandao, M., Grandi, T. M. S., Smania, E.F., Smania, S.A. and Zani, C.L. (2000). Biological screening of Brazilian medicinal plants. *Journal of Phytotherapy* **95**: 367-373.

Amandeep, K., Rajinder, K. and Uday K. (2012). Synthesis, spectral studies and biological activity of some novel biphenyl imidazo[2,1-b][1,3,4]thiadiazole derivatives. *Research Journal of Pharmaceutical, Biological and Chemical Sciences.* **3**: 1084.

Azziz-Baumgartener, E., Lindblade, K., Gieseker, K., Rogers, H. S., Kieszak, S., Njapau, H., Schleicher, R., McCoy, L. F., Misore, A., DeCock, K., Rubin, C. and Slutsker, L. (2005). Case-control study of an acute aflatoxicosis outbreak, Kenya, 2004. *Environmental Health Perspectives.* **113** :1779-1783.

Baldovini, N., Lavoine-Hanneguelle, S. and Ferrando, G. (2005). Necrodane monoterpenoids from *Lavandula luisieri*. *Phytochemistry.* **66**: 1651–1655.

Bammi, J., Khelifa, R., Remmal, A., Benjilali, B., Ettalibi, M., Ismaili- Alaoui, M. and Znira, S. (1997). Proceedings of the International Congress on Aromatic and Medicinal Plants and Essential oils, Morocco. P 502.

Bartomiej, K., Zbigniew, J. Burgie, J. J. Sepio, J. W. and Micha. K. (2006). Synthesis and fungistatic activity of aryl-substituted naphthalene- and indene-2-carbonitriles. *Environmental Biotechnology.* **2**: 20-25.

Beekrum, S., Govinden, R., Padayachee, T. and Odhav, B. (2003). Naturally occurring phenols: a detoxification strategy for fumonisin B1. *Food Additives and Contaminants.* **20**: 490-493.

Beggs, W. H. and Hughes C. E. (1987). Diagnostic Microbiology and Infectious Diseases. *Microbiology and Infectious Dis*ease. **6**, 1-3.

Ben-Ami, R., Lewis, R. E, and Kontoyiannis, D. P. (2010). "Enemy of the (immunosuppressed) state: an update on the pathogenesis of *Aspergillus fumigatus* infection". *British Journal of Haematology.* **150**: 406–417.

Bennardi, D. O., Gustavo, P. R., Jorge, L. J., Juan C. A.,Graciela, T. B. and Horacio, J. T. (2008). Synthesis of substituted flavones and chromones using a Wells-Dawson heteropolyacid as catalyst. *ARKIVOK*. **11**: 123-130.

Betran, F. J., and Isakeit, T. (2003). Aflatoxin accumulation in maize hybrids of different maturities. *Agronomy Journal* **96**: 565-570.

Bhat, R. V. and Vasanthi, S. (2003). Food Safety, Food Security and Food Trade –Mycotxin food safety risk in developing countries. International Food Policy Research Institute

Bigirwa, G., Sseruwu, G, Kaaya A. N., Adipala, E. and Okanya, S. (2006). Fungal microflora causing maize ear rots in Uganda and associated aflatoxins. *Journal of Biological Sciences* **6:** 540 – 546.

Bluma, R., Amaiden, M .R, Daghero, J. and Etcheverry, M. (2007). Control of Aspergillus section flavi growth and aflatoxin accumulation by plant essential oils. *Journal of Applied. Microbiology*. **1723**: 1364-5072.

Buchbuaer, G. (1993). Biological effect**s** of fragrance and essential oils. Proceedings of 12th intern.congr. Flav.Frag. essent.oils, Vienna. *Perfumer and flavourist* **18**: 19-24.

Chang, F. R., Wei, J.L. and Teng, C.M. (1998). Antiplatelets aggregation constituents from *annona purpurea*. *Journal of Natural products* **61**: 1457-1461.

Council for Agricultural Science and Technology (CAST) (2003). Mycotoxins – Risks in plants, animals and human systems. *Task Force Report*. **13**:1-191**.**

Cowan, M. M. (1999). Plant products as antimicrobial agents. *Clinical Microbiology Review* **12**: 564 – 582.

Desjardins, A. E and Proctor, R. H (2007). "Molecular biology of *Fusarium* mycotoxins". *International Journal of Food Microbiology.* **119**: 47–50.

Dhiman, A., Nanda, A., Ahmad, S. and Narasimhan, B. (2011). *Invitro* Antimicrobial activity of Methanol Leaf Extract of *Psidium guajara*. *Journal of Pharmacy and Bioallied Sciences*. **3**: 226-229.

Diba, K. K., Mirhendi, S. H. and Mahmoude, M. R. (2007). Identification of *Aspergillus* species using morphological characteristics. *Pakistan Journal of Medical Science* **23**: 867-872.

Diener, U. L., Cole, R. J., Sanders, T. H., Payne, G. A., Lee, L. S. and Klich, M. A. (1987). Epidemiology of Aflatoxin Formation by *Aspergillus Flavus*. *Annual Review of Phytopathology*. **25**: 249–270.

Dutton M. F. (1988). Enzymes and aflatoxin biosynthesis. *Microbiology Reviews*. **52**: 274-295.

Ebere, D. O. and Uchenna, F. N. (2012). Two novel flavonoids from *Bryophyllum pinnatum* and their antimicrobial Activity. *Journal of Chemical Pharmaceutical Research* **3***:1-10*.

European Food Safety Authority (EFSA). (2012). Scientific opinion on the risks for public and animal health related to the presence of citrinin in food and feed. *EFSA Journal*. **10**:2605.

FAO (1999). Preventing mycotoxin contamination. Food, Nutrition and Agriculture. FAO Food Nutrition Division. 23, Rome. Pg 9-28.

Ganiyat, K. O., Patricia, A. O. and Sunday, F. A. (2011). Chemical composition, toxicity, antimicrobial and antioxidant activities of leaf and stem essential oils of *Dieffenbachia picta* (Araceae). *European Journal of Scientific Research*. **49** : 567-580.

Harborne, J. B and Baxter, H. (1999). The handbook of natural flavonoids. Volumes 1 and 2. John Wiley and Sons; Chichester, UK.

Harborne, J. B and Williams, C. A. (2000). Advances in flavonoid research since 1992. *Phytochemistry*. **55**:481–504.

Hayes, A. W. and Wilson, B. J. (1970). Effects of rubratoxin B on liver composition and metabolism in the mouse. *Toxicology and Applied Pharmacology*. **17**: 481-493.

Heike, L.P., Beate, k., Klaus, S., Decker, T., Traenckner, E.B., Vogt, M., Myers, C., Parks, T., Warring, P., Muhlbacher, A., Czernilofsky, A. and Baeuer, P. (1996). The immunosuppressive fungal metabolite gliotoxin specifically inhibits transcription factor NF-kB. *Journal of experimental Medicine*. **183**:1829-1840.

Hemant, P., Nidhi, C., Sachi, S. and Shishupal, S. (2011). Anti-Inflammatory and Antimicrobial Activity of Some Novel Quinazolinones. *Rasayan Journal of Chemistry*. **4**: 498-505.

Hof, H and Kupfahl. C (2009). Gliotoxin in *Aspergillus fumigatus*: an example that mycotoxins are potential virulence factors. *Mycotoxicology Reseach. **25**:* 123–131.

Hohler. D (1998). Ochratoxin A in food and feed: Occurrence, legislation and mode of action. *Zeitschrift für Ernährungswissenschaft*. **37**: 2–12.

Holmes, R.A. and Payne G.A. (2008). Diverse inhibitors of aflatoxin biosynthesis. *Journal of Applied. Microbial Biotechnology*. **78**: 559-572.

Hopmans E. C. (1997). Patulin: a Mycotoxin in Apples. *Perishables Handling Quarterly* **91**: 5.

http://www.vicres.net/?.jc=coverage/LakeVictoriaBasinmap. Accessed on 21st June 2011 at 11a.m.

Huaiwei, D., Zhe, C., Cunlong, Z., Tian, X., Yini, W., Hongrui, S., Yuyang, J., Yuzong, C., Yongnan, X. and Chunyan T. (2012). Synthesis and Cytotoxic Activity of Some Novel *N*-Pyridinyl-2-(6-phenylimidazo[2,1-*b*]thiazol-3-yl)acetamide Derivatives. *Molecule*. **17**:4703-4716.

Ibrahim I. K, Shareef A. M. and Al-Joubory K. M. T (2000). Ameliorative effects of sodium bentonite on phagocytosis and Newcastle disease antibody formation in broiler chickens during aflatoxicosis. *Research Veterinary Science*, **69**: 119–122.

Irawan, W. K, Kazutaka, I. and Sanro, T. (2005). Antifungal Activities Against Plant Pathogenic fungi of Flavonoids Isolated from Amboyna Wood. *Pakistan Journal of Biological Science*. **8**: 136-140.

Jacob, C. (2006). A scent of therapy: pharmacological implications of natural products containing redox-active sulfur atoms. *Natural Product Reports*. **23**: 851-863.

Jong, H. K., Bruce, C. C., Noreen, M., Kathleen, L. C.,Russell J. M. and Arunmozhi, B. (2010). Augmenting the activity of antifungal agents against aspergilli using structural analogues of benzoic acid as chemosensitizing agents. *International Journal on Fungal Biology*. **144**: 817-824.

Juspin, T., Laget, M., Terme, T., Azas, N. and Vanelle P. (2010). TDAE-Assisted Synthesis of New Imidazo[2,1-b]Thiazole Derivatives as Anti-Infectious Agents. *European Journal of Medicinal Chemistry*. **45**:840-845.

Kaaya, A. N., Kyamuhangire, W. and Kyamanwa, S. (2006). Factors affecting aflatoxin contamination of harvested maize in the three agro ecological zones of Uganda. *Journal of Applied sciences.* **6:** 2401 – 2407.

Kaaya, N. A. and Warren, H. L. (2005). A review of past and present research on aflatoxin in Uganda. *African journal of food agriculture and nutritional development (AJFAND)* **5:** 1 - 18.

Kanwal, Q., Ishtiaq, H., Hamid L. S. and and Arshad, J. (2011). Antimicrobial Activity Screening of Isolated flavonoids from *Azadirachta indica* leaves. *Journal of Serbian Chemical Society*. ***76*** :375–384.

Katta S.K, Eskridge K.M and Bullerman L.B (1995). Mould content of commercial popcorn. *Journal of Food Protection*. **58**: 1014–1017.

Kaur, J. and Arora, D. (1999). Antimicrobial activities of species. *International Journal of Antimicrobial Agents*. **12**: 257-262.

Kedera C. J, Ochor T. E., Ochieng J. A. W. and Kamadi R. E. (1994). Incidence of maize ear rot in western Kenya. *International Journal of Pest Management* **40 :** 117-20.

Kenya Plant Health Inspectorate Service (KEPHIS) (2006). Mycotoxins and Food Safety. *KEPHIS headquarters*, Nairobi, Kenya.

Kimanya, M. E., De Meulenaer, B., Tiisekwa, B., Ndomondo-Sigonda, M., Devlieghere, F. van Camp, J. and Kolsteren, P. (2008). Co-occurrence of fumonisins with aflatoxins in home-stored maize for human consumption in rural villages in Tanzania. *Food Additives and Contaminants* **25**: 1353 – 1364.

Klich, M. (2002). Identification of common *Aspergillus* species. ASM Press, Washington, DC, p. 116.

Kozakiewtez, L. Z. (1989). *Aspergillus* species in stored products. *Mycological Paper*. **16**: 1-188.

Krimmel, C. P. (1968). *N*-(Dialkylaminoalkyl)adamantanecarboxamides. *Chemical Abstract.* **3**: 374,244

Kung'u J. (2005). Mould and spoilage.http://www.moldbacteria.com/ myblog/2005/07/mould-and-food-spoilage.html. Accessed on Friday, June 03, 2011, 11 am.

Lahlou, M. and Berrada, R. (2001). Composition and niticidal activity of essential oils of three chemotypes of *Rosmarinas officinalis L. Journal of Pharmaceutical Biology* **141**: 201-207.

Lanyasunya, T. P., Warnae, L. W., Musa, H. H., Olowofeso, O. and Lokwaleput, I. K. (2005). The risk of mycotoxins contamination of dairy feed and milk on smallholder dairy farms in Kenya. *Pakistan Journal of Nutrition* **4**: 162-169.

Laszlo, K., Janos, V., Sandor, K., Ilona, D., Robert, A., Samson, R. R., Venkatapathy, N., Madhavan, B., Csaba, V. and Palanisamy, M. (2007). Case of Keratitis Caused by *Aspergillus tamarii*. *Journal of Clinical Microbiology*. **7** 3464–3467.

Lee, S.E, Campbell, B.C, Moly-Neux, R.J, Hasegawa, S and Lee, H.S (2001). Inhibitory effects of naturally occurring compounds on aflatoxin B**1** biotransformation. *Journal of Agricultural Food Chemistry*. **49**: 5171-5177.

Lewis, L., Onsongo, M ., Njapau, H., Schurz-Rogers, H., Luber, G., Nyamongo, S. J., Baker, L., Dahiye, A., Misore, A. and Kevin, D. R., and the Kenya aflatoxin investigating group (2005) . Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in Eastern and Central Kenya. *Environmental Health Perspective* **113**: 1763-1767.

Liang, G. Y., Gray, A. I., Thomas, D. W. and Waterman, P. G. (1988). *Phytochemistry.* **27**: 3857–3860.

Lillard-Roberts, S. (2003). List of mycotoxins. *Mycotoxinas.* **46**:1-13.

Madigan, M. and Martinko, J. (2005). *Brocks Biology of Microorganisms* (11th edition). Prentice Hall. p 65-70.

Madkour, H. M., El-Shiekh, Y. W., Amed, A. F. and Farang, A. A. (2011). Fungicidal Efficacy of Someol New Azole towards *F. oxysporum*, *R. solani* and *S. rolfsii*. *Middle-East Journal of Scientific Research.* **9**: 520-526.

Malgorzata, M. and Irena, P. (2005). Antioxidant Activity of the Main Phenolic Compounds Isolated from Hot Pepper Fruit (*Capsicum Annuum* L.). *Journal of Agricultural and Food Chemistry*. **53**: 1750-1756.

Mantle, P., Kulinskaya, E. and Nestler, S. (2005). Renal tumourigenesis in male rats in response to chronic dietary ochratoxin A. *Food Additives and Contaminants.* **22**: 58-64.

Manwiller, A. (1987). Aflatoxin Kenya. In: Zuber, M. S., Lillehoj, E. B., and Renfro, B. L. (Eds). Aflatoxin in maize. Proceedings of Workshop, 7-11Apr. 1986. El Batan, Mexico. CIMMYT.

Matasyoh, J. C., Kiplimo, J. C., Karubiu, N. M. and Hailstorks, T. P. (2006). Chemical composition and antimicrobial activity of essential oil of *Satureja biflora* (Lamiaceae). *Bulletin of the Chemical Society of Ethiopia* **21**: 1-6.

Matasyoh, C., Wagara, I. N., Nakavuma, J. L. and Kiburai, A. M. (2010). Chemical composition of *Cymbopogon citrates* essential oil and its effect on mycotoxigenic *Aspergillus* species. *African Journal of Food Science.* **5**: 138-142.

Mishra, P. K, Sarma, B. K ., Singhai P. K and Singh, U. P. (2007). Antifungal Activity of 2-Hydroxy 4,4'6'-Trimethoxy Chalcone. *Mycobiology*. **35**: 72-75.

Moleyar, V. and Narasimham, P. (1986). Antifungal activity of some essential oil components. *International Journal of Food microbiologyl*. **3**: 331-336.

Morales, H., Marín, S., Rovira, A., Ramos, A. J. and Sanchis, V. (2007). "Patulin accumulation in apples by *Penicillium expansum* during postharvest stages". *Letter in Applied Microbiology*. **44** :122-125.

Moreno, E. C., Garciab, G. T., Onoc, M. A., Vizonid, E., kawamurae, O., Hirookaf, E. Y. and Onoa, S. Y. E. (2009). Co-occurrence of mycotoxins in corn samples from the Northern Region of Parana State, Brazil. *Journal of Food chemistry*. **10**: 1016.

Moveira, L. C., Lago, R.O. and Roque, N.D. (2005). Sesquiterpenes, steroids and alkaloids from annonaceae branches. *Biochemistry SY57 Ecology* **33**: 948-951.

Munimbazi, C. and Bullerman, L.B (1996). Molds and mycotoxins from Burundi. *Journal of food protection* **59**: 869 – 875.

Murphy, P. A., Hendrich, S., and Bryant, C. M. (2006). Food Mycotoxins – An Update. *Journal of Food Science.* **71**: 51 – 65.

Nazrul, M. D., Bhuiyan, I., Chowdhury, J. U. and Begum, J. (2009). Chemical Components in Volatile Oil from *Blumea Balsamifera* . *Bangladesh Journal of Botany*. **38**: 107-109.

Nelson, P. E., Toussoun, T. A. and Marasas, W. F. O. (1983). *Fusarium* species. An illustrated manual for identification. The Pennsylvania State University Press, University Park, PA, USA.

Nielsen K. F, Mogensen J. M, Johansen M, Larsen T. O and Frisvad J. C (2009). Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. *Analytical Bio analysis Chemistry*. **395**: 1225–1242.

Nyikal, J., Misore A., Nzioka C., Njuguna C., Muchiri E. and Njau J. (2005). Outbreak of aflatoxin poisoning in Eastern and Central Provinces, Kenya, January – July 2004. Ministry of Health Report.

Oguz, H., Hadimli H. H, Kurtoglu, V. and Erganis, O. (2003). Evaluation of humoral immunity of broilers during chronic aflatoxin (50 and 100 ppb) and clinoptilolite exposure. *Journal of Vetenary Medicine*. **154**: 483–486.

Ohat, T., Watanabe, K., Moriya, M., Shirasu, Y. and Kada, T. (2002). Antimutagenic effects of cinnamaldehyde on chemical mutagenesisin. *Escherichia coli Mutation Response***. 107**: 219-227.

Pandey, M. K., Pandey1, R., Singh1, V. P., Pandey1, V. B and Singh, U. P. (2002). Antifungal Activity of 4',7-Dimethoxyisoflavone Against Some Fungi. *Mycobiology*. **30**: 55-56.

Pankaj, G., Bipin, K. A. and Sonal, G. (2010). A Study on Combinatorial Effects of Various Flavonoids for Their Antibacterial Potential Against Clinically Significant Bacterial Species *Journal of Biological Chemistry*. **38**: 255-258.

Papadaki-Valiraki, A., Papakonstantinou-Garoufalias, S.,Makaros, P., Chytyroglou-Lada, A., Hosoya, M., Balzarini, J. and De Clercq, E. (1993). Synthesis, antifungal and antiviral effects of some adamantaneketoxime ethers. *Il Farmaco. **48***: 1091-1102.

Pitt, J. I. (1979).The genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*. Academic Press, London.

Ponce, M. A., Jose, M. S., Rosa E., Juan, A. O. and Alicia, M. G. (2004). Flavonoids from shoots, roots and roots exudates of *Brassica alba*. *Phytochemistry*. **65**: 3131–3134.

Prasanthy, G., Venkata, R. K., Koti, R. V., Nirmala, K. and Ramesh K. N. (2011). Synthesis and Biological Evaluation of 1-Substituted Imidazole Derivatives; *International Journal of Pharmaceutics*. **1**: 92-99.

Ryan, K. J and Ray, C. G. (2004). Sherris Medical Microbiology (4th edition). Mc Graw Hill. p 633-638.

Saddiq, A. A. and Khayyat, A. S. (2010). Chemical and antimicrobial studies of monoterpene: Citral. *Pesticide Biochemistry and Physiology*. **98**: 89–93.

Sartoratto, A., Lucia, A., Machado, M., Camila, D., Glyn, M. F., Martha, C., Duarte, T., Vera, L. and Rehler, G. (2004). Composition and antimicrobial activity of essential oils from aromatic plants used in Brazil. *Brazilian Journal of Microbiology* **35**: 655-658.

Satoru, M., Kiyushi, K., Yoshinori, N. and Ogihara, Y. (1997). The interaction of rubroskyrin, a modified bis-anthraquinone pigment from *Penicillium islandicum* with respiratory chain of liver mitochondrian. *Mycotoxin research*. **13**: 35-42.

Savolainen, K. and Stockmann, H. (2008.). "A review of the toxic effects and mechanisms of action of fumonisin B1". *Human & Experimental Toxicology* **27** : 799–809.

Schmale, D. G. and Munkvold, G.P. (2009). Mycotoxins in Crops: A Threat to Human and Domestic Animals Health. *The Plant Health Instructor.*

Schuster, E., Dunn-Coleman, N., Frisvad, J. C. and Van- Dijck, P. W. (August 2002). "On the safety of *Aspergillus niger* - a review". *Applied Microbiology and Biotechnology* **59:** 426–435.

Segal, B. H. (2009). "Aspergillosis". *New England Journal of Medicine.* **360**: 1870–1884.

Shanmugam P., Anura W. D., Nedra K., Gavin C., Wijesundara D., Raymond A. and Veranja K. (2008). Antioxidant Constituents from *Xylopia championii. Journal of Pharmaceutical Biology*. ***46:*** 352–355.

Shephard, G. S. (2008). Risk assessment of aflatoxins in food in Africa. *Food Additives and Contaminants* **25:** 1246 – 1256.

Sikkema, J., De Bont, J. A. M. and Poolman, B. (1995). Mechanisms of membrane toxicity of hydrocarbons. *Microbiology Revision.* **59**: 201-222.

Smith, J. E., Solomons, G. L., Lewis, C. W. and Anderson, J. G. (1994). Mycotoxins in human health. Brussels: An article of European Commission.

Sofia Vio, M., Gastón, A., Miguel, G., Peña-Vera, R. and Montenegro, G. (2012). Antifungal Activity of Three Chilean Plant Extracts on *Botrytis Cinerea. Botanical Sciences.* **90**: 179-183.

Souza, E. L., Lima, E. O, Freire K. R. and Sousa C. P. (2005). Inhibitory action of some essential oils and phytochemicals on the growth of various moulds isolated from foods. *Brazilian archives of Biology and Technology*. **48**: 245-250.

Spikes, S., Xu, R., Nguyen, C. K., Chamilos, G., Kontoyiannis, D. P. and Jacobson, R. H . (2008). "Gliotoxin production in *Aspergillus fumigatus* contributes to host-specific differences in virulence". *Journal of Infectious Diseases.* **197**: 479–486.

Stafford, H. (1991). Flavonoid evolution: An enzymatic approach. *Plant Physiology*. **96**: 680-685.

Sun, X. M., Zhang, X., Wang, H., Cao-Zuo, J. and Wang,F. (2002). Effects of Sterigmatocystin, Deoxynivalenol and Aflatoxin G1on: Apoptosis of Human Peripheral. *Biomedical and Environmental Sciences* **15:***1*45-152.

Townsend C. A. (1997). Progress towards a biosynthetic rationale of the aflatoxin pathway. *Pure and Applied Chemistry.* **58**:227-238.

Vallejo, G. I, Vallejo, G. C. and Sanz, J. (1994). Necrodane derivatives in *Lavandula luisieri*, new compounds to the plant kingdom. *Phytochemistry*. **36**: 43–45.

Varga, J., Frisvad, J.C. and Samson, R.A. (2009). A reappraisal of fungi producing aflatoxins. *World Mycotoxin Journal*. **2**: 263-277.

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

Weiming, X., Jiang, H., Ming, H., Feifei, H., Xuehai, C., Zhaoxi, P., Jian ,W., and Maoguo, T. (2011). Synthesis and Antifungal Activity of Novel Sulfone Derivatives Containing 1,3,4-Oxadiazole Moieties. *Molecules.* **16**: 9129-9141.

WHO. (2008). World Health Statistic (2008). WHO Press, Geneva, http://www.who.int/whostat/EN WHS08.

Wilamowski, J., Kulig, E., Sepioł, J. J. and Burgieł, Z. J. (2001). Synthesis and in *vitro* antifungal activity of 1-amino-3,4-dialkylnaphthalene-2-carbonitriles and their analogues. *Journal of Pest Management Science.* **57** :625-632.

Wild, C. P. and Gong, Y. Y. (2010). Mycotoxins and human disease: a largely ignored global health issue. *Carcinogenesis.* **31**: 71-82.

Winkel-Shirley, B. (1999). Evidence for enzyme complexes in the phenylpropanoid and flavonoid pathways. *Plant Physiology*. **107**: 142-149.

Winkel-Shirley, B. (2001). Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology and biotechnology. *Plant Physiology.* **126**: 485-493.

Xiang, L. , Zhi, L., Xin-feng, Z., Li-juan Wang 1, Yi-nan, Z., Chang-chun, Y. and Guang-zhi, S. (2008). Isolation and Characterization of Phenolic Compounds from the Leaves of *Salix matsudana*. *Molecules*. **13***:* 1530-1537.

Zahra, R., Soghra, K., Kamiar, Z., Kyvan, P., Giti, K., Narges, S. and Sanaz, G. (2011). Design, Synthesis and Antifungal Activity of Some New Imidazole and Triazole Derivatives. *Pharmaceutical & Medicinal Chemistry***. 344**: 658-665.

Zheng, Q. R., Zhang, Y. J. and Yang, C. R. (2006). New meta-homoisoflavone from the fresh stems of *Dracaena coochinchinensis*. *Journal of Asian Natural Products research*. **8**: 571-577.

APPENDICES

Appedix 1: Tables of t-test paired means for the 7th and 14th day means of *Aspergillus* species

T-Test

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	SEVEN	12.9792	12	6.67548	1.92704
	FOURTEEN	11.9500	12	6.40273	1.84831

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	SEVEN & FOURTEEN	12	.995	.000

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	SEVEN - FOURTEEN	1.0292	.70725	.20417	.5798	1.4785	5.041	11	.000

Appedix 2: Tables of t-test paired means for the 7th and 14th day means of *Fusarium* species

T-Test

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	SEVEN	13.3333	9	3.88194	1.29398
	FOURTEEN	11.0333	9	3.26930	1.08977

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	SEVEN & FOURTEEN	9	.977	.000

Paired Samples Test

	Paired Differences					t	df	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1 SEVEN - FOURTEEN	2.3000	.97368	.32456	1.5516	3.0484	7.087	8	.000

Appedix 3: Tables of t-test paired means for the 7th and 14th day means of *Penicillium* species

T-Test

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	SEVEN	14.5364	14	9.32042	2.49099
	FOURTEEN	13.4521	14	9.35762	2.50093

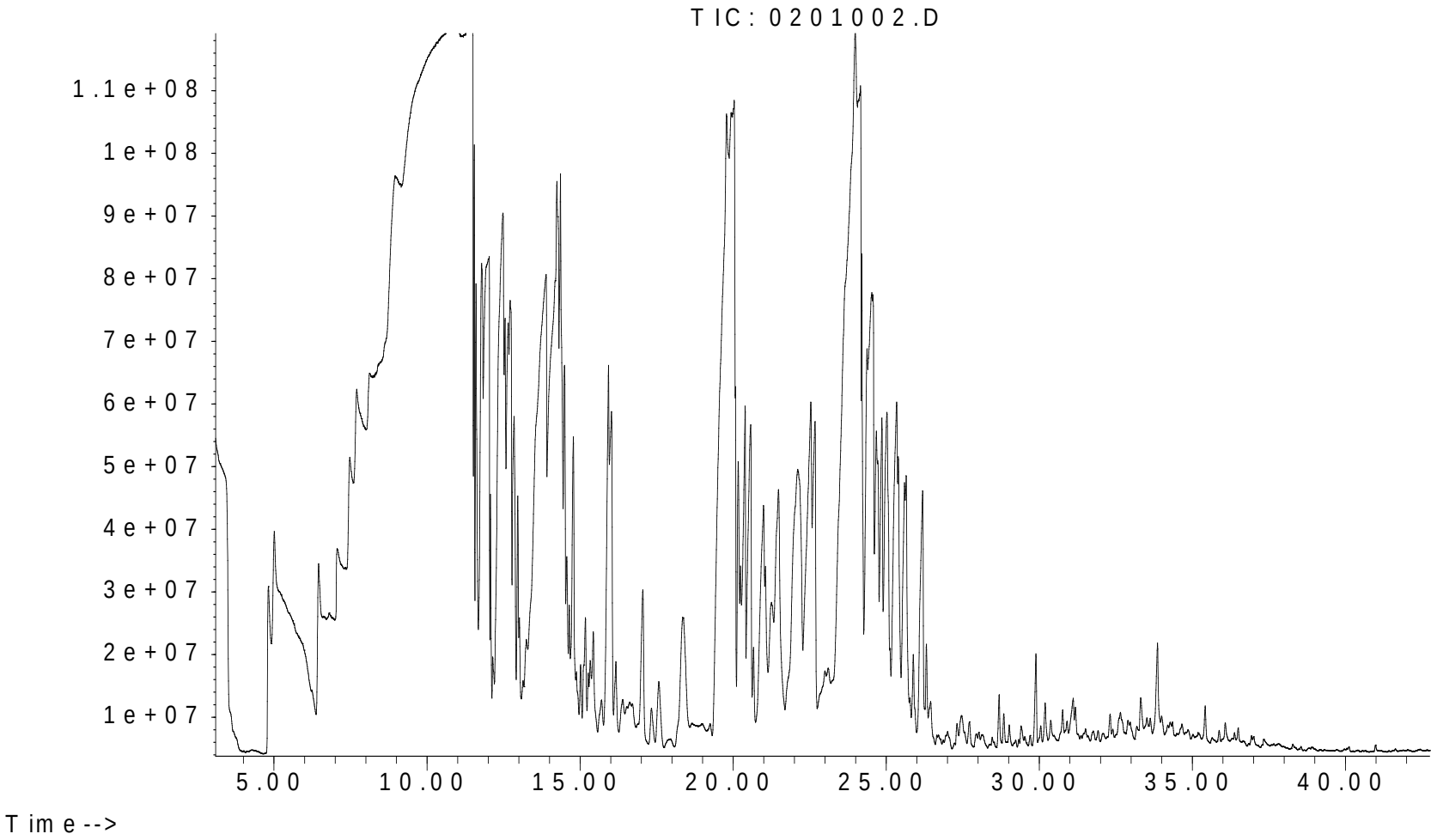
Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	SEVEN & FOURTEEN	14	.999	.000

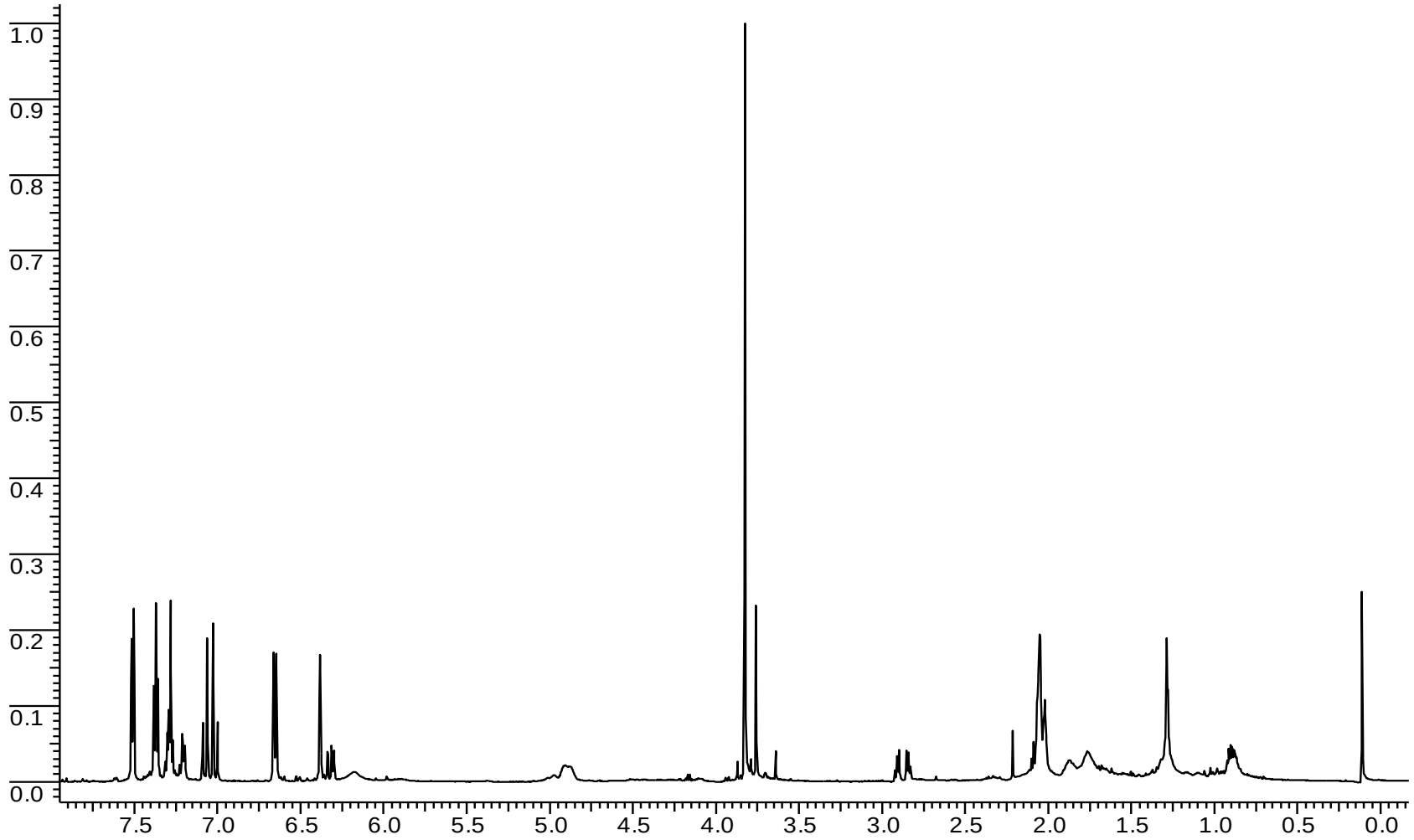
Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	SEVEN - FOURTEEN	1.0843	.42282	.11300	.8402	1.3284	9.595	13	.000

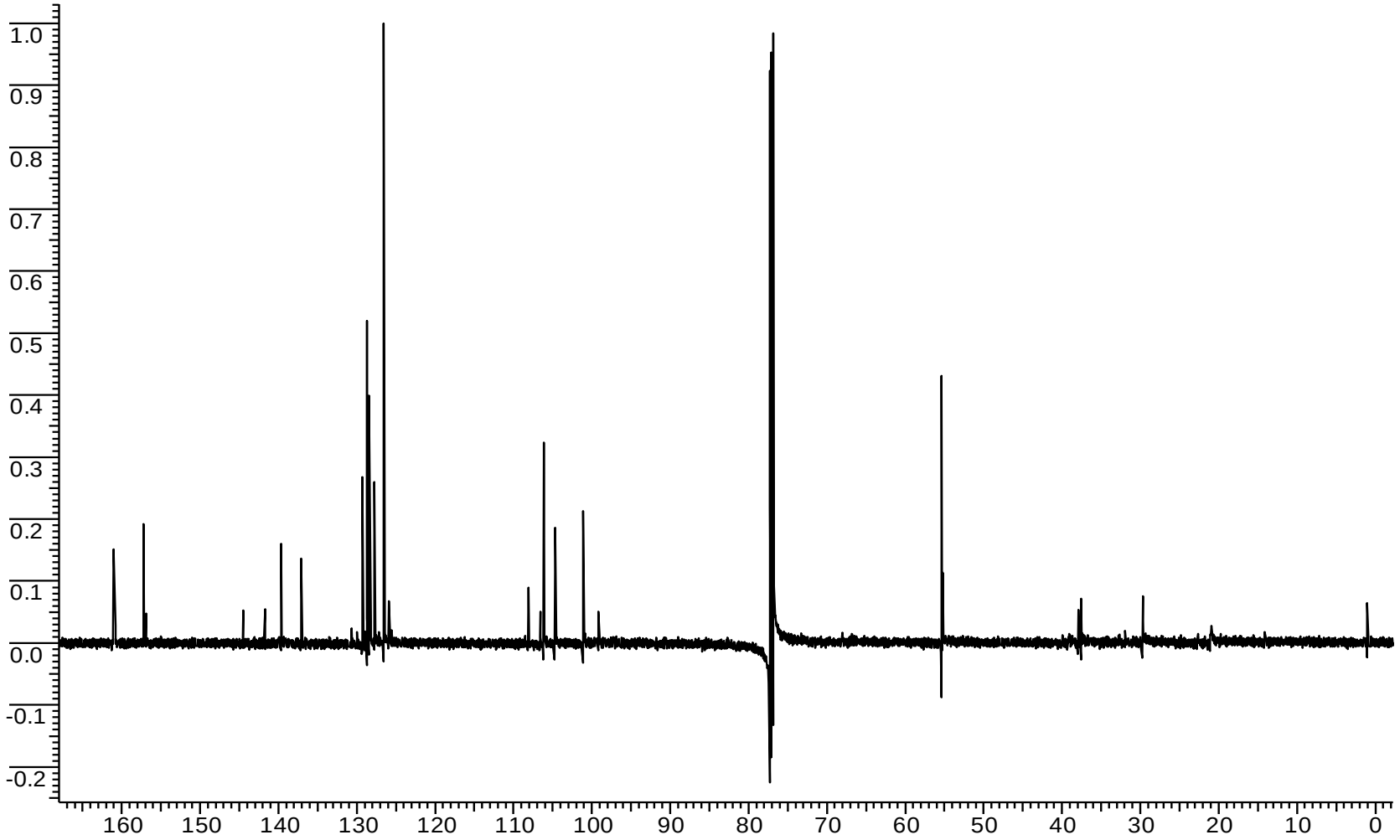
Appedix 4: GC-MS spectra of the oil



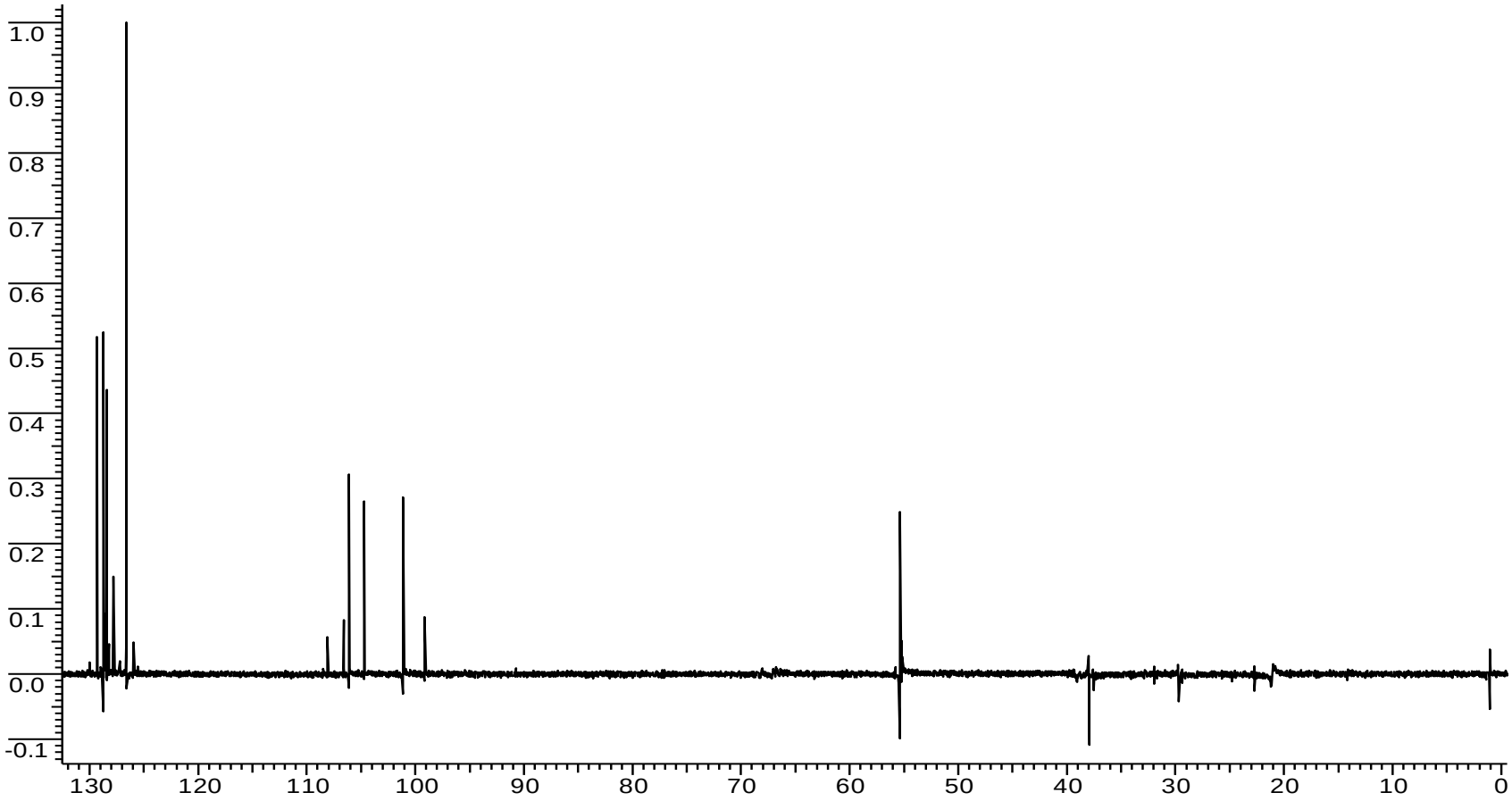
Appedix 5: ^{1}H NMR spectrum for compound X



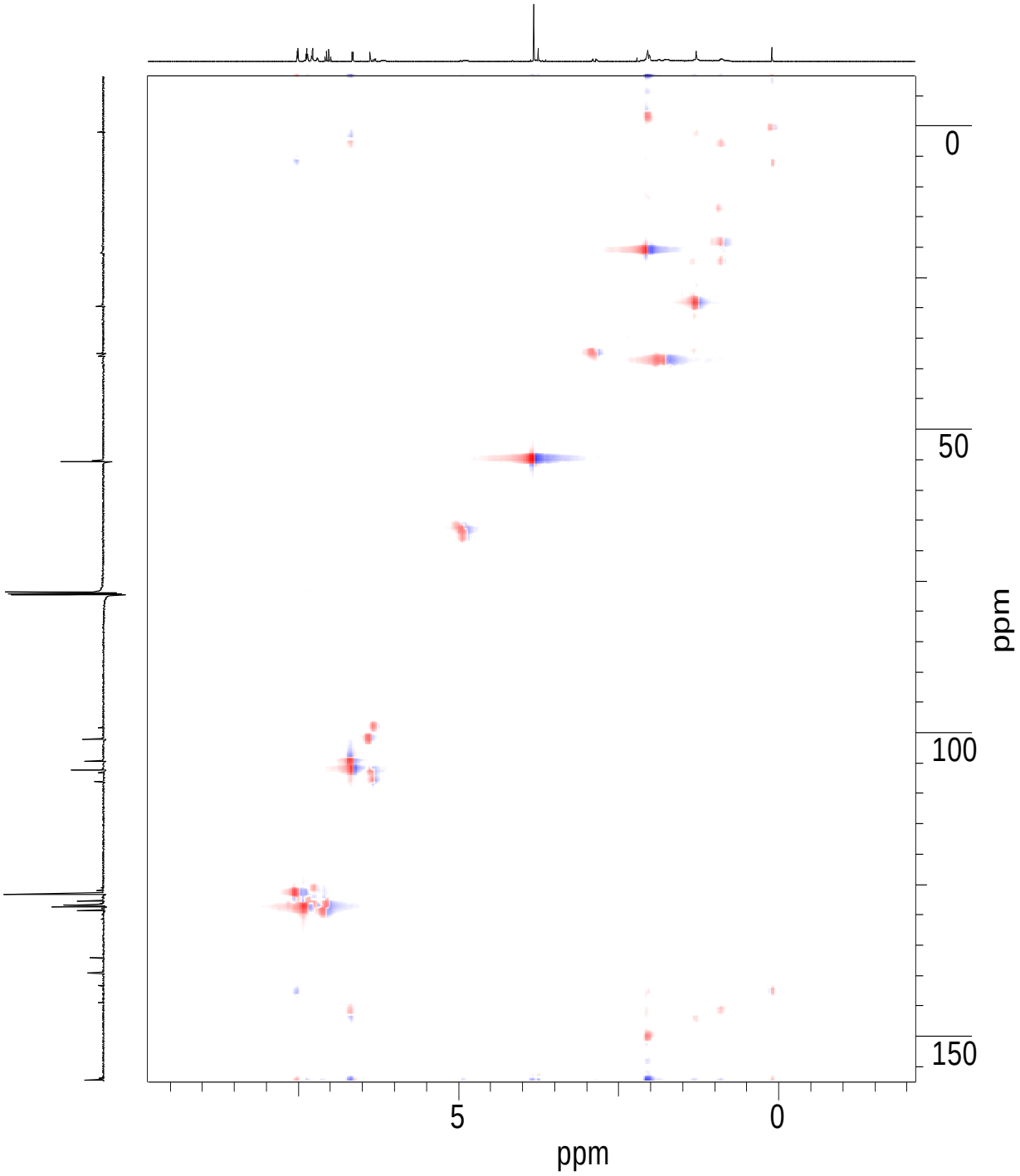
Appedix 6: ^{13}C NMR spectrum for compound X



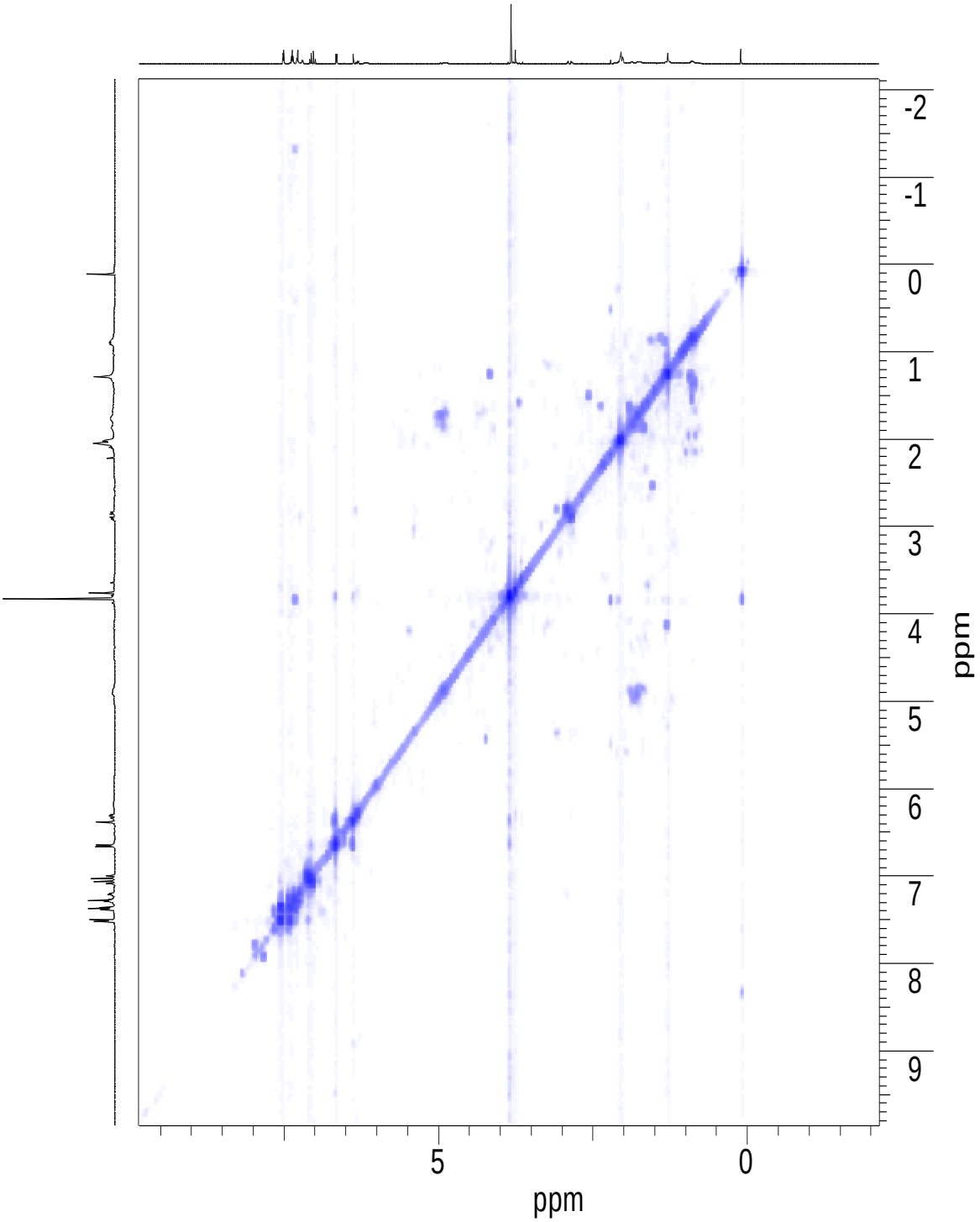
Appedix 7: DEPT NMR spectrum for compound X



Appedix 8: HSQC NMR spectrum for compound X



Appedix 9: COSY NMR spectrum of compound X



Appedix 10: HMBC NMR spectrum for compound X