MYCOTOXIN LEVELS IN CHICKEN FEEDS AND PRODUCTS AND EFFECTS OF EXPOSING ASSOCIATED FUNGI TO SELECTED PLANT ESSENTIAL OILS IN KENYA

GITHINJI IRENE MUENI (M.Sc.)
I84/30671/2015

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY OF IN THE SCHOOL OF PURE AND APPLIED SCIENCES OF KENYATTA UNIVERSITY

DECEMBER, 2020

DECLARATION

I, Irene Mueni Githinji, hereby declare that this thesis is my original work and has not been presented in any other institution for the award of a degree or any other award.

Signature.....Irene..... Date ……18/03/2021……..

Supervisors:
We confirm that the work reported in this thesis was carried out by the candidate under our supervision.

Dr. Margaret Mwihaki Ng'ang'a
Department of Chemistry
Kenyatta University

Signature ---------- Date ……18/03/2021……..

Prof. Ethel O. Monda
Department of Biochemistry, Microbiology and Biotechnology
Kenyatta University

Signature Date ……18/03/2021……..

Prof. Ahmed Hassanali
Department of Chemistry
Kenyatta University

Signature ---------- Date 18/01/2021

DEDICATION

This thesis is dedicated to my loving family for their support and understanding.

ACKNOWLEGMENTS

First, I would like to give thanks the Almighty God for blessing me with the strength, good health and determination to undertake the research. I do immensely appreciate the effort of my academic supervisors Dr. Margaret Ng'ang'a, Prof. Ethel Monda and Prof. Ahmed Hassanali of Kenyatta University, for their guidance, timely response and support in every step of the study that led to the successful completion of this thesis. I convey my thanks to Dr. Janet Irungu of ICIPE, Department of Bee Health, where part of the analyses were conducted.

I appreciate the technical staff laboratory of the Departments of Chemistry and Microbiology for the assistance offered during the research. I acknowledge the Kenyatta University, School of Pure and Applied Sciences Dean's research grant for the financial support to conduct this research. Special thanks go to my family for their love, encouragement and resilience during my study.

TABLE OF CONTENTS

FIGURE

LIST OF PLATES

LIST OF TABLES

LIST OF ABBREVIATIONS AND ACRONYMS

AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin-G1
AFG2	Aflatoxin-G2
AFPA	*Aspergillus flavus* and *Parasiticus* agar
AFTs	Aflatoxins
ANOVA	Analysis of Variance
CZ	Czapek Dox agar
ESI	Electronspray ionization
EU	European Union
FAO	Food and Agriculture Organization
FB1	Fumonisin B1
FDA	Food and Drug Administration
GC-MS	Gas chromatography–mass spectrometry
HPLC	High performance liquid chromatography
IARC	International Agency for Research on Cancer
JECFA	Joint Expert Committee on Food Additives
LC-MS	Liquid chromatography-mass spectrometry
MEA	Malt extract agar
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
NIST	National Institute of Standards and Technology
OTA	Ochratoxin A
OTB	Ochratoxin B
OTC	Ochratoxin C
OTD	Ochratoxin D
PCNB	Pentachloronitrobenzene
PDA	Potato dextrose agar
PPA	Peptone-pentachloronitrobenzene agar
WHO	World Health Organization

ABSTRACT

Mycotoxins are secondary metabolites produced by fungi. The most toxic mycotoxins are particular secondary metabolites of *Penicillium*, *Aspergillus* and *Fusarium* fungi species that infect a variety of human food and animal feeds. Poultry farming has become one of the most profitable businesses in Kenya because of the growing demand of chicken and their eggs by households, hotels and restaurants. Besides creating employment, poultry meat and eggs are an alternative source of proteins for many people in both urban and rural areas. However, there is increased risk that poultry feeds may contain potential microbiological and toxicological contaminants that may compromise their safety and nutritional value. This study sought to quantify the levels of ochratoxin A (OTA), fumonisin B1 (FB1) and aflatoxin B1 (AFB1) in poultry feeds and chicken products using the Liquid chromatography-mass spectrometry technique, and to screen essential oils of selected ethnobotanical plants, individually and in different blends as potential inhibitors of fungi produced by *Fusarium* and *Aspergillus* species. Chicken feeds and chicken products were sampled from different agro-ecological zones in Kenya, where poultry farming is widely practiced. From the sampled chicken products and poultry feeds, 13.3 % and 46.9 % respectively were found to be OTA contaminated. All the poultry feed samples were found to be FB1 contaminated, while all the chicken products showed no detectable FB1 levels. AFB1 contamination occurred in 43.3% and 59.3 % of the sampled chicken products and poultry feeds, respectively. Contamination levels of OTA, AFB1 and FB1 differed significantly within the study regions and in different chicken feeds and products. Chicken feed and products from Kakamega County had the highest levels of OTA and AFB1 contamination. The mean levels of OTA in samples from Kakamega were 165.33±0.42 and 7.12±1.25 ng/g for chicken feeds and chicken products respectively. Kakamega had average levels of AFB1 of 32.44±1.54 ng/g in chicken feed and 4.10±1.33 ng/g in chicken products. Makueni County had the highest level of FB1 contamination in chicken feed (27700.00 ng/g). FB1 was not detected in all the chicken products. The mycelial diameter of *A. niger*, *A. flavus* and *F. verticillioides* decreased with increase in the concentration of each of the three plant essential oils, *Lippia javanica, Ocimum gratissimum* and *Toddalia asiatica*, from 1 µL/mL to 32 µL/mL. Essential oil of *T. asiatica* were most effective, followed by that of *O. gratissimum,* and lastly that of *L. javanica*. Of the blends of two essential oils, that of *O. gratissimum* and *T. asiatica* resulted in the least mycelia diameter, thus the best inhibitory activity against the growth of *A. niger, A. flavus* and *F. verticillioides*. On the other hand, the blend of *O. gratissimum* and *L. javanica* showed the least inhibition activity. A blend of all the three essential oils gave the highest growth inhibitory activity against the fungi, showing synergistic effects of various constituents of the three oils. Thus, this blend of *L. javanica, O. gratissimum* and *T. asiatica* at 32 µL/mL shows potential for use in the preparation of an efficient fungi inhibitor. Essential oils of *L. javanica*, *O. gratissimum* and *T. asiatica* contain phytochemicals that can be explored for mycotoxin causing fungi growth inhibition. However, further studies on the duration of efficacy, its toxicity, and constituents associated with the synergistc effect needs to be carried out.

CHAPTER ONE

INTRODUCTION

1.1 Background information

About 25 % of annual agricultural products in the world, amounting to 1 billion metric tons, go to waste as a result of contamination with mycotoxins (FAO, 2004; World Bank, 2011). Mycotoxins contamination leads to reduced agricultural output value, which occurs as a result of toxigenic fungi induced diseases. The mycotoxin induced diseases are responsible for increased human and animal health cost as well as losses in animal productivity. In other scenarios, high costs are incurred in mycotoxin management such as research cost, mitigation, sampling, control and prevention. Therefore, mycotoxins contamination also has economic implications which concern multiple sections of a society.

Mycotoxin contamination occurs as a result of improper drying of agricultural harvests and poor storage. In Africa, most of the contaminated products are grains, which are fed to farm animals and poultry (FAO, 2004). Feed contamination with fungi increases poisoning risk of animal products, decreases their nutritional value and damages its organoleptic properties. The level of mycotoxin toxicity after consumption of contaminated feeds and foods is determined by the sensitivity of the animal, number of metabolites formed, amount of absorption and the exposure period (FAO, 2004).

Poultry are more susceptible to mycotoxin contamination than other farm animals (Iqbal *et al.*, 2014). Mycotoxins like T-2 toxin, deoxynivalenol, aflatoxins, fumonisins and ochratoxin A

(OTA) affect poultry health and productivity. Some of the most abundant food contaminants that thrive well in the hot and humid environment of Africa's sub-Saharan region are aflatoxins, fumonisins and ochratoxin A. Aflatoxins are produced by fungi of the *Aspergillus* species; OTA is produced by *Penicillium* and *Aspergillus* species, while fumonisins are produced by *Fusarium* species.

In Kenya, the second largest contributor of the gross domestic product after the service sector is agriculture (Ministry of Agriculture Livestock and Fisheries, 2015). Agriculture accounts for 25 % of the gross domestic product with poultry representing a third of the agricultural contribution (Ministry of Agriculture Livestock and Fisheries, 2015). Poultry products, eggs and meat, serve as a major alternative source of proteins. According to a report by the Netherlands-African Business Council (2015), poultry consumption in Kenya is expected to triple by the year 2030 from a combination of population growth and increasing consumption among the growing middle class. Poultry contributes 3.7 % of per capital animal protein that is consumed annually.

Kenya has a poultry population of approximately 37.3 million birds. Indigenous chicken account for 84.1 %, while layers and broilers represented 14 % (Omiti, 2007). Other poultry species like turkeys, ducks, quails, ostriches, geese, pigeons and guinea fowls account for 1.9 % of poultry population (Omiti, 2007). Layers and broilers are mainly reared in the urban areas while the indigenous chicken set ups are common in the rural areas (Miriam *et al.,* 2014).

1.2 Statement of the problem

Today, the poultry diet is complemented or at times supplemented with nutritionally balanced commercial feeds that are often bought in bulk and stored by suppliers and famers (Ministry of Agriculture Livestock and Fisheries, 2015). Improper storage of feeds is associated with a number of mycotoxins, the most abundant being aflatoxin B1 (AFB1), fumonisin B1 (FB1) and OTA. Consumption of AFB1, FB1 and OTA in poultry diet is associated with deleterious effects on health and productive parameters of chicken. The chicken experience nephrotoxicity, poor egg shell quality and reduced egg production (Oliveira *et al*., 2018). According to Oliveira *et al.* (2018) the three mycotoxins are responsible for decreased daily average weight gain due to poor feed conversion and excessive diarrhea, resulting in high mortality rate.

These mycotoxins are not only a menace to poultry farming but also to human health. The FB1 and OTA have been classified by the International Agency for Research on Cancer (IARC) as group 2B possible human carcinogen (Pfohl-Leszkowicz and Manderville, 2007). The AFB1 is a confirmed group 1 carcinogen known to cause liver cancer and human esophageal cancer in addition to other diseases (Alakonya *et al.*, 2009). Cancer remains a global public health issue accounting for more deaths than malaria, TB and HIV combined with more than two thirds of the cases being reported in Africa (WHO, 2011).

Cancer arises when external agents, which include chemical carcinogens such as OTA, AFB1 and FB1, interact with genetic factors within a cell (Iqbal *et al.*, 2014). Currently, Kenya does not have strict measures to regulate or monitor OTA, AFB1 and FB1 levels in poultry feeds and

poultry products. According to a case study in Kenya by Alakonya *et al*. (2009), maize, the main ingredient in poultry feed, was found to be contaminated with FB1. There is limited documented data on the levels of OTA, AFB1 and FB1 in poultry products and feeds in Kenya and the surrounding regions.

1.3 Justification

Agriculture is one the major contributors of Kenya's gross domestic product, poultry farming accounts for more than 30 % of the total agricultural contribution (Ministry of Agriculture Livestock and Fisheries, 2015). Previous studies have shown that OTA, AFB1 and FB1 contamination can cause detrimental effects to both birds and human beings. According to Ostry *et al.* (2017) IARC has classified OTA and FB1 as possible carcinogens while AFB1 is classified as a group one carcinogen.

More than a third of the global cancer burden is in Africa (Jemal *et al.*, 2012). Cancer accounts for approximately 7 % of the deaths in Kenya, equivalent to 27,000 lives (Jemal *et al.*, 2012). There is need for African countries such as Kenya to invest in scientific research aimed at curbing the production of carcinogenic mycotoxins, such as OTA, AFB1 and FB1, in poultry products and stored agricultural feeds. This study aimed at assessing levels of the mycotoxins in poultry feeds and chicken products, and exploring growth inhibitory activities of selected essential oils and their blends against OTA, AFB1 and FB1 mycotoxin producing fungi.

Kenya has diverse and rich floras which are a vital natural resource that form the backbone of traditional medicines. Essential oils from various medicinal plants contain phytochemicals which

can be explored for their ability to inhibit the growth of *A. niger* and *A. ochraceus* which are responsible for the production of OTA in Saharan and sub-saharan climatic conditions; *A. flavus* and *F. verticillioides* which are responsible for the production of AFB1 and FB1 respectively. Growth inhibition of the fungi will positively be impacting the levels of OTA, AFB1 and FB1 in stored poultry feeds.

Medicinal plants produce chemical compounds that can be extracted for various biological functions. Ethno-medicinal plants of the Ocimum genus have been studied and found to have a range of properties, including insecticidal activity against post-harvest insect pests such as *Rhyzopertha dominica* and *Sitophilus zeamais* (Bekele and Hassanali, 2001). In previous research, ethno-medicinal plants from the Lippia genus have been reported on to show pesticidal activities (Anjarwalla *et al*., 2015). The Rutaceae family has few known species most of which are effective in the treatment of various human ailments (Orwa *et al*., 2008). Ethno-medicinal plants have numerous compounds grouped in different biochemical classes: terpenes, alkaloids, glycosides and polyphenols (Kokwaro, 2009). The plants range from edible to non-edible trees and shrubs. Trees with medicinal properties can easily be overexploited thus threatened with extinction. Greater attention has shifted to medicinal shrubs such as *T. asiatica, L. javanica* and *O. gratissimum,* which mature within shorter periods (Orwa *et al.,* 2008).

Some mycotoxins such as OTA are not metabolized in mono-gastric animals and poultry, and are thus thransmitted into poultry products, such as eggs and meat, which are an important source of proteins consumed by human beings (Iqbal *et al.*, 2014). Studies by Omiti (2007) reported that South West region has the highest poultry population, accounting for 33.6 % of the total

population followed by Rift Valley, Eastern and Central regions, which account for 26.9 %, 11.2 % and 8.9 % of Kenya's total poultry population, respectively.

Filazi *et al.* (2018) found out that specific, continuous, effective counteraction and control of mycotoxins in poultry feeds offers an opportunity to significantly improve poultry health, productivity, performance and profit. In addition to improving poultry health and enhancing food security in the country and beyond, this research aimed at documenting a detailed study on the quantification of OTA, AFB1 and FB1 levels in poultry feeds and chicken products in Kenya as a way of creating awareness on the safety of foods derived from these raw products.

1.4 Hypotheses

i. Commercial chicken feeds and chicken products (liver, eggs and meat) from Kakamega, Kiambu, Baringo and Makueni County are significantly contaminated with *A. niger, A. ochraceus, A. flavus* and *F. verticillioides.*

ii. Essential oils of *Lippia javanica, Ocimum gratissimum* and *Toddalia asiatica* can inhibit the growth of *A. niger, A. ochraceus, A. flavus* and *F. verticillioides.*

iii. Activities of the essential oils of *L. javanica, O. gratissimum* and *T. asiatica* and /or blends *on A. niger, A. ochraceus, A. flavus* and *F. verticillioides* growth inhibition are due to synergistic or additive effects of the major constituents.

1.5 Objectives

1.5.1 General objective

To quantify the levels of OTA, AFB1 and FB1 in chicken feeds and chicken products, and to screen essential oils of selected plants individually and in different combinations as poultry feed protectants against the production of OTA, AFB1 and FB1.

1.5.2 Specific objectives

i. To quantify the levels of OTA, AFB1 and FB1 in chicken feeds and products (liver, eggs and meat) obtained from Kakamega, Kiambu, Baringo and Makueni County.

ii. To evaluate the effects of varying doses and subtractive bio-assays of the essential oils of *Lippia javanica, Ocimum gratissimum* and *Toddalia asiatica* on *A. niger, A. ochraceus, A. flavus* and *F. verticillioides* and the production of OTA, AFB1 and FB1 in chicken feeds.

iii. To undertake GC-MS analyses of the essential oils and identify their major constituents.

iv. To carry out subtractive bio-assays with combinations of major constituents of the essential oils of *Lippia javanica, Ocimum gratissimum* and *Toddalia asiatica* to identify the most effective combination for inhibiting the growth of *A. niger, A. ochraceus, A. flavus* and *F. verticillioides.*

1.6 Significance of the study

This study sought to provide useful information on the levels of OTA, AFB1 and FB1 in chicken feeds and chicken products in Kenya. This information would serve as a basis for creating

awareness on food safety. The results obtained from OTA, AFB1 and FB1 quantification are expected to serve as a potential platform for Kenya and the entire East Africa to set up OTA, AFB1 and FB1 monitoring and control units within the existing standards bodies such as the Kenya Bureau of Standards and set maximum allowed levels in feeds and in chicken products. The set maximum levels would serve as a reference point in monitoring OTA, AFB1 and FB1 levels in chicken feeds and which would lay down the bases of putting in place practices and interventions that would reduce levels of the mycotoxins.

Thus, the study also sought to identify safe and readily available phytochemicals for the control of production of selected mycotoxins in stored chicken feeds. The development of specific phytochemicals with inhibitory activities against *A. niger*, *A. ochraceus*, *A. flavus* and *F. verticillioides* would promote higher productivity and better health in poultry as well as human beings.

1.7 Limitation and scope of the study

This study focused on three mycotoxins only, OTA, AFB1 and FB1. Other possible sources of OTA, AFB1 and FB1 in chicken products, apart from chicken feeds were not considered in the study. Possible effects of seasonal weather pattern variations in different agro-ecological zones on the levels of the mycotoxins in chicken feeds and products, and essential oil constituents from the selected plants were not monitored. Moreover, in this research, only chicken feeds and products were analyzed for OTA, AFB1 and FB1.

CHAPTER TWO

LITERATURE REVIEW

2.1 Mycotoxins

Mycotoxins are toxic secondary metabolites of low molecular weight produced by filamentous fungi and over 200 fungal species are known to produce mycotoxins (Carballo *et al*., 2018). The most toxic mycotoxins associated with vital diseases causing effects that affect both human and animal health, are particular secondary metabolites of *Penicillium*, *Aspergillus* and *Fusarium* fungi species (Filazi *et al.*, 2018). When these secondary metabolites are introduced into a living organism, they are capable of accumulating causing certain diseases, disorders or death (Carballo *et al*., 2018).

Mycotoxins have varied chemical structures and biological activities which include immunosuppressive, dermatoxic, oestrogenic, neurotoxic, nephrotoxic, hepatotoxicity and carcinogenic (esophagus, uterus and liver) activities. The structural diversity is associated with chemical reactions such as halogenation, alkylation, condensation, reduction and oxidation (Carballo *et al*., 2018; Filazi *et al.*, 2018). Mycotoxins are classified based on their effects in plant and animal health, bio-origin and chemical composition. Mycotoxins are complex in nature; a particular type can be produced by more than one fungal species from vast genera. One mycotoxin can be associated with adverse effects on multiple organs as well as diverse symptoms and varying levels of toxicity on different animal species (Filazi *et al*., 2018).

Incidences of feed and food contamination with mycotoxins vary from one geographical region to another. It is determined by multiple factors including chemical composition of the feed or

food substrate, climatic conditions and implementation of local and regional regulations on food quality control (Pitt *et al.,* 2012). The main determinants of mycotoxins production are a favorable climatic condition and biotic factors that favour the growth of specific toxigenic fungi (Iqbal *et al.*, 2014).

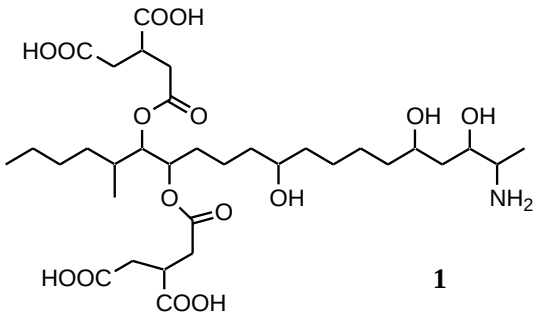
Mycotoxins poisoning in animals and humans is caused by consumption of contaminated foods and feeds. According to a study by WHO (2011), improperly stored foodstuffs and feed my have more than one mycotoxin contaminant. It is possible to have only one or more mycotoxin contamination in raw feed material and co-contamination in mixed feeds. Generally, poultry feeds comprise of vegetable substances, most of which are cultivated in different climatic conditions that vary from one region to another.

In Kenya, the main chicken feed ingredient is maize which is mixed with other cereals and in some cases fishmeal (Mutiga *et al*., 2015). Mycotoxin production and fungal growth are initiated in the fields before harvest, during storage or transportation and are mostly influenced by environmental conditions. Farming in Kenya is practiced under agro-climatic conditions that favor both mycotoxin accumulation and fungal colonization (Mutiga *et al.*, 2015).

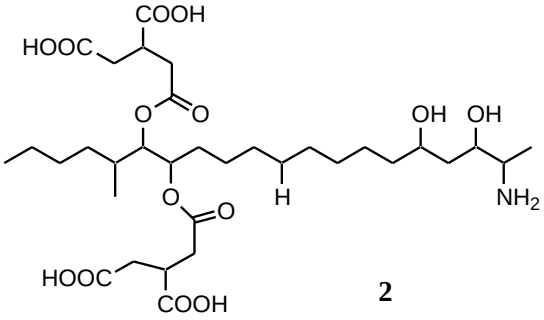
2.2 Fumonisins

Fumonisins are mycotoxins produced naturally by fungi of the fusarium species. They occur in almost all parts of the world but are most common in warm tropical and warm climate regions where maize is grown. High amounts of fumonisin contamination are common in maize and maize-based products (WHO, 2018). The main producers of fumonisins are *Fusarium*

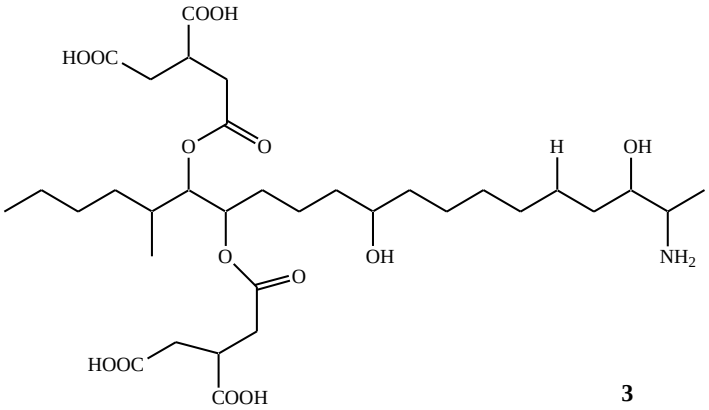
proliferatum and *F. verticillioides* (which was previously known as *F. moniliforme*). There are different types of fumonisins that are known, but fumonisins B1 **(1)**, B2 **(2)** and B3 **(3)** are the major forms found in food. Fumonisin B1 is potentially carcinogenic to humans as its intake has been linked to carcinogenic properties in various rodent species and acute poisoning in farm animals (horses and swine) including hepatotoxicity and nephrotoxicity. The different types differ in the level of toxicity with FB1 being the most toxic (Henry and Wyatt, 2001).



1



2



3

2.2.1 Fumonisin B1 (FB1)

Fumonisin B1 ($C_{34}H_{59}NO_{15}$, 721.838 g·mol^{-1}) is the most common member from the family of fumonisins, and which occurs in cereals, wheat and maize. It appears as a white to off-white

mould. Maize, a main ingredient in chicken feed is one of the crops that is most affected by FB1 contamination. The FB1 contamination has been reported in various regions at mg/kg level (Alakonya *et al.,* 2009; Mutiga *et al.,* 2015). According to IARC FBI contamination is commonly found in maize in high levels and it has been reported to be a possible carcinogen to humans (Ostry *et al.,* 2017).

2.2.2 Impact of FB1 ingestion on human health

The FB1 is classified as a class 2B carcinogen; its ingestion has a great potential to stimulate oesophageal regenerative cell proliferation that can lead to cancer of the oesophagus in humans (Ostry *et al*., 2017). It is also correlated with disruption of fat metabolism (WHO, 2018). A study conducted by Yu *et al*. (2009) in Tanzania on dietary exposure to mycotoxins from maize based foods indicated that FB1 exposure is associated with impairment in human growth; it leads to stunted growth and underweight in children. The FB1 exposure in pregnant women is associated with risks of neural tube defects in infants (Missmer *et al*., 2006; WHO, 2018).

2.2.3 Impact of FB1 on poultry health

The presence of fumonisins in poultry diet is one of the major causes of deleterious effects on health and productive parameters in chicken. Moreover, co-contamination is known to increase the level of toxicity in poultry (Filazi *et al*., 2018). Poultry rations with high FB1 contamination are associated with liver necrosis, feed refusal, poor performance, high mortality, oral lesion, weak legs and diarrhea in addition to poor feed conversion, which result in economic losses for

the farmers. The contamination blocks the synthesis of a class of lipids known as sphingosine, 2-amino-4-trans-octadecene-1,3-diol, which plays a vital role in membrane, muscle and nerve protection (Henry and Wyatt, 2001).

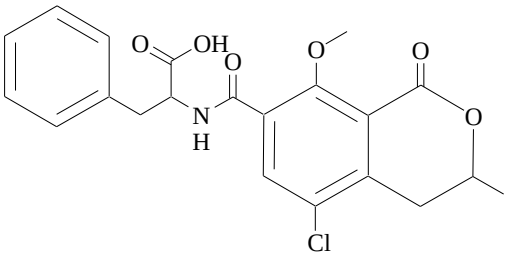
2.2.4 FB1 regulation

The joint Expert Committee on Food Additives (JECFA), formed from collaboration between FAO and WHO, recommends FB1 maximum tolerable daily intake of 2 mg/Kg body weight in human beings. The JECFA also recommends observation of maximum levels for FB1 in animal and poultry feeds. The European Union (EU) also has set maximum allowed levels for FB1 in animal feeds and foodstuff (FAO, 2004). The EU has standards of FB1 adopted based on a 2006 opinion by the Scientific Panel on Contaminants in Food (Banks *et al.*, 2004). According to EU (Commission Recommendation 2016/1319/EC) regulations, food intended for human consumption should not exceed 5 mg/Kg while poultry feed containing maize by products should not exceed 20 mg/Kg (Pitt *et al*., 2012).

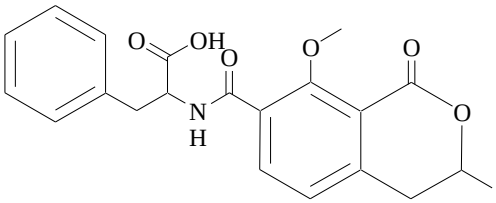
2.3 Ochratoxins

Ochratoxins are secondary metabolites produced by fungus of the Penicillium and Aspergillus species. Biosynthetically, they have pentaketide structures derived from isocoumarins family of compounds. The isocoumarins are linked to a β-phenylalanine. There are four known ochratoxin metabolites, namely ochratoxin A (OTA), B (OTB), C (OTC) and D (OTD), the molecular formulae of which are $C_{20}H_{18}ClNO_6$ **(4)**, $C_{20}H_{19}NO_6$ **(5)**, $C_{22}H_{22}ClNO_6$ **(6)** and $C_{20}H_{16}ClNO_7$ **(7)**

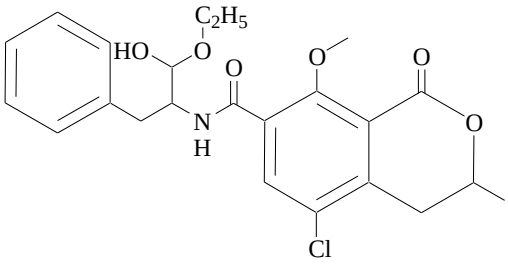
respectively. The four metabolites slightly differ from each other in their chemical structures and have varying levels of toxicity. OTB is the non-chlorinated form of OTA, OTC is OTA's ethyl ester while OTD is a 4-hydroxyl form of OTA. OTA is the most abundant and most toxic form of the OT family (Nuhu, 2015).



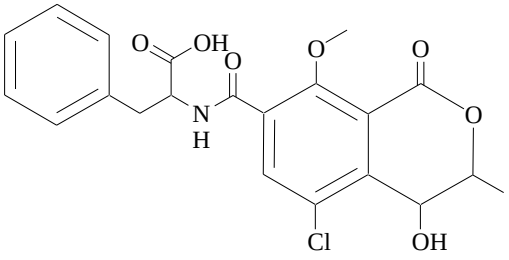
4



5



6



7

2.3.1 The OTA

Ochratoxin A (*N*-[(3*R*)-(5-chloro-8-hydroxy-3-methyl-1-oxo-7-isochromanyl)carbonyl]-L-phenylalanine) is a fungal toxin that occurs naturally and is a colorless crystal under normal light at room temperature with a melting point of 168 ◦ C (el Khoury and Atoui, 2010). It is produced by a variety of fungal species, which include *Aspergillus ochraceus, A. carbonarius, A. niger* and *penicillium verrucosum*. *A. carbonarius* only infects cocoa, coffee and cereal grains (Nuhu, 2015). *P. verrucosum* infects stored feeds and cereal grains in temperate climate experienced in

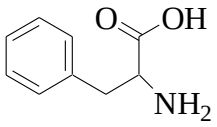
northern Europe, Canada and parts of South America. *P. verrucosum* is able to survive, grow and produce OTA at temperatures as low as 0°C (Nuhu, 2015). *A. niger* and *A. ochraceus* are found in tropical and sub-tropical climatic regions and grows to produce OTA at optimum temperatures of between 13 °C and 37 °C. *P. verrucosum, A ochraceus* and *A. niger* are storage fungi (Terra *et al.*, 2013).

OTA exhibits blue and green fluorescence in ultraviolet light. It moderately dissolves in organic solvents such as xylene, chloroform, methanol and ethanol but it is soluble in water. It has a molecular weight of 403.81 g/mol and is a heat stable compound that has been reported to maintain its structure after autoclaving for duration of up to three hours (el Khoury and Atoui, 2010).

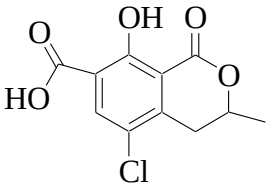
The OTA toxicity is a result of its chemical structure with a phenol group in addition to its isocoumarin structure and its amide linkage to phenylalanine (el Khoury and Atoui, 2010). Its metabolites include OTB, OTC and OTD. The main source of OTA in both animal and human body is dietary. Human beings ingest OTA through the consumption of OTA contaminated meat products, legumes, cereals, grapes, coffee-beans, cocoa-beans, spices, nuts, dried vine fruits products (el Khoury and Atoui, 2010). Animals ingest OTA through the consumption of OTA-contaminated feeds.

Previous studies show that ruminants such as cattle are rarely affected by consumption of ochratoxin contaminated feeds (Yasushi *et al*., 2015). In ruminants, the ochratoxins are detoxified naturally during digestion. Ruminants have microorganisms within their

gastrointestinal tract which have the ability to hydrolyze the amide bond that links the L-β-phenylalanine to the 7-carboxy group. Hydrolysis of the OTA **(4)** leads to production of a non-toxic phenylalanine **(8)** and OTα **(9)**.



8



9

The intake of OTA contaminated feeds by farm animals only affects poultry and mono-gastric animals like pigs (Yasushi *et al.*, 2015). When ingested by poultry or mono-gastric animals, OTA can be present in the end products such as meat (Yasushi *et al*., 2015). According to Sherazi *et al.* (2015), OTA has a strong affinity to proteins, which promotes bioaccumulation in animal organs, and thus the contamination is carried over along the food chain.

The WHO describes the occurrence of OTA in human food products of animal and vegetal origin as a potential international health hazard (WHO, 2011). Acidic properties of the stomach aid in the absorption of OTA found in human diet. Absorption of OTA can also occur in the gastrointestinal tract and the small intestine (Pfohl-Leszkowicz and Manderville, 2007). After absorption into the blood stream, the OTA is distributed to the body fat, muscles, liver and kidneys. The mycotoxin has the ability to bind to the human serum albumin. Besides being detected in adult human beings' blood and milk, the mycotoxin has been found in infants' sera (Pfohl-Leszkowicz and Manderville, 2007).

2.3.2 Impact of OTA on human health

According to the International Agency for Research on Cancer (IARC), OTA is categorized as a group 2B possible carcinogen (Kunio and Uetsuka, 2011). The OTA causes renal carcinoma in human beings and is considered teratogenic and genotoxic as it damages both the foetus and DNA (Kunio and Uetsuka, 2011). It has been reported in previous studies to be weakly mutagenic by initiation of oxidative DNA damage (Bezencon *et al*., 2007). The mycotoxin is a potent nephrotoxin that causes a severe kidney disease, Balkan endemic nephropathy, and the development of tumors in the human urinary tract (Fuchs and Peraica, 2005). Moreover, it has also been associated with nutritional disorders such as stunted growth, immune suppression, hepatic damage, reproductive disorders and hepatocarcinoma (Fuchs and Peraica, 2005). The extent of the health effects correlates to the levels of OTA exposure (Government of Hong Kong, 2006).

2.3.3 Impact of OTA on poultry health and productivity

Consumption of OTA contaminated feeds in poultry causes ochratoxicosis. Ochratoxicosis in poultry is accompanied by a reduction in weight gain due to poor feed conversion, excessive urine excretion and diarrhea. Chicken suffering from ochratoxicosis experience nephrotoxicity and poor egg shell quality in addition to a reduced egg production. The consumption of OTA in chicken increases the embryonic mortality thus reduces the chicken’s hatchability.

Presence of OTA in chicken feed at 1 mg to 5 mg/Kg alters the biochemistry of the birds' serum (Iqbal *et al*., 2014). The alteration includes a decrease in the total protein, cholesterol, globulin, triglyceride levels, potassium and albumin. The OTA triggered serum biochemistry alteration in chicken can lead to increases in creatine levels and uric acid as well as in gamma glutamine transpeptidase and serum alkaline phosphate activities. Effects of OTA in poultry depend on the time exposure and concentration of the toxin per kg feed (Saleemi *et al.,* 2010).

2.3.4 The OTA regulation

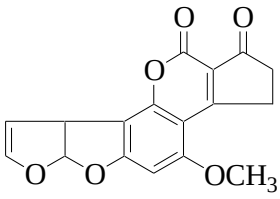
In the last decades, the knowledge that OTA can cause serious health and economic effects on humans and animals has led various countries and regions to regulate its occurrence in feed and food (WHO, 2018). The OTA regulations do not only protect human beings from the harmful effects but also harmonize fair practices in food trade and engender well-being and, increase a nation's economic viability (FAO, 2004). Kenya and East Africa as a region has not adopted any set limits on OTA. Relatively few countries around the world, such as Uruguay, Switzerland, Israel, Brazil and the European Union (EU) have set maximum allowed levels for OTA in feeds and foodstuff (FAO, 2004).

The EU recommends a maximum of 100 ng/g of human body weight as the tolerable weekly intake. Specific food commodities have selected numerical values for the maximum OTA levels (Banks *et al.,* 2004). The set limits for OTA in commodities intended for direct human consumption range between 2-10 ng/g while those intended for feed preparation have a limit of 100 ng/g (Banks *et al*., 2004; FAO, 2004).

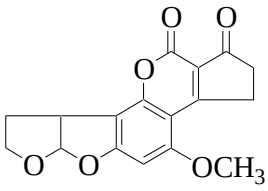
2.4 Aflatoxins

Aflatoxins (AFTs) are toxic metabolites produced by *A. parasiticus*, *A. flavus* and *A. nomius* which are abundant in humid and warm climatic regions. There are different types of AFTs, classified according to their level of toxicity. There exists more than 16 AFTs, of which aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin-G1 (AFG1) and aflatoxin-G2 (AFG2) are the most toxic homologues. The level of toxicity increases from AFG2, AFB2, AFG1 to AFB1. The AFTs are difuranocoumarin derivatives with a bifuran group and either a six-membered lactone ring or a pentanone ring attached on opposite sides of the coumarin nucleus. The AFB1 **(10)** and AFB2 **(11)** have a bifuran group and a pentanone ring attached on the coumarin nucleus while AFG1 **(12)** and AFG2 **(13)** have a bifuran group and a six-membered lactone ring attached on the coumarin nucleus (Grío *et al*., 2010).

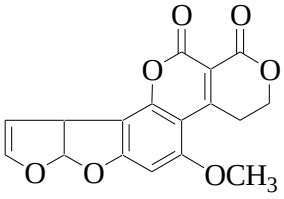
The AFTs producing fungi contaminate improperly stored farm produce like grains and cereals, feeds, soil and decaying vegetation. When animals are fed with AFTs contaminated feeds, the contaminants can be passed to the products such as meat, milk and eggs. The AFTs are major food contaminants that harm human and almost all animal species' health. The AFTs are potent toxins, carcinogens and mutagens (Grío *et al*., 2010). *A. nomius* is a non-agricultural contaminant (Grío *et al*., 2010). *A. parasiticus* and *A. flavus* are abundant staple food contaminants. They contaminate crops like nuts and maize during drying, storage or in the farms during growth. *A. parasiticus* is responsible for the production of AFTs in groundnuts while *A. flavus* is associated with the production of AFTs in maize (Monda and Alakonya, 2016). *A. parasiticus* produces AFG1, AFG2, AFB1 and AFB2, while *A. flavus* produces AFB1 and AFB2.



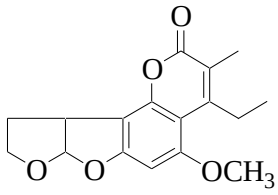
10



11



12



13

2.4.1 Aflatoxin B1

The AFB1 is the most toxic aflatoxin associated with various health defects in all species tested though the degree of sensitivity varies from one species to another (Sarma *et al*., 2017). It is produced by *A. parasiticus* and *A. flavus*. It is a common contaminant in various foods such as milk, meat, cereal grains and animal feeds. The AFB1 is the most hepatocarcinogenic and hepatotoxic mycotoxin that has been reported and affect the health of all species tested (Grío *et al*., 2010).

2.4.2 Impact of AFB1 on human health

Human beings can be exposed to AFTs through the consumption of contaminated milk, meat or plant products. The AFTs can also penetrate into the body through the skin or by inhalation of dust generated when handling contaminated feeds and crops (Sarma *et al*., 2017). Ingestion of small AFB1 doses for a prolonged duration is carcinogenic. According to IARC, AFB1 is the most potent natural human carcinogen (WHO, 2011).

The AFB1 is known to cause liver cancer, known as hepatocellular carcinoma. According to global risk assessment studies, AFT exposure results to about 25,200 to 155,000 human liver cancer diagnosis annually where 40 % of the cases are reported in Africa (Monda and Alakonya, 2016). Besides being carcinogenic, chronic AFB1 exposure can result in immune suppression and nutritional deficiency which at a later stage results in morbidity and mortality especially in children. The AFT exposure is associated with low birth weight of babies and stunted growth in children. Acute exposure of AFB1 contamination is characterized by acute liver damage, edema, haermorrage and death in humans (WHO, 2018).

Ingestion of large doses from highly contaminated foods or feeds by humans and animals respectively results in decreased liver function, which can lead to decreased serum proteins, jaundice, blood clotting mechanism, abdominal pain, vomiting, edema and death (Sarma *et al.*, 2017). In Kenya, aflatoxicosis related deaths have been reported; the first report was in 1982 where 12 people lost their lives due to chronic AFT exposure (Probst *et al*., 2007). The worst known case of aflatoxicosis was reported in Kenya in 2004; 317 people suffered from AFT

toxicity where more than 125 of them died (Probst *et al*., 2007). Reports on mycotoxin analyses revealed that *A. flavus* in maize as the cause of the aflatoxicosis (Probst *et al*., 2007).

2.4.3 Impact of AFB1 on poultry health and productivity

The AFB1 contaminated poultry feed can lead to various effects which affect the entire body system. The AFB1 is associated with interference in bone metabolism thus resulting into various deformities as a result of decreased bone diameter and strength. It also disrupts muscle arrangement resulting into an increased weight in thigh, wings, and back and decreased weight in breast (Filazi *et al.*, 2018).

Clinical signs in poultry feeding on AFB1 contaminated feeds include decreased growth and appetite, decreased egg hatchability, discoloration of feet and legs, picking of feathers, convulsions, lameness and death (Filazi *et al.*, 2018). The AFB1 causes deficiency of fat-soluble vitamins (K, E, D and A) and also inhibits synthesis and transportation of lipids in the poultry (Iqbal *et al*., 2014).

2.4.4 The AFB1 regulation

In addition to the quality and health of chickens, AFB1 contaminants can be passed on to humans along the food chain. Various studies have confirmed the presence of aflatoxin residues in chicken muscles and eggs (Iqbal *et al*., 2014). The AFB1 regulations are important in the

control of aflatoxicoses. AFB1 toxicology in humans can vary depending on age, health status, length and degree of exposure.

Kenya has an AFT regulation similar to that set by the Food and Drug Administration and the US department of Agriculture where the tolerance limit in food meant for human consumption set at 20 µg/Kg (FAO, 2004). Various foods have varying limits in different nations. The European Commission has maximum acceptable limit for AFTs in animal feeds set at 2 $X10^{-2}$ mg/Kg (Pitt *et al*., 2012).

2.5 Methods of controlling OTA, AFB1 and FB1

Fungal growth can either take place during storage, harvesting or before harvesting. *A. niger* and *A. ochraceus* are predominantly storage fungi while *F. verticillioides* grows in the field environment. *A. flavus* has a potential to contaminate farm produce during storage and, before and during harvesting. A variety of control strategies can be adopted either for fungal growth inhibition or mycotoxin elimination. Growth inhibition for specific mycotoxin producing fungi is an efficient approach in the management of OTA, AFB1 and FB1 production in animal and poultry feeds, thus minimizing entry of the mycotoxin into higher levels of the food chain (Cicoňová *et al*., 2010).

2.5.1 Physical control

This involves removal of OTA, AFB1 and FB1 contaminated portions by solvent extraction, mechanical separation, density segregation and heat treatment (Varga *et al.*, 2010). Physically damaged and infected grains can be sorted out thus significantly reducing mycotoxins contamination.

Dehulling can be carried out as a control mechanism of mycotoxins in whole grains. Dehulling has been practiced in Kenya, where the impermeable maize testa is removed (Mutiga *et al*., 2015). This method has proved to be effective, but time consuming. In some instances, physical control method can be very expensive thus limiting its access and increasing the cost of production especially where the employment of radiations is required. Radiations have also been utilized in mycotoxin control due to their ability to convert toxins into innocuous compounds (Varga *et al.*, 2010). Adjuvants can also be added to contaminated feeds to minimize the toxic effects of the mycotoxins (Varga *et al.*, 2010).

Processes such as nixtamalization lead to the production of less toxic foods and feeds. Nixtamalization is a traditional food preparation method common in Central America and Mexico, where maize is treated with lime before cooking then dried and ground into flour. Nixtamalization has been proven effective in the reduction of fumonisins and AFTs contamination as well as their toxic effects by over 45 % (Standard Digital Media, 2018). In Kenya, Food Security and Nutrition has listed it as one of the four primary development agendas dubbed ‘the big four’. Nixtamalization is currently practiced in Kenya, after the launch of a

Nixtamalization machine, as a mycotoxins control measure and enhancement of food security (Standard Digital Media, 2018).

2.5.2 Chemical method

This involves the use of chemicals to inactivate and destroy FB1, AFB1 and OTA producing fungi. Alkaline chemicals such as bicarbonate salts of potassium and sodium, ammonium hydroxides, ammonium carbonates, potassium carbonates, sodium carbonates and sodium hydroxides have the capacity to degrade various mycotoxins (Varga *et al.*, 2010). Most proposed chemical treatments are not attractive as they lead to depletion of food quality (Faruk, 2009). The chemicals used for mycotoxins control in food need to be removed by rinsing thoroughly before consumption, a practice that does not guarantee 100 % chemical elimination (WHO, 2018). When used for mycotoxins control in feed, then washing may not be practical, thus the chemicals are ingested by animals despite their negative health effects (WHO, 2018).

2.5.3 Biological control

Biological control method utilizes the use of microorganisms to inhibit OTA, AFB1 and FB1 producing fungi. Yeasts such as epiphytic, have the ability to colonize plant wounds or surfaces for lengthy periods especially under extremely low moisture levels. Some bacteria, such as lactic acid bacteria, produce antifungal properties that have been reported to control OTA, AFB1 and FB1 production (Probst *et al*., 2011).

Some fungi, such as *A. flavus* are saprophytic in soil. Biocontrol can also be achieved via introduction of an atoxigenic strain to the soil to outcompete the toxigenic strain. Atoxigenic strains refer to non-mycotoxin producing fungi strains. One of the atoxigenic strains, NRRL 21882 – Syngenta, has been developed and currently being tested for efficacy in various countries, including Kenya, Senegal, Zambia and Nigeria (Dorner, 2010). The NRRL 21882 - syngenta is designed to out-compete AFTs thus reducing AFT contamination by 80-95 %. Apart from being very costly, the main potential drawback is the possibility of nuclear fusion and cytoplasm fusion between atoxigenic and toxigenic strains, which is capable of exacerbating the specific mycotoxin contamination problem (Dorner, 2010). Some ethno-medicinal plants have antifungal and antimicrobial activities. Studies on ethno-medicinal plants and especially their essential oils are gaining popularity due to the high degree of efficacy reported from previous research.

2.6 Ethno-medicinal plants

Africa is gifted with world-renowned biodiversity constituting a vast range of food plants used for therapeutic purposes, as food and for medicinal purposes (Ng'ang'a *et al*., 2011). Medicinal plants produce chemical compounds that can be extracted for various biological functions. Ethno-medicinal plants of the Ocimum genus have been studied and found to have a range of properties, including insecticidal activity against post-harvest insect pests such as *Rhyzopertha dominica* and *Sitophilus zeamais* (Bekele and Hassanali, 2001).

In previous research, ethno-medicinal plants from the Lippia genus have been reported on to show pesticidal activities (Anjarwalla *et al*., 2015). The Rutaceae family has few known species most of which are effective in the treatment of various human ailments (Orwa *et al*., 2008). Ethno-medicinal plants have numerous compounds grouped in different biochemical classes: terpenes, alkaloids, glycosides and polyphenols (Kokwaro, 2009). The plants range from edible to non-edible trees and shrubs. Trees with medicinal properties can easily be overexploited thus threatened with extinction. Greater attention has shifted to medicinal shrubs such as *T. asiatica, L. javanica* and *O. gratissimum,* which mature within shorter periods (Orwa *et al.*, 2008).

2.6.1 *Toddalia asiatica* L.

T. asiatica (common name, orange climber) is flowering plant belonging to the Rutaceae family and the only species of the Toddalia genus. It is an endangered species in Kenya because of its widespread use by different communities for its medicinal properties (Kokwaro, 2009). *T. asiatica* is a liana with thorny stems whose length can reach upto 10 m. It grows as a bush in non-forested areas. It bears orange coloured fruits with a citrus scent; its flowers are yellow-green and the leaves appear shiny green (Plate 2.1).

T. asiatica contains various phytochemicals, including tannins, flavonoids, alkaloids and glycosides which are responsible for its pharmacological properties (Praveena and Suriyavathana, 2013). *T. asiatica* has been used in the past to treat and manage various diseases and ailments such chest pain, malaria, stomach problems, sore throat, coughs and food poisoning (Orwa *et al.*, 2008).

Plate 2.1: Leaves and fruits of *Toddalia asiatica* L. (Anjarwalla *et al*., 2015)

2.6.2 *Ocimum gratissimum* L.

O. gratissimum (common name, African basilica) belongs to the lamiaceae family which has more than 150 species (Prabhu *et al.*, 2009). It is a drought resistant shrub with branched stems and can grow up to a height of 1.9m. Its leaves have a serrated margin, a dotted surface and ovate in shape (Plate 2.2).

O. gratissimum is a traditional herb used by various communities in Kenya. Each community has a vernacular name by which it is referred; for example, in Kenya the Kamba call it *mutaa* (Kokwaro, 2009). It is used in the treatment of fever, coughs, blocked nostrils, diarrhea, regulation of menstruation and barrenness, abdominal pains, ear infection, prolapse of the rectum and sore eyes (Kokwaro, 2009).

Previous studies on the antibacterial activities of *O. gratissimum* have shown its active inhibition on a number of disease-causing organisms such as *Staphylococcus aureus, Shigella sonnei, Escherichia coli* and *Bacillus subtilis. O. gratissimum* has also been reported to have antifungal activities on various organisms, which include *Microsporum canis, Epidermophyton floccosum, Trichophyton rubrum* and *Trichophyton mentagrophyte* (Prabhu *et al.*, 2009). The gratissimum species is an effective growth inhibitor for many microbes and fungus though some studies have reported its inactivity on some strains such as *Klebsiella pneumonia* which causes meningitis and blood stream infections, and *Pseudomonas aeruginosa* which causes various infections in AIDs and cancer patients, as well as those with severe burns (Kokwaro, 2009).

Plate 2.2: Leaves of *Ocimum gratissimum* L. (Anjarwalla *et al*., 2015)

2.6.3 *Lippia javanica* (Burm. f.)

L. javanica belongs to the verbenaceae family and it is commonly referred to as the lemon bush. It is one of the approximately 200 species of the lippia genus. It grows as a 1 to 2m shrub on hillsides, forest fringe and grasslands. The stems of *L. javanica* are heavily branched. The leaves of the shrub have a hairy surface and elliptical shape with a dentate margin. *L. javanica* is common in southern and eastern Africa where it has different vernacular names. In eastern Africa, the plant is used by various communities associated with its pharmacological properties. Each community refers to it by a vernacular name; for example, the Maasai call it *oreitai* (Kokwaro, 2009). The javanica species grow in various soil types and are drought resistant.

In previous studies, *L. javanica* has been found to have pesticidal properties: it is used as an insect repellant, especially against the bark beetle, a wood borer that can cause tree mortality (Anjarwalla *et al.*, 2015). In ethnomedicine, *L. javanica* is used in the treatment of bites and stings, skin rash and malaria, and also as a parasite repellant. It is also used as a cosmetic especially by the Maasai community of Kenya, who decorate their bodies with its red ointment. Traditionally, its leaves are mixed with hot tea and used as a skin moisturizer (Kokwaro, 2009).

Plate 2.3: Leaves and flowers of *Lippia javanica* (Anjarwalla *et al*., 2015).

2.7 Essential oils

Essential oils are concentrated hydrophobic liquids that exist naturally in plants. Essential oils extracted from medicinal and aromatic plants are volatile and each has a characteristic pleasant scent. They are mainly extracted from the leaves, fruit rinds, seeds, flowers, stems, bar or roots of various plants (Thiem *et al*., 2011). Most essential oils exist as mixtures of diverse chemical compounds that belong to different families including ketones, ethers, phenolic, esters, alcohols, aldehydes and terpenes (Thiem *et al*., 2011).

Essential oils have complex chemical compositions with most of them exhibiting more than 20 bioactive components, with the chemical characterization revealing only 2 to 3 major

components at 20-70 % concentration while others appear in smaller or trace amounts (Matasyoh *et al*., 2007). Studies by Bekele and Hassanali (2001) on essential oils against post-harvest insects revealed that either one main component can be responsible for the oil's medicinal effect, or the effect can occur as a result of different combinations of the chemical constituents in the essential oils. Essential oils find broad use in the manufacture of consumer products such as cosmetics, perfumes, soaps and candles.

Various essential oils from medicinal plants have been studied for their various effects, including antimalarial, antifungal, antiviral and antibacterial activities among others (Orwa *et al.*, 2008). Selected essential oil constituents have been incorporated in the manufacture of herbal cosmetics, soaps, oils, toothpaste and traditional medicines such as the Ayurvedic medicine due to their pharmacological activities.

2.8 Antifungal effects of essential oils

In search for antifungal constituents that are active against disease causing fungal strains, new compounds have been identified in plant extracts including essential oils. Some compounds extracted from essential oils have been found to exhibit antifungal activities either by destruction of the fungi cells or by inhibiting the growth of specific fungi, which is difficult to distinguish. Antifungal activity is measured as the minimum inhibitory concentration (MIC) or the minimum fungicidal concentration (MFC) (Swamy *et al*., 2016). Numerous essential oils have been studied extensively for their inhibition properties against mycelium growth of fungal pathogens (Swamy *et al*., 2016). In the past, researchers have carried out studies on essential oils, where some of

them including those from clove and garlic have exhibited antifungal activities of against *Cladosporium*, *Penicillium*, *Botrytis* and *Fusarium* species (Swamy *et al*., 2016). These findings suggest that essential oils of some plants can be effective in controlling fungal species that produce mycotoxins.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample collection

Chicken feed and chicken product samples were obtained from Kakamega, Makueni, Baringo and Kiambu counties located in different agro-ecological regions, where poultry farming is widely practised with highest poutry production in Kenya (KNBS, 2017; Omiti, 2007). Sampling was done systematically according to the protocol proposed by Kothari (2004), where equation 3.0 below was applied in the calculation of the sample size.

$$n=\frac{Z^2pq}{e^2} \quad \text{……..…….Equation 3.0}$$

Where: e is the desired level of precision, q is 1 – proportion of the population, p is the (estimated) proportion of the population which has the attribute in question, the Z-value was obtained from the Z table

The four counties experience varying climatic conditions (KNBS, 2017). Kakamega County is characterized by both high rainfall and temperature, which range between 1250 mm – 1750 mm and 25 ˚C – 30 ˚C respectively. Baringo County, the south western, receives an average rainfall of 1000 mm and maximum temperature of 30 ˚C. Kiambu County has a cool and wet climate with maximum temperatures of 20 ˚C and average rainfall of 962 mm. Makueni County receives the least amount of rainfall (800 mm) and experiences the highest temperatures with a maximum of 35 ˚C.

The chicken products that were used included; eggs, liver and meat. A 10 g samples of meat and liver from six months old birds, one egg per farm and 100 g sample of chicken feed, were randomly sampled from different farmers in the four counties. A total of 111 samples were collected where chicken feed and products accounted for 81 and 30 samples respectively. Samples from the different counties were stored separately in well labeled and sealed paper bags at 2 ˚C until when required for laboratory use. Bulk feeds collected were categorized based on the sampling region, particle size and composition as pellets, crumb or mash. Bulk chicken product samples were prepared based on the sampling regions. The different feed types collected were layer pellets and broiler pellets, layer chick starter crumb and broiler chick starter crumb, layer finisher mash and broiler finisher mash, and layer starter mash and broiler starter mash. Chicken products sampled included liver, muscle and eggs.

Bulk samples based on the feed type were prepared by separately mixing 10 g of each feed type from the sampling regions to obtain representative samples. Bulk feed samples based on the sampling county were prepared by homogeneously mixing 10 g of all the feeds (irrespective of their type) to obtained representative samples for Kakamega, Makueni, Baringo and Kiambu County, which were used in mycotoxin quantification.

Fresh leaves, flowers and succulent stems of *T. asiatica, O. gratissimum and L. javanica* were collected from their natural habitat from different geographical regions of Kenya and each was used in the extraction of essential oils. The *T. asiatica* was collected randomLy from Kakamega forest which covers Lurambi sub County. The sampling region lies between 0˚16’0” N and 34˚52’60” E. The *O. gratissimum* was sampled from Ngulini Forest in Machakos County. The

sampling region for *O. gratissimum* is located 1°21’0” S and 37°22’0” E. The *L. javanica* was collected from Katimok forest in Baringo County. The sampling region lies between 0°37’0” N and 35°46’60” E.

The choice of plants was informed on ethnobotanical information and reported pharmacological uses (Kokwaro, 2009). The fresh samples were transported to Kenyatta University for authentication by a taxonomist at the Department of Plant Sciences, where a voucher specimen was deposited (voucher numbers MD 3/2017, MD 4/2017 and MD 5/2017 for *T. asiatica, O. gratissimum* and *L. javanica* respectively).

3.2 The OTA, FB1 and AFB1 extraction from chicken feed and products

The quick, easy, cheap, effective, rugged, and safe (QuEChERS) extraction procedure of mycotoxins was adopted from Paíga *et al*. (2012) and modified with an aim of eliminating matrix interferences. Chicken feed and products bulk samples (categorized based on the sampling region and sample type) were separately ground and each homogenized to a powder-like consistency. A 5 g portion of each homogeneous sample was accurately weighed and transferred into 50 mL centrifuge tubes. 15 mL of deionized water was added into the tube to hydrate the samples and hand-shaken for 1 minute, and then vortexed for 5 minutes. To the mixture, 15 mL of 2 % (v/v) formic acid in acetonitrile was added and stirred for 30 minutes at 300 rpm.

A salt comprising of 0.5 g of sodium citrate dibasic sesquihydrate, 1.0 g of sodium citrate tribasic dihydrate, 1.0 g of sodium chloride, and 4.0 g of anhydrous magnesium sulphate were

added into the mixture to induce phase separation as well as increase the mixture's ionic strength. The content in the centrifuge tube was then hand shaken for 1 minute followed by centrifuging using an eppendorf Centrifuge **(**Eppendorf® Centrifuge 5920 R, Merck, Germany) at 14,000 rpm for 5 minutes, after which 5 mL of the supernatant was filtered using clean up columns. For the chicken products (meat, liver and eggs), the supernatant was added to a sorbent material prior to filtration. The sorbent comprised of 0.25 g of C_{18} silica, 0.75 g of anhydrous magnesium sulphate and 0.25 g of graphitized carbon black, to facilitate removal of excess fat droplets and other non-polar interferences, water and pigmentation respectively (Carballo *et al*., 2018).

3.2.1 Cleaning of mycotoxins extracts

Multistep cleanup column, MultiSep® 229 Ochra was used in the cleanup of OTA quantification sample extracts while Mycospin clean up column, MycoSpin® 400 Multitoxin was used in the cleanup of AFB1 and FB1 quantification sample extracts. A stream of nitrogen was used to evaporate an aliquot (3 mL) of each cleaned extract to dryness which was later re-dissolved in 300 µL methanol-water (20:80, v/v) and centrifuged for 5 minutes at 14,000 rpm prior to LC-MS analysis.

3.3 The LC-MS analysis of OTA, FB1 and AFB1

The LC-MS analysis of OTA, FB1 and AFB1 was performed in the electronspray ionization (ESI) mode using an Agilent 1100 (Agilent Technologies, Waldbronn, Germany) HPLC series fitted with a temperature-controlled oven, an auto sampler and a binary pump. The HPLC system was coupled to an Agilent 1100 MS detector with an electron-spray interface. A ZORBAX Eclipse Plus RRHT C18 column maintained at 40 ˚C was used for chromatographic separation

during the analysis. Nitrogen was used as the drying gas set at a temperature of 330 ˚C, a nebulizer pressure of 50 psig, a flow rate of 12 L/min, fragmentor voltage of 60 eV and capillary voltage of 3 kV. The mobile phase elute A and B consisted of 0.2 % acetic acid with 1 mM ammonium acetate in water and 0.2 % acetic acid in acetonitrile.

The mobile phase had a flow rate of 0.5 mL/min and the gradient was such that the proportion of elutent A was 90 % during the initial 1 minute, decreased from 90 % to 20 % in 5 minutes then maintained constant for 10 minutes. An infusion study was carried out to determine each mycotoxin's fragmentation pattern in negative and positive modes so as to generate $[M-H]^-$ or $[M+H]^+$ ions which were monitored during quantification and screening of samples.

3.4 The LC-MS method validation

The LC-MS method for quantification of mycotoxins was validated by evaluating the linearity, limit of detection (LOD) and limit of quantification (LOQ), and reproducibility, repeatability and recovery as per the April 2017 European Commission regulation for analytical methods (De Santis *et al*., 2017).

3.4.1 Linearity

For each mycotoxin a calibration curve was developed using standard solutions of six concentrations each eluted in the mobile phase, which were used as the basis for the development

of calibration curve. The analyses were carried out in triplicates. Linear regression analyses of dose-response data were then carried out.

3.4.2 Recovery

Method repeatability was evaluated by analyzing poultry feeds, chicken muscle, liver and egg samples separately spiked with OTA, AFB1 and FB1. On a particular day, all samples were measured and spiked in triplicates using five different concentrations. Reproducibility of the method was evaluated by testing the various spiked samples over duration of five days. The recovery of OTA, AFB1 and FB1 is the response detected due to an amount of the mycotoxin standard added to a matrix and extracted in comparison to the detector response from the same concentration of the spiked mycotoxins in a solvent. The data obtained was used in the determination of the method recovery during extraction of OTA, AFB1 and FB1 by application of equation 3.1 below.

$$\% \text{ Recovery} = \frac{(C1 - C2)}{C3} \qquad \textbf{Equation 3.1}$$

C1 = Concentration determined on spiked sample

C2 = Concentration determined on blank sample

C3 = Spiking concentration

3.4.3 Limit of detection (LOD) and limit of quantification (LOQ)

The LOQ and the LOD were determined by using the signal-to-noise approach at the levels 10 and 3.3 respectively. Calculations were internally done by the LC-MS equipment by application of International Conference of Harmonization (ICH) equations expressed as equation 3.2 and 3.3.

$$LOD = \frac{3.3 \times SD}{m} \qquad \textbf{Equation 3.2}$$

$$LOQ = \frac{10 \times SD}{\square} \qquad \textbf{Equation 3.3}$$

Where *SD* is the residual standard deviation of the linear regression or Standard deviation of the blank *m* the Slope of the regression line.

3.5 Isolation and sub-culturing of *Aspergillus* species in chicken feed

A 10 g portion of each sample was separately ground into fine powder and placed in a conical flask. 90 mL of sterile distilled water with 0.1 % peptone was added into the flask and horizontally shaken for 30 minutes. Serial dilutions of 10^{-1} up to 10^{-4} mixture were prepared. 100 µL of each dilution was spread plated using a sterile bent glass rod onto potato dextrose agar (PDA) amended with 0.005 % chloramphenicol in triplicates. The plates were incubated for 7 days at 25 ˚C (Klich, 2002).

Discrete mold colonies, based on their colour and texture were sub-cultured and purified three times to obtain pure cultures. A seven-day old mycelia plug on PDA was transferred onto Czapek Dox agar (CZ), which is selective for the *Aspergillus* species. CZ media was amended with 0.005% chloramphenicol, an antibiotic to prevent bacterial contamination. All colonies were sub-cultured in triplicates. The plates were incubated at 25 ˚C for seven days under alternating 12 hours regime of light and darkness.

Seven-day old mycelia plugs were obtained from pure mold colonies suspected to be of *A. flavus* and transferred onto malt extract agar (MEA) plates and incubated for seven days and the physical characteristics observed. The mold colonies were incubated for additional three days to monitor any physical changes on the colonies as they aged. The physical characteristics of *A. flavus* resemble those of *A. parasiticus* in MEA. *Aspergillus flavus* and *Parasiticus agar* (AFPA) was used as a differentiating medium. The seven-day old mycelia plugs were sourced from MEA plates and inoculated on AFPA in triplicates and incubated at 30 ˚C for three days.

3.6 Isolation and sub-culturing of *Fusarium* species in chicken feed

Ten grams of each sample was separately ground into fine powder and each placed in a conical flask. A 90 mL portion of sterile distilled water with 0.1 % peptone was added into the flask and placed on a horizontal shaker for 30 minutes. Serial dilutions of 10^{-1} up to 10^{-4} mixture were prepared. 100 µL of each dilution was spread plated using a sterile bent glass rod onto potato dextrose agar (PDA) amended with 0.005 % chloramphenicol in triplicates. The plates were incubated for 7 days at 25 ˚C where a mixed culture of fungi was obtained.

Peptone pentachloronitrobenzene Agar (PPA) was used for the selective growth of Fusarium species. The PPA was prepared by mixing 15 g of peptone with potassium dihydride phosphate (1 g), hydrated magnesium sulphate (0.5 g), agar (20 g) and 750 g of pentachloronitrobenzene (PCNB) in 1 L of distilled water (Leslie and Summerell, 2006). Discrete mold colonies, based on their colour and texture were sub-cultured and purified three times to obtain pure cultures on PPA. Each seven-day old mycelia plug on PDA was transferred onto PPA growth media amended with 0.005 % chloramphenicol and incubated for 7 days at 25 ˚C.

3.7 Identification *of A. niger, A. ochraceus, A. flavus* and *F. verticillioides* from stored chicken feeds

The colony morphology on culture media inoculated plates were observed, with special emphasis on mycelium size, colour, texture and nature of colony to isolate the selected study fungi from the mixed cultures. Identification of *F. verticillioides* was done according to features described in a laboratory manual by Leslie and Summerell (2006), while *Aspergillus* mold colonies (*A. niger*, *A. ochraceus* and *A. flavus*) on CZ and MEA were identified based on the physical, microscopic and macroscopic features according to keys by Klich (2002).

Microscopy observation of fungi characteristics was carried out; lactophenol cotton blue stain was put on a glass slide where the unidentified fungal colony from the petri-dish was placed using a sterilized platinum inoculating pin. The colony was teased out then covered and pressed down slightly to enhance observation by disintegrating the hypal growth and expelling air

bubbles. The slide was then observed under a light microscope at x40 magnification for identification and analysis of microscopic features as shown in Figure 3.1.

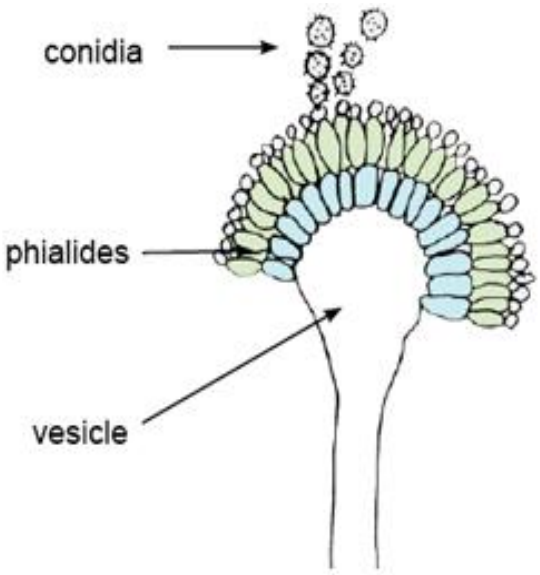


Figure 3.1: Microscopic morphological features used in aspergillus fungi identification (The University of Adelaide, 2017)

3.8 Extraction of essential oils by steam distillation

Extraction of the essential oils from the aerial parts, leaves, flowers and succulent stems of the three selected plant species was carried out separately by steam distillation using a modified Clevenger apparatus. 200 g of the plant sample was added to 200 mL of water and put in a 1000 mL round bottom flask. The flask was then connected with a double pocket condenser and a Clevenger apparatus and steam distillation was done for four hours for each sample. The essential oils obtained from steam distillation were mixed with n-hexane and filtered through anhydrous sodium sulphate to remove water. The n-hexane extract was later removed by use of a rotary evaporator and the oils stored in amber coloured vials at -20 °C.

3.9 Determination of Chemical constituents of essential oils using GC-MS

The chemical composition of the essential oils was determined by using a Gas Chromatography-Mass Spectrometry (GC-MS). The GC-MS analysis was carried out using a fused silica capillary column (DB-1, J and W Scientific, film thickness 0.52 µm, 0.32 mm x 50 m i.d) connected to a cool on column injector which was coupled to a mass spectrometer detector (HP 5972). The analysis was carried out in a splitless mode and helium was used as the carrier gas.

Electron impact was used for ionization (source temperature of 250 ° C, 70 eV). The oven column was programmed at 5 to 250 °C and retained at 30 °C for duration of 5 minutes. Retention indexes were calculated via co-injection with an n-alkenes series. The identification of the chemical constituents of the essential oils was based on comparison of the mass spectra with computer search using Adams, NIST107 and NIST21 libraries (Adams, 1995; NIST, 2005). The concentration of the individual constituents of each of the essential oils were calculated from the gas chromatography peak area and displayed in order of GC elution.

3.10 Fungal inhibition assays

Fungal inhibition assays were carried out to investigate the inhibition activities of *T. asiatica, O. gratissimum* and *L. javanica* plant essential oils and their blends. Antifungal activities of individual major constituents of the essential oil of each plant and various blends were also investigated. During the fungal inhibition assays potato dextrose agar (PDA) plates amended with 0.005% chloramphenical were used as a negative control. PDA amended with a synthetic

fungicide (ROYALCAP 500 FS Flowable concentrate for seed treatment) that contains 500 g/L of captan was used as the positive control.

Fungal inhibition assays were carried out according to a method described by Mi-jeong *et al*. (2013). For every inoculation, a 4 mm mycelia plug was cut using a corkborer from an actively growing seven-day-old colony. The mycelia plug was inoculated at the center of a petri dish containing PDA culture medium amended with 0.005 % chloramphenicol and the essential oil or compound of interest in 5 replicates on a completely randomized design and incubated for 7 days at 25 ˚C. The mycelia diameter was measured in mm and used to calculate the percentage inhibition as depicted in Equation 3.4.

$$\% \text{ inhibition} = \frac{(C-T)}{(C-M)} \text{ X } 100 \qquad \textbf{Equation 3.4}$$

Where,

C = Diameter of mycelia plug in negative control plates

T = Diameter of mycelia plug in essential oil or compound treated plates

M = Initial diameter of mycelia plug (4 mm)

3.11 Bioassays on the effects of the essential oils and their major constituents against the growth of *A. flavus, A. niger* and *F. verticillioide*s

Equal portions (ranging from 1 µLmL^{-1} - 32 µLmL^{-1}) of the essential oils of *T. asiatica*, *O. gratissimum* and *L. javanica* were measured and blended together. Inhibition activities of a blend of the three essential oils against the growth of *A. flavus*, *A. niger* and *F. verticillioides*, at

various concentrations ranging from 1 µLmL^{-1} to 32 µLmL^{-1} were studied. Subtractive bioassay experiments were carried out by using equal portions of various blends of two oils to study their growth inhibition activities against the selected fungi. The essential oils of *T. asiatica, O. gratissimum* and *L. javanica* were also individually tested on the growth inhibition of each fungus and their dose response data compared to the various blends for any synergistic effects. A protocol by Langeveld *et al*. (2014) on synergy between essential oil components was adopted.

Identification of the major constituents was done based on each component's percentage constituent in the essential oils. Available standards of the major constituents of the essential oils (Limonene, Artemisia ketone, Myrcene, Sabinene Eugenol, Terpinen-4-ol, β-Elemene, Methyl eugenol, Linalool and Terpinolene) based on their relative abundance were purchased. Various synthetic blends were prepared and their inhibition activities against the growth of *A. flavus, A. niger* and *F. verticillioides* studied. Synthetic blends of the four main constituents from each study plant's essential oils were separately prepared in the relative proportions of occurrence and their antifungal activities were tested. From the blend of four, each compound was subtracted and the resulting blends were also tested. Inhibition activities of the individual oil components against the growth of *A. flavus, A. niger* and *F. verticillioides* were also tested, and their activities compared with the different blends to determine any synergistic effects.

3.12 Data analysis

Statistical analysis was done using SPSS Statistics software version 25. Average zones of inhibition of *A. niger, F. verticillioides* and *A. flavus* exposed to different blends and

concentrations of the essential oils were analyzed by one-way Analysis of Variance (ANOVA). Data of control cultures and those treated with either synthetic constituents or plant essential oils were compared for antifungal activity evaluation. Obtained data was expressed as mean ± SD, $P<0.05$ was considered statistically significant. The data obtained was presented in tabular and graphical form.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Validation of LC-MS parameters

a) Linearity

The correlation coefficient (R^2), ranged from 0.9924 to 0.9998 as presented in Table 4.1.

Table 4.1: The LC--S method validation parameters

Mycotoxin	R^2	% recovery	LOQ (ng/g)	LOD (ng/g)
OTA	0.9998	89	0.39	0.13
FB1	0.9924	105	59.97	14.51
AFB1	0.9960	94	0.22	0.04

The value of R^2 serves as an indication of how accurate the mycotoxin concentrations can be predicted by the LC-MS method responses. This means that from the R^2 values obtained 99.98% of OTA, 99.24 % of AFB1 and 99.60 % of FB1 can be predicted without error by the detector response. The statistical significance was based on *p*-value< 0.05 which is an indication of a model that can be described by a regression at 95 % confidence interval. The R^2 values of OTA, AFB1 and FB1 were not less than 0.99, which indicates homogeneity of variance in the distribution of replicates and a good correlation of linearity in the concentrations used to prepare the calibration curve.

b) Percentage recovery

Each of the three mycotoxins analyzed had varying percentage recovery levels. OTA had the lowest recovery at 89 % while FB1 had the highest recovery at 105 %. AFB1 had a recovery of 94 % as expressed in table 4.1. The mycotoxins recovery experiments yielded values that ranged from 89 to 105 %. Percentage recoveries indicate ion enhancement or suppression during the LCMS technique where ESI was applied. A recovery of 100 % is an indication their was no ion enhancement or suppression during the analysis. A percent recovery of less than 100 is an indication of ion suppression while a percent recovery greater than 100 is an indication of ion enhancement. From table 4.1, FB1 had a percent recovery greater than 100 while OTA and AFB1 had recoveries of 89 % and 94 % respectively. The FB1 recovery of 105 % is due to ion enhancement due to coelution of compounds in the sample. The OTA and FB1 % recoveries were less than 100 due to partial fragmentation during ESI.

The results obtained in the recovery experiments lie within the acceptable international standard limit that range between 80 and 120 % for ion enhancement or suppression in analytical method validation (Andreasson *et al*., 2015; ISO 5725-5, 1998). The results suggest minimal interference in the sample matrices that could otherwise mask the determination of the study mycotoxins. The recovery experiment outcome within the acceptable range indicates that the concentration-response relationship is similar in the samples and the calibration curve (Andreasson *et al*., 2015).

c) L imit of detection (LOD) and limit of quantification (LOQ)

The LOQ and LOD for the three mycotoxins are presented in table 4.1. The AFB1 had the lowest LOD and LOQ values followed by OTA. The FB1 had the highest values for both the LOD and LOQ. The LOQ and LOD of AFB1 was 0.22 and 0.04 ng/g respectively. The OTA had a LOQ of 0.39 ng/g and a LOD of 0.13 ng/g while FB1 had LOQ value of 59.97 ng/g and LOD of 14.51 ng/g. Previous studies of LC-MS multi-toxin analysis by Spanjer *et al*. (2008) yielded a similar trend where AFB1 from different matrices had the lowest LOD and LOQ values followed by OTA then FB1.

4.2 The LC-MS analysis of OTA, FB1 and AFB1

The LC-MS method was used to detect OTA, AFB1 and FB1 in sampled chicken feed and chicken products. The three mycotoxins were detected and quantified based on their reference chromatograms, retention time and mass measurement. The LC-MS chromatograms of OTA are shown in appendix 1.

Table 4.2 shows the mean levels of OTA, FB1 and AFB1 in chicken feed and chicken products from the selected counties. Scanning the mass to charge ratio between 100 and 1000 m/z showed molecular ions of OTA, FB1 and AFB1 at 404.0, 723.2 and 312.9 respectively. Precursor ions of OTA, FB1 and AFB1 exhibited responses in ESI positive mode to produce $[M+H]^+$ ions which were monitored during screening and quantification to determine mycotoxin concentration in chicken feed and chicken products. Previous studies on multi-mycotoxins determination by De Santis *et al*. (2017) yielded comparable results when conducted in positive ionization where the

precursor ions were 313.2[M+H]+, 723.3[M+H]+ and 404.1[M+H]+ for AFB1, FB1 and OTA respectively.

Table 4.2: Mean levels of OTA, FB1 and AFB1 in chicken feed and products from the selected counties in Kenya

Mycotoxin	m/z	Retention time (min)	County	Mean±sd in chicken feeds (ng/g)	Mean±sd in chicken product (ng/g)
OTA	404.0	4.1	Kakamega	165.33±0.45^{d}	7.12±1.25^{d}
			Kiambu	121.02±1.33^{b}	4.35±1.42^{b}
			Makueni	93.67±0.53^{a}	2.03±0.93^{a}
			Baringo	138.5±1.67^{c}	5.81±1.27^{c}
FB1	723.2	4.5	Kakamega	24100±1.33^{d}	< LOD
			Kiambu	15900±1.45^{a}	< LOD
			Makueni	27700±0.67^{c}	< LOD
			Baringo	19200±1.44^{b}	< LOD
AFB1	312.9	5.6	Kakamega	32.44±1.54^{c}	4.1±1.33^{c}
			Kiambu	25.08±0.89^{a}	2.0±0.75^{a}
			Makueni	24.39±0.67^{a}	1.8±0.46^{a}
			Baringo	28.63±1.73^{b}	3.4±1.35^{b}

Mean values± sd with the same small lettering each column per mycotoxins are not significantly different $P < 0.05$ (SNK).

From table 4.2, the mean OTA concentration in the analyzed chicken feed and products ranged from 93.67 to 165.33 ng/g and 2.03 to 7.12 ng/g respectively. The AFB1 levels ranged from 24.39 to 32.44 ng/g in the chicken feed and from 1.8 to 4.1 ng/g in the chicken products. Samples obtained from Kakamega County had the highest OTA and AFB1 contamination in the chicken feed and products. Samples from Makueni County had the least OTA and AFB1 contamination. The mean FB1 contamination levels in chicken feed from the four counties ranged from 15900 to 27700 ng/g. The FB1 levels was not detected in all the chicken products

analyzed. Makueni County accounted for the highest mean FB1 contamination in chicken feed (27700 ng/g) while Kiambu County had the least mean FB1 contamination levels (15900 ng/g).

Mycotoxin levels in the four study regions differed significantly except for AFB1 levels in samples from Kiambu (25.08 ng/g) and Makueni (24.39 ng/g) counties. The difference in mycotoxins contamination level can be partly attributed to the climatic conditions of the specific counties. Mycotoxins causing fungi thrive well in hot and wet climatic conditions. This results correlate with reported data in previous study by Atela *et al*. (2016). Atela *et al*. (2016) also confirmed the presence of mycotoxins such as aflatoxins in commercial chicken feeds in Kenya.

4.2.1 Quantification of OTA in chicken feeds and chicken products

The occurrence of OTA in various chicken products and chicken feeds is presented in table 4.3.

Table 4.3: The OTA contamination in chicken feeds and products in Kenya

Substrate	Samples analyzed	Positive samples	% positive samples	Maximum level (ng/g)
Chicken product	**30**	**4**	**13.3**	**10.14**
Egg	10	0	0	ND
Liver	10	3	33.3	10.14
Muscle	10	1	10.0	0.54
Chicken feed	**81**	**38**	**46.9**	**202.00**
Layer (pellet)	8	5	62.5	197.80
Layer Chick Starter (crumb)	11	6	54.5	98.10
Layer Finisher (Mash)	11	5	45.5	56.00
Layer Chick Starter (Mash)	11	4	36.4	49.80
Broiler Chick Starter (crumb)	11	5	45.5	120.40
Broiler Finisher (Mash)	11	3	27.3	50.20
Broiler Starter (Mash)	11	6	54.5	64.00

Broiler (pellet)	7	4	57.1	202.00

Out of a total 30 chicken products that were analyzed, the percentage occurrence of OTA contamination was 13.3 % while the percentage occurrence of OTA contamination in 81 samples of chicken feeds was 46.9 %. Maximum OTA contamination level was 10.14 ng/g and 202.00 ng/g in chicken products and chicken feed respectively.

The chicken product with the highest OTA contamination was the liver (10.14 ng/g) followed by meat (6.54 ng/g) while egg samples had OTA level below the LOD (< 0.13 ng/g), thus could not be quantified. A similar trend was observed in a previous study by Iqbal *et al*. (2014) where eggs and different chicken parts were analyzed for OTA and the liver was found to have the highest contamination while the eggs had the least OTA level. Comparable results have been reported in similar studies by Lee *et al*. (2016) where OTA contamination was not detected in eggs.

Both broiler and layer chicken feeds were quantified for OTA contamination. Broiler pellets had the highest level of OTA contamination 202.00 ng/g, followed by the layer pellets 197.80 ng/g. Chicken feed in the form of pellets had the highest number of OTA contaminated samples where the layer pellets had 62.5 % and the broiler pellets had 57.1 % of the samples with levels > 0.39 ng/g, the LOQ. The mash form of feed had the lowest OTA contamination level. The layer starter mash had 49.80 ng/g and the broiler finisher mash had 50.20 ng/g.

For layer and broiler feeds, the pellet form had the highest OTA contamination followed by the crumb and mash form. This can be attributed to the fact that large pellets compared to small crumbs and mash require more water and a longer duration to form and these conditions favor

mycotoxins production. Similar trends were reported by Sherazi *et al.* (2015) where chicken feeds with the largest particles size were found to have the highest levels of mycotoxins contamination.

The level of OTA contamination in chicken feeds exceeded the maximum regulatory limit of 100.00 ng/g recommended by European Union (EU) (Lee *et al*., 2016). It is evident that regulatory measures as well as fungi inhibition mechanisms need to be employed in Kenya so as achieve <100.00 ng/g OTA mycotoxins contamination levels in chicken feeds.

4.2.2 Quantification of FB1 in chicken feed and chicken products

The FB1 contamination in various chicken products and chicken feeds is presented in Table 4.4.

Table 4.4: The FB1 contamination in chicken feeds and products in Kenya

Substrate	Samples analyzed	Positive samples	% positive samples	Maximum level (ng/g)
Chicken product	**30**	**0**	**0.0**	< LOD
Egg	10	0	0.0	< LOD
Liver	10	0	0.0	< LOD
Muscle	10	0	0.0	< LOD
Chicken feeds	**81**	**81**	**100.0**	**28900.0**
Layer (pellet)	8	8	100.0	28900.0
Layer Chick Starter (crumb)	11	11	100.0	19200.0
Layer Grower (Mash)	11	11	100.0	15900.0
Layer Chick Starter (Mash)	11	11	100.0	15300.0
Broiler Chick Starter (crumb)	11	11	100.0	17300.0
Broiler Finisher (Mash)	11	11	100.0	16600.0
Broiler Starter (Mash)	11	11	100.0	17100.0
Broiler (pellet)	7	7	100.0	28200.0

From table 4.4, out of 30 chicken products that were analyzed, the percentage occurrence of FB1 contamination was found to be 0 %. All the chicken products analyzed were found to have levels below 14.51 ng/g, the LOD, thus were not quantized. Chicken feed samples analyzed for FB1 contaminations were found to have a maximum levels 28900 ng/g. The percentage occurrence of FB1 in chicken feeds was 100 %; all the 81 samples that were analyzed had detectable quantities of FB1 contamination. Though FB1 was detected in large amounts in chicken feeds, it was below detectable levels in chicken products. The absence of the mycotoxin in chicken products can be explained by the fact that FB1 is poorly absorbed in chicken metabolic system and is rapidly degraded to a hydrolyzed form which is less toxic (Marasas *et al*., 2000).

Similar studies on mycotoxin analysis in chicken feeds confirmed FB1 as dominant in 100 % of all the samples analyzed (Greco *et al*. 2014; Mokubedi *et al*. 2019). In addition, previous studies on the occurrence of mycotoxigenic fungi in chicken feed in Nairobi also reported fusarium as the most prevalent species (Gathumbi, 1993). The detection of FB1 in all the chicken feed samples was attributed to the presence of the fusarium species which thrives under warm and moist conditions to produce the mycotoxins.

Maize is the main ingredient in chicken feed formulation in Kenya (KMT, 2016). Various studies by Alakonya *et al*. (2009) have reported high levels of FB1 in Kenyan maize. The presence of FB1 in chicken feed can as well be attributed to its presence in the main ingredient.

Analyzed chicken feed samples in the pellet form had levels that exceeded the FAO and EU, FB1 limit of 20,000 ng/g in chicken feed. This can be attributed to the fact that pellets are larger

in size and ingredients remains in a moist condition for longer during pellet formation thus leading to higher levels of mycotoxins (Sherazi *et al*., 2015).

4.2.3 Quantification of AFB1 in chicken feeds and products in Kenya

The AFB1 contamination in various chicken products and chicken feeds are presented in Table 4.5.

Table 4.5: The AFB1 contamination in chicken feeds and products in Kenya

Substrate	Samples analyzed	Positive samples	% positive samples	Maximum level (ng/g)
Chicken product	**30**	**13**	**43.3**	**2.03**
Egg	10	3	30.0	0.72
Liver	10	8	80.0	2.03
Muscle	10	2	20.0	0.54
Chicken feeds	**81**	**48**	**59.3**	**34.52**
Layer (pellet)	8	7	87.5	34.01
Layer Chick Starter (crumb)	11	7	63.6	26.05
Layer Grower (Mash)	11	4	36.4	16.20
Layer Chick Starter (Mash)	11	5	45.5	18.93
Broiler Chick Starter (crumb)	11	8	72.7	29.71
Broiler Finisher (Mash)	11	5	45.5	18.64
Broiler Starter (Mash)	11	5	45.5	19.00
Broiler (pellet)	7	7	100.0	34.52

From table 4.5, out of 30 chicken products that were analyzed, the total percentage occurrence of AFB1 contamination was found to be 43.3 %. Liver samples were the most contaminated chicken product accounting for 26.7 % of the total AFB1 contamination. Chicken muscle had the least AFB1 contamination where the level ranged between 0.48 and 0.54 ng/g. Chicken liver had

the highest maximum level of 2.03 ng/g among the chicken products, while eggs had the lowest level of 0.72 ng/g.

Chicken feed samples analyzed for AFB1 contaminations were found to have contamination levels ranging between 0.72 ng/g and 34.52 ng/g. The total percentage occurrence of AFB1 in all the analyzed chicken feeds was 59.3 %. The pellet form of feeds had the highest AFB1 contamination where 100.0 % of the broiler pellet feeds analyzed were contaminated. The layer grower mash had the least AFB1 occurrence of 36.4 % and the lowest level of contamination among the feeds.

In the feeds, the pellet form had the highest AFB1 contamination followed by the crumb and mash form. This can be attributed to the fact that the fact that large pellets compared to small crumbs and mash require more water and a longer duration to form, these conditions favor mycotoxins production. Similar trends were reported by Sherazi *et al.* (2015) where chicken feeds with the largest particles size were found to have the highest levels of mycotoxins contamination.

In previous research by Herzallah (2013) on AFB1 contamination in chicken products, muscles were found to have the lowest level of contamination while liver samples were recorded with the highest AFB1 contamination. Levels of AFB1 in the samples are comparable to previous studies carried out by Atela *et al*. (2016) where most of the study samples collected from Baringo and Kisumu County, Kenya were AFB1 contaminated. In similar studies conducted in Kenya, 52.5 %

of the study samples exceeded 20 ng/g, the food and drug administration (FDA) limit in chicken feeds (Thuita *et al*., 2019).

4.3 Isolation of toxigenic fungi from stored chicken feed

Suspected typical colonies of *A. flavus* and *A. niger* were identified among mixed fungal species in the potato dextrose agar (PDA) media. Suspected colony of *F. verticillioides* was identified in the PPA cultures which are selective for the fusarium species from which it was isolated and purified. Plate 4.1 shows a mixed culture of fungi from which *A. flavus*, *A. niger* and *F. verticillioides* were isolated.

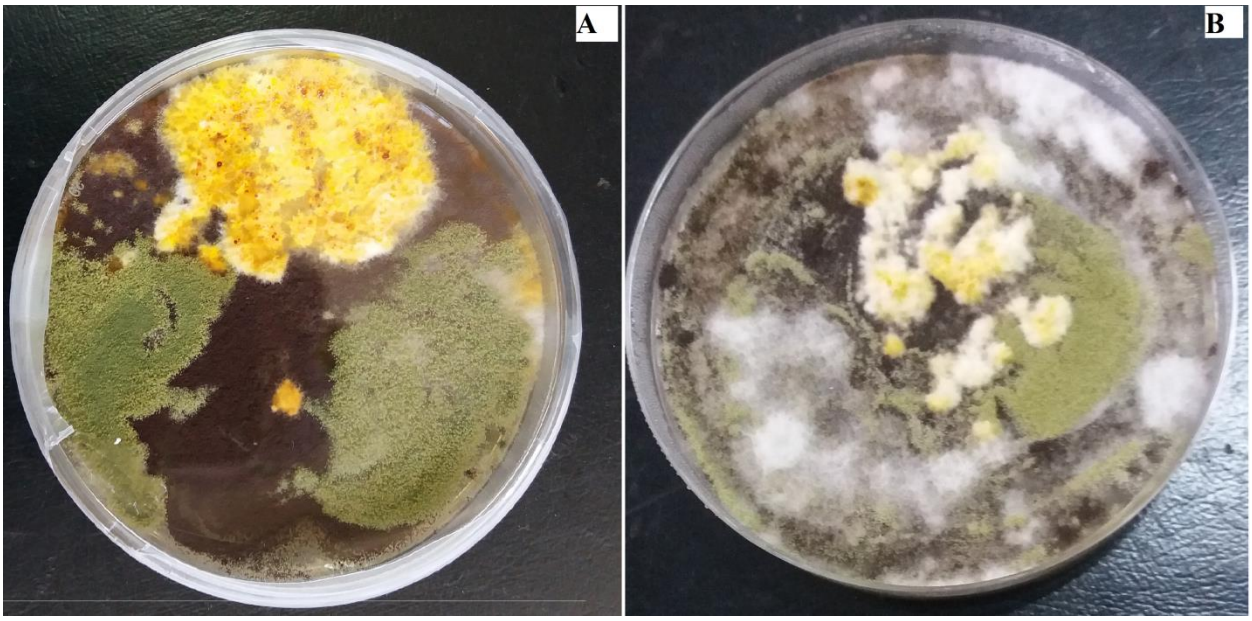


Plate 4.1: (A) Mixed culture of fungal sp. on PDA; (B) Mixed culture of fungal sp. on PPA

The presence of a mixed culture of fungi is an indication that the chicken feed had multiple mycotoxins contamination. Reports from similar studies yielded comparable results (Ghaemmaghami *et al*., 2016; Ibrahim *et al*., 2017). In previous studies on mycoflora of poultry feed in Iran, eight mycotoxin causing fungi were identified from sample cultures (Ghaemmaghami *et al*., 2016). Other studies by Ibrahim *et al*. (2017) where multiple distinct

colonies were identified indicated the presence of multiple mycotoxigenic species in poultry feed.

4.3.1 Identification of *A. niger*

A pure mold colony represented in plate 4.2 A was identified after multiple isolation and sub-culturing on CZ.

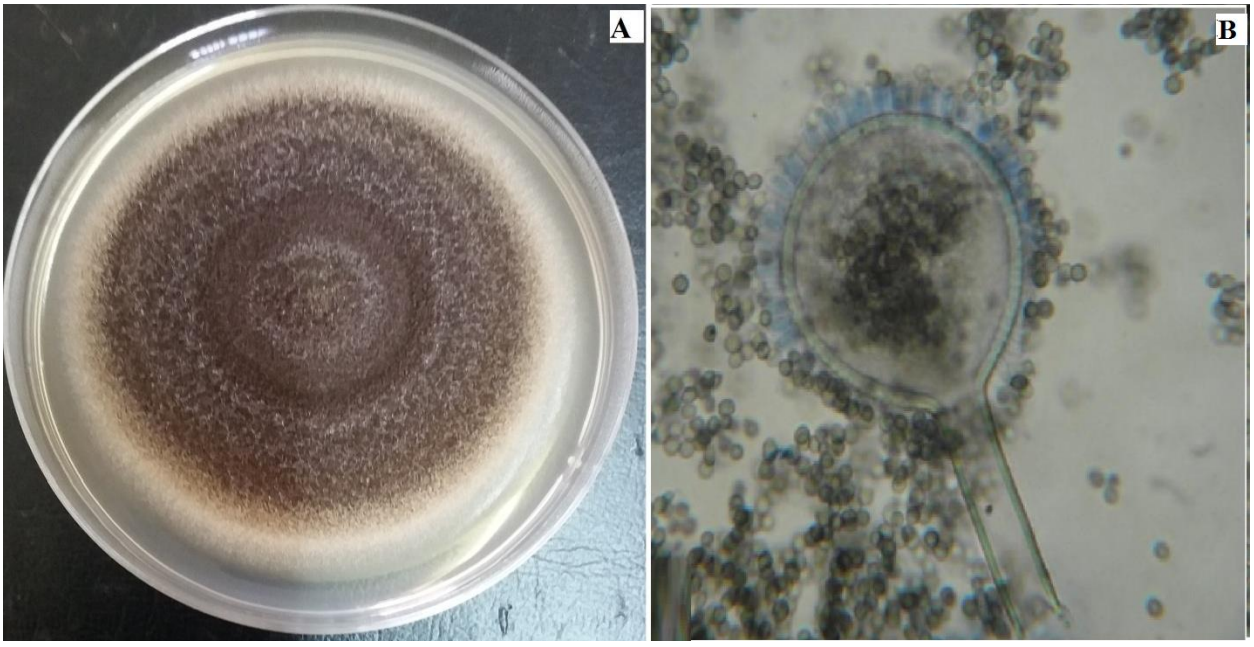


Plate 4.2A: *Aspergillus niger* colony morphology figure; B: X40 magnification of *A. niger* under microscope

The *A. niger* colony was a white mycelia surface extending to the edge with the center covered by a dense rough walled layer of black conidial heads. The microscopic observation under X40 magnification is represented in plate 4.2 (B). The conidia were globose while the conidial heads appeared to be dark brown in colour with biseriate conidiophores. The conidiophores and spores appeared smooth and colourless with a light blue colouration from the lactophenol cotton blue stain that was used.

4.3.2 Identification of *A. ochraceus*

A. ochraceus was not identified from the cultures. Similar studies by Greco *et al*. (2014) on mycotoxigenic fungi in chicken feeds reported negative results for *A. ochraceus*. However, *A. niger* was identified as the only OTA causing fungi during mycobiota determination studies. Research by Ariyo *et al*. (2013) where both *A. ochraceus* and *A. niger* were isolated from chicken feeds noted that *A. ochraceus* recorded a lower frequency of occurrence of 1 % while that of *A. niger* was 7 %. This was an indication that a greater percentage of OTA levels quantified in chicken feeds were more likely to be secondary metabolites of *A. niger*. The absence of *A. ochraceus* in this study suggests that the quantified OTA levels in the chicken feeds could have been produced by *A. niger* only.

4.3.3 Identification of *A. flavus*

A pure mold colony represented in plate 4.3A was identified after multiple isolations and purification on malt extract agar (MEA).

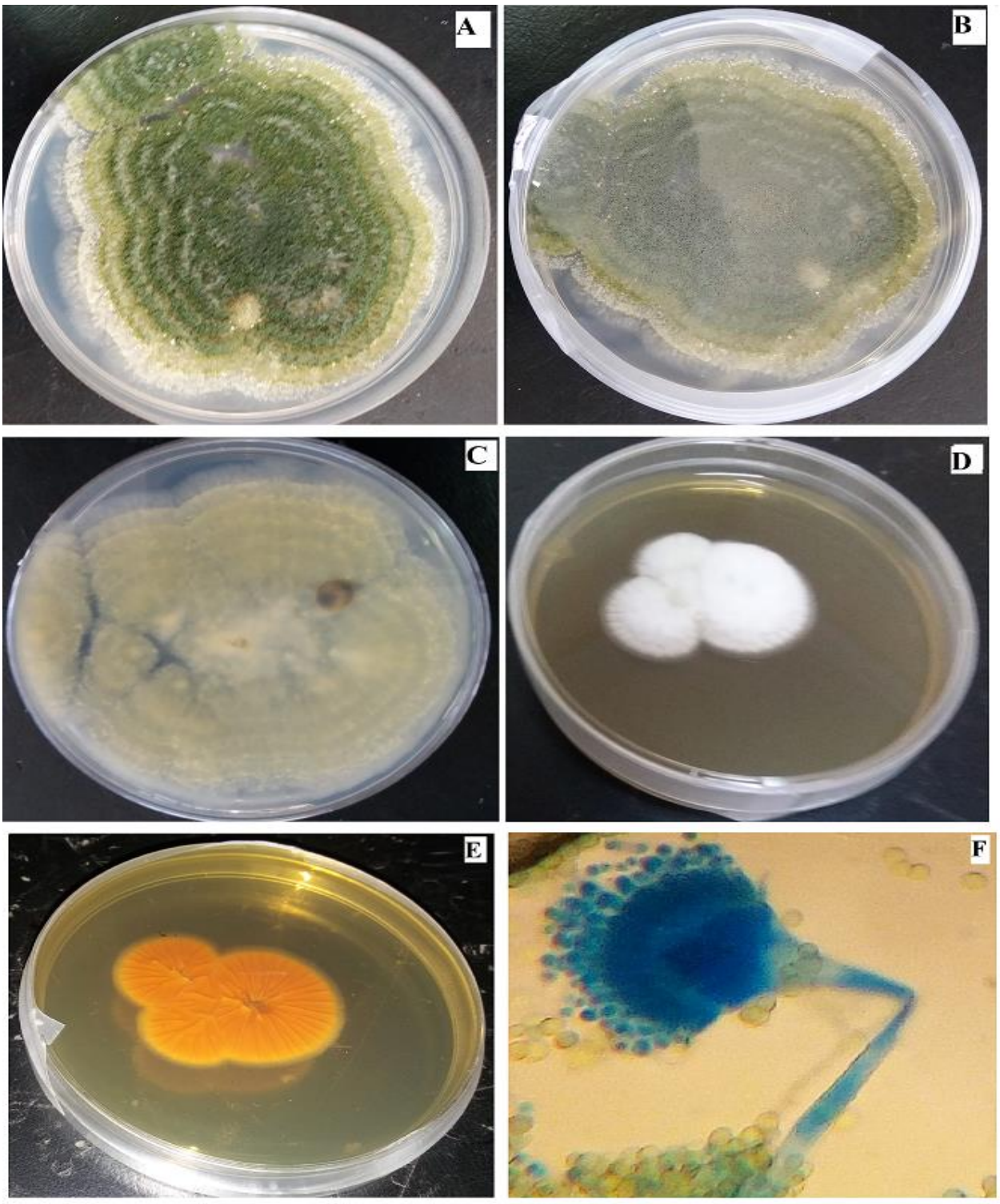


Plate 4.3A: 7-day old *A. flavus* colony; B: 10-day old *A. flavus* colony; C: reverse *A. flavus* colony; D: *A. flavus* colony on AFPA; E: reverse colony morphology of *A. flavus* on AFPA; F: X40 microscopic of *A. flavus*

The *A. flavus* colonies were green in colour with white mycelia around the edge. The colonies developed a brown shade as they aged (plate 4.3B). The colonies maintained a cinnamon brown colour on the reverse and sporulation rings were visible (plate 4.3C). The colony had a loose, velvety texture with visible furrows. The mycelia were submerged and the media remained colourless. Plate 4.3D and 3E show confirmation of *A. flavus* by inoculating on AFPA. The fungi physical appearance on AFPA was as expected for positive identification of *A. flavus*. The colony was characterized by white mycelium with buff spores and a bright orange underside.

Under microscope observation, the conidial heads had radiate vesicles, with a colourless conidiophores and a roughened end. The vesicles were biseriate, globose in shape and were covered with phialides on the surface. Plate 4.3F shows the microscopic features used in the identification of *A. flavus*.

4.3.4 Identification of *F. verticilloides*

A pure mold colony is represented in plate 4.4A.

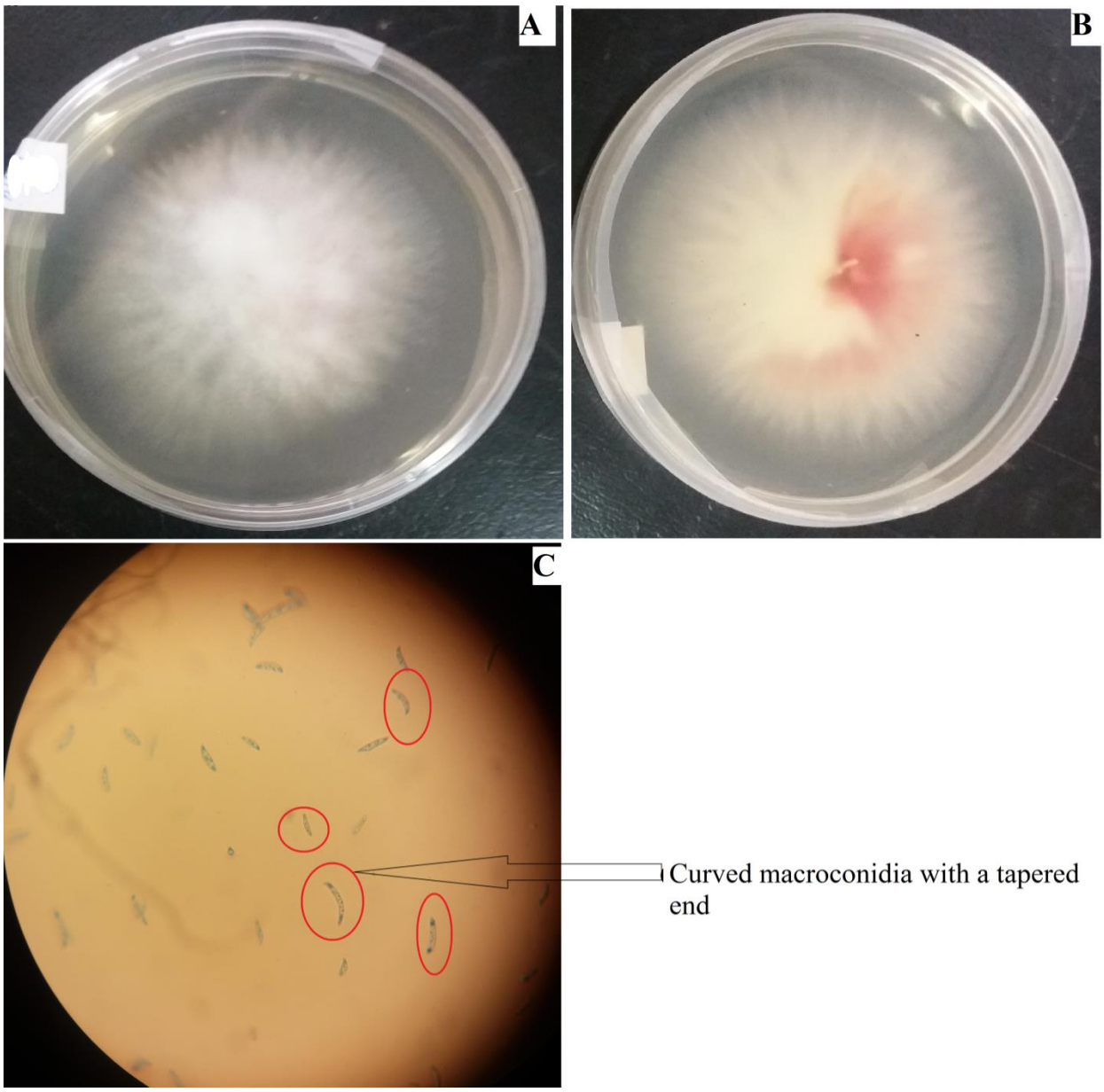


Plate 4.4A: 4-day old *F. verticilloides* colony; B: 6-day old *F. verticilloides* colony; C: X40 microscopic features of *F. verticilloides* culture

The pure colony was identified after multiple isolation and purification on PPA. A four-day old *F. verticilloides* culture had white mycelia. On the sixth day of incubation, the culture developed a purplish magneta pigment that originated from the center.

Under microscope observation of X40 magnification, the features were as described by Leslie and Summerell (2006) for the identification of *F. verticilloides*. The macroconidia were long, thin and divided into five septate. They had a curved shape and the ends were tapered to a point.

4.4 Identification of chemical constituents of essential oils using GC-MS

Various chemical constituents of *T. asiatica, O. gratissimum* and *L. javanica* plant essential oils were identified using GC-MS analysis. The constituents were numbered in order of their relative abundance starting with the highest. The compounds were identified by comparing the fragmentation patterns with those in the NIST and Adam libraries. The concentration of the individual constituents in each essential oil was calculated from the gas chromatography peak area and displayed in order of GC elution.

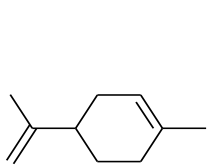
4.4.1 The GC-MS analysis of essential oils of *L. javanica*

Aerial parts of *L. javanica* used yielded an average of 2.3 % essential oil which was collected as a yellow-greenish oil. Appendix 2 shows the chromatogram obtained from GC-MS analysis of *L. javanica* essential oils. From the analysis, 15 compounds were identified constituting 97.51 % of the compounds present in the essential oil. Limonene **(14)**, artemisia ketone **(15)**, myrcene **(16)** and sabinene **(17)** accounted for the highest compound composition in the essential oils which was 31.06 %, 14.91 %, 9.87 % and 8.6 % respectively (Table 4.6).

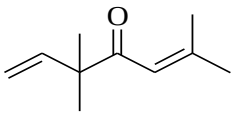
Table 4.6: Compounds identified from GC-MS analysis of *L. javanica* essential oils

Peak number	Retention time (minutes)	Compound	% composition	CAS registry number
1	13.200	Limonene	31.06	138-86-3
2	13.444	Artemisia ketone	14.91	546-49-6
3	10.403	Myrcene	9.87	123-35-3
4	9.501	Sabinene	8.60	3387-41-5
5	14.502	Terpinolene	7.17	586-62-9
6	14.633	Linalool	6.03	78-70-6
7	17.164	(E)-Carvyl acetate	4.51	1197-07-5
8	8.879	Camphene	3.39	79-92-5
9	14.795	Terpinene-4-ol	2.15	562-74-3
10	12.412	α-Phellandrene	1.96	99-83-2
11	13.897	(Z)-Sabinene hydrate	1.88	546-79-2
12	17.956	β-Caryophyllene	1.83	87-44-5
13	7.412	α-Thujene	1.54	5/2/2867
14	12.473	α-Terpinene	0.98	99-86-5
15	17.612	α-Cubebene	0.72	17699-14-8
16	15.900	α-Terpineol	0.68	98-55-5
17	8.625	α-Pinene	0.23	80-56-8
		17 compounds	**97.51**	

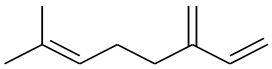
The obtained mass spectrum of the major compounds: limonene, artemisia ketone, myrcene, and sabinene and their comparison with the NIST and Adams libraries are shown in appendices 5,6, 7 and 8 respectively. All the four major compounds identified in *L. javanica* essential oils are monoterpenes. This data is in agreement with previous research by Kosgei *et al*. (2017) on the chemical composition of *L. javanica* where all major compounds were monoterpenes. The percentage composition of the chemical constituents of the essential oils of *L. javanica* differed based on the soil and climatic condition of the geographical location where harvesting was done (Kosgei *et al*., 2017; Viljoen *et al*., 2005).



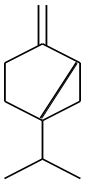
14



15



16



17

4.4.2 The GC-MS analysis of essential oils of *O. gratissimum*

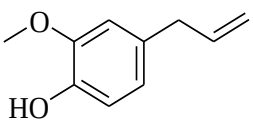
Aerial parts of *O. gratissimum* yielded an average of 1.8 % essential oil which was collected as a white oil. Appendix 3 shows the chromatogram obtained from GC-MS analysis of *O. gratissimum* essential oils. From the analysis, 15 compounds were identified constituting 97.16% of the compounds present in the essential oil. Eugenol **(18)**, terpinen-4-ol **(19)**, *β*-elemene **(20)** and methyl eugenol **(21)** accounted for the highest compound composition in the essential oils which was 23.42 %, 16.08 %, 11.35 % and 9.73 % respectively (Table 4.7). The obtained mass spectra of the major compounds and their comparison with the NIST and Adams libraries are shown in appendices 9, 10, 11 and 12.

Table 4.7: Compounds identified from GC-MS analysis of *O. gratissimum* essential oils

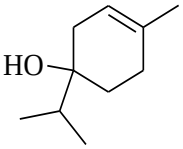
Peak number	Retention time (minutes)	Compound	% composition	CAS registry number
1	11.824	Eugenol	23.42	97-53-0
2	11.717	Terpinen-4-ol	16.08	562-74-3
3	11.900	β-Elemene	11.35	515-13-9
4	12.249	Methyl eugenol	9.73	93-15-2
5	12.414	β-Caryophyllene	8.12	87-44-5
6	11.540	β-Ocimene	5.89	13877-91-3
7	11.643	Linalool	5.01	78-70-6

8	10.211	α-Pinene	4.96	80-56-8
9	11.319	β-Pinene	4.14	127-91-3
10	11.179	Myrcene	3.80	123-35-3
11	11.297	α-Terpinene	1.43	99-86-5
12	11.320	p-Cymene	1.14	99-87-6
13	11.442	1,8-Cineole	0.99	470-82-6
14	10.318	Sabinene	0.73	3387-41-5
15	12.553	α-Humulene	0.37	6753-98-6
		15 compounds	**97.16**	

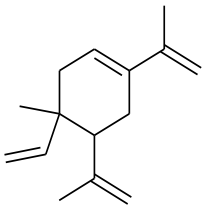
In previous studies on the chemical composition of essential oils of *O. gratissimum,* the percentage composition of the compounds varied but eugenol was noted to account for the highest chemical composition (Matasyoh *et al*., 2007; Prabhu *et al*., 2009). This was in agreement to the chemical composition of essential oils of *O. gratissimum*.



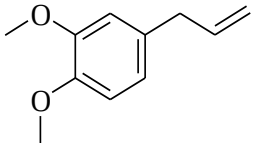
18



19



20



21

4.4.3 The GC-MS analysis of essential oils of *T. asiatica*

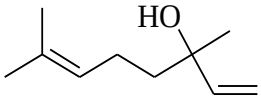
T. asiatica yielded 0.9 % essential oil which was collected as pale yellow oil above water. Appendix 4 shows the chromatogram obtained from GC-MS analysis of *T. asiatica* essential oils. From the analysis, 15 compounds were identified accounting for 98.36 % of the compounds present in the essential oil. The most abundant chemical compounds identified in *T. asiatica*

were monoterpene hydrocarbons. Sabinene **(17)**, linalool **(22)**, terpinen-4-ol **(19)** and terpinolene **(23)** accounted for the highest percentage compound composition in the essential oils which was 22.19 %, 17.19 %, 11.93 % and 9.73 % respectively (Table 4.8).

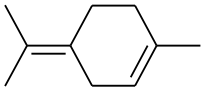
Table 4.8: Compounds identified from GC-MS analysis of *T. asiatica* essential oils

Peak number	Retention time (minutes)	Compound	% composition	CAS registry number
1	9.399	Sabinene	22.19	3387-41-5
2	11.220	Linalool	17.19	78-70-6
3	11.117	Terpinen-4-ol	11.93	562-74-3
4	11.003	Terpinolene	9.01	586-62-9
5	9.105	α-Thujene	8.71	2867-05-2
6	9.301	α-Pinene	7.85	80-56-8
7	9.849	Myrcene	6.03	123-35-3
8	10.629	(Z)-β-Ocimene	5.32	3338-55-4
9	12.714	Spathulenol	3.18	6750-60-3
10	10.235	δ-2-Carene	2.09	554-61-0
11	9.700	β-Pinene	1.64	127-91-3
12	11.499	Bornyl acetate	1.08	76-49-3
13	10.408	β-Phellandrene	0.97	555-10-2
14	10.731	γ-Terpinene	0.62	99-85-4
15	12.410	Linalool propanoate	0.55	144-39-8
		15 compounds	**98.36%**	

The mass spectra of the major compounds **(17, 19, 2** and **23)** and their comparison with the NIST and Adams libraries are shown in appendices 9, 11, 13 and 14 respectively. In previous studies by Gakuubi (2016) on the characterization of *T. asiatica*, sabinene was identified as the most abundant chemical compound. Linalool has also been identified as a major constituent of the essential oils of *T. asiatica* (Nattudurai *et al*., 2014)



22



23

4.5 Effects of individual essential oils at different concentrations on fungal growth

Fungal assays were carried out to investigate the effects of three selected essential oils at different concentrations on the growth of *F. verticillioides*, *A. niger* and *A. flavus*. *T. asiatica*, *O. gratissimum* and *L. javanica* exhibited varying responses on different fungi growth.

4.5.1 Percentage inhibition of individual essential oils at different concentrations against the growth of *F. verticillioides*

The percentage inhibition of the three essential oils at different concentrations against the growth of *F. verticillioides is* given in Table 4.9.

Table 4.9: Percentage inhibition of *F. verticillioides* in media treated with different concentrations of essential oils of *T. asiatica* (T), *O. gratissimum* (O) and *L. javanica* (L)

	Percentage inhibition (mean±SD)		
Concentration (µLmL^{-1})	***T. asiatica***	***O. gratissimum***	***L. javanica***
1	18.70±2.48^{bA}	14.86±2.72^{bA}	12.11±1.90^{Aa}
2	29.14±1.61^{bB}	23.65±0.73^{bB}	21.18±1.83^{Ab}
4	42.33±1.40^{bC}	35.19±1.12^{bC}	33.26±1.86^{aC}
8	47.82±1.19^{bD}	42.33±2.78^{bD}	40.13±2.87^{Ad}
16	62.11±2.26^{bE}	56.62±1.56^{aE}	55.52±2.31^{aE}
32	75.85±1.28^{bF}	72.00±2.99^{bF}	68.70±2.30^{aF}
Positive control:	(Captan 32 µLmL^{-1}) 60.62±2.28 %	**Negative control** (PDA) 0%	

Mean values± SD with the same small letterin each row and with the same capital letter in each column are not significantly different $P < 0.05$ (SNK).

According to table 4.9, the inhibitory activities of all the three essential oil increased with increased concentrations. The percentage inhibition of the essential oils increased significantly at higher concentrations. Of the three oils, those of *T. asiatica* and *O. gratissimum* showed comparable levels of inhibitory activity.

The percentage inhibition of *T. asiatica* and *O. gratissimum* essential oils did not statistically differ significantly at lower concentrations (< 16 µLmL^{-1}). At 16 µLmL^{-1}, statistically different inhibition activities of *T. asiatica* (62.11 %) and *O. gratissimum* (56.62 %) were observed. *T. asiatica* (62.11 %) at 16 µLmL^{-1}, showed inhibition activity that was comparable to that of the positive control, captan (60.62 %) at 32 µLmL^{-1}.

At 32 µLmL^{-1}, the individual essential oils had better inhibition activity than captan. When 32 µLmL^{-1} of the essential oils were separately incorporated in the growth media comparable activities of 75.85 % (*T. asiatica*) and 72.00 % (*O. gratissimum*) fungal inhibition were observed. When 32 µLmL^{-1} of the essential oils *L. javanica* was used, a statistically lower percentage growth inhibition (68.70 %) was achieved. *L. javanica* demonstrated 75 % inhibition activity when 35 µLmL^{-1} of the essential oil was incorporated in the culture media. The percentage inhibitions achieved when essential oils of *L. javanica* were used were significantly lower than those of *T. asiatica* and *O. gratissimum.* At a concentration of 16 µLmL^{-1}, comparable growth inhibition activities of 56.62 % (*O. gratissimum*) and 55.52% (*L. javanica*) were observed.

An inhibition activity of 90 % against the growth of *F. verticillioides* was achieved when concentrations of 92, 90 and 110 µLmL^{-1} of essential oils of *T. asiatica*, *O. gratissimum* and *L. javanica* respectively were separately incorporated in the culture media. Essential oils of *L. javanica* had the lowest inhibitory effect, compared to *T. asiatica* and *O. gratissimum,* on the growth of *F. verticillioides.* Graphical presentation of the inhibitory trends of the three essential oils at different concentrations against the growth of *F. verticillioides* is shown in appendix 15.

4.5.2 Percentage inhibition of individual essential oils at different concentrations against the growth of *A. niger*

The percentage inhibition of the three essential oils at different concentrations against the growth of *A. niger* is given in Table 4.10.

Table 4.10: Percentage inhibition of *A. niger* in media treated with different concentrations of essential oils of *T. asiatica* (T), *O. gratissimum* (O) and *L. javanica* L)

	Percentage inhibition (mean±sd) N=5		
Concentration (µLmL^{-1})	***T. asiatica***	***O. gratissimum***	***L. javanica***
1	21.99±1.28^{cA}	15.60±2.56^{bA}	11.25±1.72^{aA}
2	29.41±1.40^{cB}	25.32±2.14^{bB}	19.18±2.46^{aB}
4	39.13±1.46^{cC}	33.76±1.90^{bC}	28.13±1.90^{aC}
8	60.10±2.29^{cD}	51.92±2.14^{bD}	43.99±2.29^{aD}
16	63.94±2.29^{cE}	59.85±2.49^{bE}	55.54±2.92^{aE}
32	84.40±2.29^{cF}	75.70±0.90^{bF}	70.59±1.28^{aF}
Positive control:	(Captan 32 µLmL^{-1}) 87.60±1.14 %		**Negative control** (PDA) 0%

Mean values± SD with the same small letterin each row and with the same capital letter in each column are not significantly different $P < 0.05$ (SNK).

From table 4.10, the percentage inhibition of each of the three essential oils increased with increased concentrations. An increase in the concentration of the essential oil incorporated in the PDA growth media resulted to a significantly higher inhibition against the growth of *A. niger*. A comparison of the percentage inhibition activity of the individual essential oils at a constant concentration differed significantly from each other.

Of the three essential oils, those of *T. asiatica* had higher inhibition activity against the growth of *A.niger* compared to *O. gratissimum* and *L. javanica*; 75 and 90 % fungal inhibitions were achieved with treatments of of 22 and 36 µLmL^{-1} respectively. *O. gratissimum* demonstrated a 75 % inhibition activity at 32 µLmL^{-1} and 90 % inhibition activity at 81 µLmL^{-1}. The positive control, captan, demonstrated 75 % and 90 % fungal inhibition against *A. niger* with treatments of of 20 µLmL^{-1} and 34 µLmL^{-1} respectively. All percentage inhibitions demonstrated against

the growth of *A. niger* at 32 µLmL^{-1} significantly differed from each other. The positive control demonstrated a higher inhibition activity, against the growth of *A. niger*, than the individual essential oils. At 32 µLmL^{-1} *T. asiatica*, *O. gratissimum*, *L. javanica* and captan had a percentage inhibition of 84.40, 75.70, 70.59 and 87.60 % respectively.

Essential oils of *L. javanica* had the lowest growth inhibitory effect against *A. niger* where 75 % and 90 % percentage inhibitions were achieved at 34 µLmL^{-1} and 93 µLmL^{-1} respectively. Graphical presentation of the inhibitory trends of the three essential oils at different concentrations against the growth of *A. niger* is shown in appendix 16.

4.5.3 Percentage inhibition of individual essential oils at different concentrations against the growth of *A. flavus*

The percentage inhibition of the three essential oils at different concentrations against the growth of *A. flavus* is given in Table 4.11.

Table 4.11: Percentage inhibition of *A. flavus* in media treated with different concentrations of essential oils of *T. asiatica* (T), *O. gratissimum* (O) and *L. javanica* (L)

	Percentage inhibition (mean±sd) N=5		
Concentration (µLmL^{-1})	***T. asiatica***	***O. gratissimum***	***L. javanica***
1	19.01±1.17^{bA}	18.14±1.96^{bA}	15.49±1.86^{aA}
2	24.41±1.05^{bB}	22.54±2.35^{bB}	19.72±1.34^{aB}
4	36.15±2.43^{cC}	30.99±2.68^{bC}	26.53±1.05^{aC}
8	51.17±1.96^{cD}	43.43±1.74^{bD}	38.73±1.29^{aD}
16	66.20±1.53^{cE}	61.03±2.68^{bE}	55.87±1.78^{aE}
32	80.75±2.57^{cF}	74.65±1.96^{bF}	69.01±1.96^{aF}
Positive control:	(Captan 32 µLmL^{-1}) 78.34±1.33 %	**Negative control** (PDA) 0%	

Mean values± SD with the same small letter in each row and with the same capital letter in each column are not significantly different $P < 0.05$ (SNK).

From table 4.11, fungal assays were carried out to investigate the effects of *T. asiatica*, *O. gratissimum* and *L. javanica* at different concentrations on the growth of *A. flavus*. The inhibitory activities of all the three-essential oil against *A. flavus*, increased with increased concentration.

Essential oils of *L. javanica* had significantly lower inhibitory effects compared to the essential oils of *T. asiatica* and *O. gratissimum*. A 75 % fungal growth inhibition was achieved at 28 µLmL^{-1}, 32 µLmL^{-1}, 34 µLmL^{-1} and 30 µLmL^{-1}, when using *T. asiatica, O. gratissimum, L. javanica* and captan respectively. Fungal inhibition growth of 90 % for the essential oils of *T. asiatica, O. gratissimum, L. javanica* and captan was achieved at concentrations of 68 µLmL^{-1}, 71 µLmL^{-1}, 104 µLmL^{-1} and 70 µLmL^{-1}, respectively. *T. asiatica* demonstrated better growth inhibition against *A. flavus* than captan and essentials oils of *O. gratissimum* and *L. javanica*.

At lower concentrations, < 4 µLmL^{-1}, *T. asiatica* and *O. gratissimum* exhibited comparable effects on their inhibition activities against *A. flavus.* When a concentration of 1 µLmL^{-1} essential oil was used, there was no significance difference in the percentage inhibition exhibited by *T. asiatica* (19.01 %) and that exhibited by *O. gratissimum* (18.14 %). At 1 µLmL^{-1}, *L. javanica* had a lower percentage inhibition activity (15.49 %). When a concentration of 2 µLmL^{-1} essential oil was used, *L. javanica* exhibited a significantly lower inhibition activity of 19.72 % compared to *T. asiatica* (24.41 %) and *O. gratissimum* (22.54 %) whose inhibition activity had no significance difference. At higher concentrations, >4 µLmL^{-1}, the inhibition activities of the three essential oils (*L. javanica, T. asiatica* and *O. gratissimum*) differed significantly. At higher concentrations (>4 µLmL^{-1}) *T. asiatica* showed a higher percentage growth inhibition of *A. flavus* than *O. gratissimum*.

At 32 µLmL^{-1} *T. asiatica*, *O. gratissimum*, *L. javanica* and captan had a percentage inhibition of 80.75, 74.65, 69.01 and 78.34 % respectively. The percentage inhibitions demonstrated by the three essential oils against the growth of *A. flavus* at 32 µLmL^{-1} significantly differed from each other. Among the essential oils, *T. asiatica,* had the highest inhibition activity (80.75 %). The positive control demonstrated an inhibition activity of 78.34 %, against the growth of *A. niger*, compared to the essential oils of *O. gratissimum* (74.65 %) and *L. javanica* (69.01 %). There was no statistical difference between the inhibition activities of captan (78.34 %) and that of essential oils of *T. asiatica* (80.75 %). Graphical presentation of the inhibitory trends of the three essential oils at different concentrations against the growth of *A. flavus* is shown in appendix 17.

4.6 Percentage inhibition of different blends of essential oils against fungal growth

4.6.1 Percentage inhibition of different blends of essential oils at different concentrations against the growth of *F. verticillioides*

Tthe percentage growth inhibition of *F. verticillioides* in media treated with different blends (1:1 and 1:1:1 blends) of essential oils at different concentrations is given in Table 4.12.

Table 4.12: Percentage growth inhibition of *F. verticillioides* in media treated with different blends of essential oils at different concentrations

	Percentage inhibition (mean±sd) N=5			
Concentration (µLmL^{-1})	**T+L**	**T+O**	**O+L**	**T+O+L**
1	32.99 ± 1.48^{bA}	35.74 ± 2.44^{bA}	24.20 ± 2.45^{aA}	55.52 ± 1.24^{cA}
2	43.98 ± 1.11^{bB}	48.10 ± 2.07^{cB}	39.03 ± 2.27^{aB}	65.41 ± 1.49^{dB}
4	55.79 ± 2.00^{bC}	63.48 ± 3.13^{cC}	42.64 ± 2.24^{aC}	78.59 ± 1.22^{dC}
8	67.88 ± 1.87^{bD}	70.35 ± 2.49^{cD}	62.66 ± 1.54^{aD}	82.44 ± 1.18^{dD}
16	76.78 ± 0.96^{bE}	78.32 ± 2.03^{cE}	71.73 ± 1.25^{aE}	95.08 ± 1.22^{dE}
32	86.56 ± 1.77^{bF}	82.16 ± 2.21^{aF}	81.62 ± 1.27^{aF}	100.00 ± 0.00^{cF}
Positive control:	(Captan 32 µLmL^{-1}) 60.62±2.28 %		**Negative control** (PDA) 0 %	

Mean values± SD with the same small letter in each row and with the same capital letter in each column are not significantly different $P < 0.05$ (SNK).

From table 4.12, the percentage inhibition of *F. verticillioides* in media treated with different blends of essential oils increased with increased oil concentration and increased number of plant essential oils incorporated in the growth media. A blend of all the three essential oils: *T. asiatica* (T), *O. gratissimum* (O) and *L. javanica* (L) (T+O+L) showed higher inhibitory activity than blends of two essential oils.

In subtractive bio-assays, blends of two essential oils were used. When a concentration of 1 μLmL^{-1} was used, there was no significant difference in the growth inhibition activity exhibited by *T. asiatica* and *O. gratissimum* (35.74 %) blend and that exhibited when using a blend of *T. asiatica* and *L. javanica* (32.99 %). A 1 μLmL^{-1} blend of *O. gratissimum* and *L. javanica* exhibited a lower percentage growth inhibition of 24.20 %. At 1 μLmL^{-1}, a blend of three essential oil (1:1:1) had the growth highest inhibition activity of 55.52 %.

When concentrations ranging between 1 and 32 μLmL^{-1} (>1 μLmL^{-1} and < 32 μLmL^{-1}) were used, a blend of *T. asiatica* and *O. gratissimum* showed comparable inhibitory activity to the blend of *T. asiatica* and *L. javanica.* At these concentrations, there was no significant difference in the inhibition activities of the blend containing *T. asiatica* and *O. gratissimum* and that of *T. asiatica* and *L. javanica.* At concentrations >1 μLmL^{-1} and < 32 μLmL^{-1}, a blend of *O. gratissimum* and *L. javanica* exhibited a lower percentage growth inhibition in comparison to other blends.

At higher essential oil concentration, 32 μLmL^{-1}, there was no significant difference in the inhibition activity exhibited by the blend of *T. asiatica* and *O. gratissimum* (82.16 %) and that exhibited by a blend of *O. gratissimum* and *L. javanica* (81.62 %). At 32 μLmL^{-1}, among the 1:1 blend, a combination of *T. asiatica* and *L. javanica* had a higher inhibition activity of 86.56 %.
At 32 μLmL^{-1}, captan, the positive control demonstrated the lowest inhibition activity (60.62 %) while a 1:1:1 blend of the three essential oils exhibited the highest inhibition activity of 100.00 % inhibition. A graphical presentation of the inhibitory trends of media treated with different blends against the growth of *F. verticillioides* is presented in appendix 16.

4.6.2 Percentage inhibition of different blends of essential oils at different concentrations against the growth of *A. niger*

The percentage growth inhibition of *A. niger* in media treated with different blends (1:1 and 1:1:1 blends) of essential oils at different concentrations is given in Table 4.13.

Table 4.13: Percentage growth inhibition of *A. niger* in media treated with different blends of essential oils at different concentrations

	Percentage inhibition (mean±sd) N=5			
Concentration (µLmL^{-1})	**T+L**	**T+O**	**O+L**	**T+O+L**
1	34.78±1.81^{bA}	37.08±2.29^{cA}	28.64±1.40^{aA}	56.01±2.14^{dA}
2	48.85±1.81^{bB}	53.96±2.02^{cB}	40.66±1.94^{aB}	67.52±2.14^{dB}
4	58.82±1.07^{bC}	61.89±1.90^{cC}	48.08±2.14^{aC}	74.94±2.14^{dC}
8	67.77±1.40^{bD}	77.49±1.46^{cD}	61.64±1.81^{aD}	85.42±2.14^{dD}
16	76.73±1.90^{bE}	82.10±2.02^{cE}	69.05±2.92^{aE}	96.42±2.29^{dE}
32	87.47±1.40^{bF}	92.33±1.81^{cF}	82.86±1.46^{aF}	100.00±0.00^{dF}
Positive control:	(Captan 32 µLmL^{-1}) 87.60±1.14 %		**Negative control** (PDA) 0 %	

Mean values± SD with the same small letter in each row and with the same capital letter in each column are not significantly different $P < 0.05$ (SNK).

From table 4.13, the percentage inhibition of *A. niger* in media treated with different blends of essential oils increased with increased oil concentration and increased number of plant essential oils incorporated in the growth media.

A blend of all the three essential oils (*T. asiatica* (T), *O. gratissimum* (O) and *L. javanica* (L)) showed the better inhibitory activity than blends of two essential oils. Combining all the essential oils increased the growth inhibition of *A. niger*. Subtraction of one essential oil from the 1:1:1

blend reduced the inhibitory effect. The inhibition activity of the different blends differed significantly from each other at the various concentrations. Among the blends of two, *T. asiatica* and *O. gratissimum* exhibited a higher inhibition activity followed by a blend of *T. asiatica* and *L. javanica.* A blend of *O. gratissimum* and *L. javanica* exhibited the lowest percentage growth inhibition at different concentrations.

Elimination of *T. asiatica* demonstrated the highest effect on percentage inhibition. A 1:1 blend of *O. gratissimum* and *L. javanica* had the least inhibition activities among the blends of two. Elimination of *L. javanica* demonstrated the least effect on percentage inhibition. A 1:1 blend of *O. gratissimum* and *T. asiatica* had the highest inhibition activities among the 1:1 blend.

A blend of the three essential oils at 32 μLmL^{-1} demonstrated the highest inhibition activity (100 %). Inhibition activities of the blend of three essential oils (100 %) and that of the blend of *T. asiatica* and *O. gratissimum* (92.33 %) were higher than that exhibited by captan, the positive control (87.60 %). A graphical presentation of the inhibitory trends of media treated with 1:1:1 blend and 1:1 blends of the essential oils against the growth of *A. niger* is presented in appendix 17.

4.6.3 Effects of a blend of all the selected essential oils and their subtractive bioassays at different concentrations on *A. flavus*

The percentage growth inhibition of *A. flavus* in media treated with different blends (1:1 and 1:1:1 blends) of essential oils at different concentrations are shown in Table 4.14.

Table 4.14: Percentage growth inhibition of *A. flavus* in media treated with different blends of essential oils at different concentrations

	Percentage inhibition (mean±sd) N=5			
Concentration (µLmL^{-1})	**T+L**	**T+O**	**O+L**	**T+O+L**
1	31.92±2.87^{bA}	34.27±2.87^{cA}	24.18±1.57^{aA}	48.36±1.66^{dA}
2	38.26±1.34^{bB}	41.78±1.96^{cB}	33.80±1.96^{aB}	51.64±1.53^{dB}
4	46.24±1.74^{bC}	49.77±2.10^{cC}	41.78±2.57^{aC}	61.27±1.17^{dC}
8	63.85±1.53^{aD}	67.14±1.66^{bD}	61.03±2.54^{aD}	71.83±1.44^{cD}
16	76.53±1.86^{aE}	80.28±2.68^{bE}	75.82±2.29^{aE}	87.79±1.96^{cE}
32	84.27±1.96^{aF}	88.50±1.74^{bF}	82.63±1.29^{aF}	100.00±0.00^{cF}
Positive control:	(Captan 32 µLmL^{-1}) 78.34±1.33 %		**Negative control** (PDA) 0 %	

Mean values± SD with the same small letter in each row and with the same capital letter in each column are not significantly different $P < 0.05$ (SNK).

From table 4.14, the percentage inhibition of *A. flavus* in media treated with different blends of essential oils increased with increased oil concentration and increased number of plant essential oils incorporated in the growth media.

A blend of the three oils showed the highest inhibitory activity among the various blends. Subtractive bioassays, where only blends of two essential oils were used the inhibition activity of all the blends differed significantly from each other at concentrations of 1, 2, and 4 µLmL^{-1}. A blend of *T. asiatica* and *O. gratissimum* (T+O) had a higher inhibition activity at 1, 2, and 4 µLmL^{-1} followed by a blend of *T. asiatica* and *L. javanica* (T+L). At low concentrations, a blend of *O. gratissimum* and *L. javanica* (O+L) had the lowest percentage inhibitions.

At higher concentrations, >4 µLmL^{-1}, a blend of T+O exhibited the highest inhibition activity among the blends of two essential oils. Blends O+L and T+L did not have any significant

difference in the exhibited percentage inhibitions. At concentrations >4 µLmL^{-1}, a blend of the three oils had the highest inhibition activity among the various blends. A maximum inhibition activity of 75, 90 and 100% was achieved by combing all the three oils at a concentration of 9, 17 and 32 µL/mL. Blends of *T. asiatica* and *L. javanica* (T+L), *T. asiatica* and *O. gratissimum* (T+O) and *O. gratissimum* and *L. javanica* (O+L) demonstrated percentage inhibitions of 84.27, 88.50 and 82.63 % respectively. At 32 µL/mL, the positive control demonstrated the lowest inhibition activity (78.34 %) compared to the different blends tested. A graphical presentation of the inhibitory trends of media treated with 1:1:1 blend and 1:1 blends of the essential oils against the growth of *A. flavus* is presented in appendix 18.

4.7 Percentage fungal growth inhibition in media treated with different blends of the major components of plant essential oils

The major compounds of each plant essential oils were identified based on their percentage composition. Major components identified from *O. gratissimum* essential oils were Eugenol (E1), terpinen-4-ol (T1), *β*-elemene (E2) and methyl eugenol (ME). A blend of these major components in the ratio 1:1:1:1 (E1+T1+E2+ME) and its subtractive bioassays were separately studied for their inhibition activities.

4.7.1 Percentage fungal growth inhibition in media treated with the individual and different blends of major compounds of essential oils of *T. asiatica*

The percentage inhibition of *A. niger*, *F. verticillioides* and *A. flavus* in media treated with a blend of four major compounds of essential oils of *T. asiatica* (Sabinene, Linalool, Terpinen-4-ol and Terpinolene) and their subtractive bioassays are given in Table 4.15.

Table 4.15: Percentage inhibition of *F. verticillioides*, *A. niger* and *A. flavus* in media treated with a blend of major compounds of essential oils of *T. asiatica* and blends with each constituent subtracted

		Percentage inhibition (mean±sd) N=5		
Treatment	**Compound**	***A. niger***	***F. verticillioides***	***A. flavus***
1	S+L1+T1+T2	58.69 ± 2.26^{bH}	67.84 ± 1.57^{cI}	52.41 ± 2.57^{aJ}
2	S+L1+T1	52.58 ± 1.78^{bG}	64.79 ± 1.44^{cH}	49.77 ± 2.10^{aI}
3	S+L1+T2	49.77 ± 1.53^{bF}	56.81 ± 1.53^{cG}	44.84 ± 1.17^{aH}
4	S+T1+T2	47.18 ± 1.17^{bE}	51.64 ± 1.29^{cF}	39.91 ± 3.04^{aG}
5	L1+T1+T2	46.48 ± 1.05^{bE}	49.53 ± 1.17^{cEF}	36.85 ± 1.74^{aF}
6	S+L1	42.96 ± 1.34^{bD}	48.36 ± 1.66^{cE}	33.10 ± 0.83^{aE}
7	S+T1	41.08 ± 1.53^{bCD}	47.89 ± 1.78^{cE}	32.39 ± 1.78^{aDE}
8	L1+T1	39.44 ± 1.34^{bC}	45.54 ± 1.05^{cD}	31.92 ± 1.86^{aD}
9	T1+T2	37.79 ± 1.44^{bC}	42.25 ± 0.52^{cC}	27.00 ± 1.29^{aC}
10	Sabinene (S)	35.92 ± 1.57^{bB}	38.50 ± 1.34^{cB}	24.88 ± 1.44^{aB}
11	Linalool (L1)	35.45 ± 1.86^{bB}	38.50 ± 0.64^{cB}	24.41 ± 1.78^{aB}
12	Terpinen-4-ol (T1)	34.27 ± 0.83^{bB}	37.32 ± 1.34^{cB}	23.94 ± 1.93^{aB}
13	Terpinolene (T2)	32.16 ± 1.74^{Ba}	35.21 ± 0.98^{cA}	20.42 ± 0.98^{aA}

Mean values± SD with the same small letter in each row and with the same capital letter in each column are not significantly different $P < 0.05$ (SNK).

From table 4.15, varying inhibition activities were demonstrated when different blends of major compounds in *T. asiatica* essential oils were used as culture media treatments. The percentage inhibition increased with increased number of compounds in the blend. A 1:1:1:1 blend of the four major compounds of *T. asiatica* (treatment 1) demonstrated the highest inhibition activity against the fungus growth. The inhibition activity exhibited by treatment 1 on the three fungus differed signifintly with *F. verticillioides* (67.84 %) showing the highest percentage inhibition in comparison to *A. niger* (58.69 %), and *A. flavus* (52.41 %).

A compound's percentage composition in the essential oil significantly impacted the fungus percentage inhibition during subtractive bioassays. Elimination of major compounds that account for the highest chemical composition in the essential oil (Treatment 4 and Treatment 5) had an impact on fungal growth inhibition. Treatment 4 and 5, without L1 and S respectively, exhibited the least percentage inhibition among the blends comprising of three compounds. The percentage inhibitions of treatment 4 (*A. niger* (47.18 %), *F. verticillioides* (51.64 %) and *A. flavus* (39.91 %) and those of treatment 5 (*A. niger* (46.48 %), *F. verticillioides* (49.53 %) and *A. flavus* (36.85 %) did not differ significantly. Subtractive bioassays of different 1:1 blend (treatment 6, 7, 8 and 9) demonstrated lower inhibition activities compared to the blend of four compounds (treatment 1) and the blends of three compounds (treatment 2, 3, 4 and 5).

Fungal assays using individual compounds of *T. asiatica* essential oils demonstrated the least inhibition activities in comparison to the blends. The mean inhibition activities of the sabinene, linalool and terpinen-4-ol when used individually (treatment 10,11 and 12) against the growth of *F. verticillioides*, *A. niger* and *A. flavus* did not differ significantly. This is an indication that the high percentage inhibition exhibited by various blends was due to synergistic effects of the compounds. The percentage inhibitions increased with increased number of compounds in the treatments. These results are in agreement with data from previous studies which attribute antibacterial and antifungal properties of essential oil constituents to synergistic properties of the blends (Nikkhah *et al*., 2017).

4.7.2 Percentage fungal growth inhibition in media treated with the individual and different blends of major compounds of essential oils of *L. javanica*

The percentage inhibition of *F. verticillioides*, *A. niger* and *A. flavus* in media treated with a blend of major compounds of essential oils of *L. javanica* (limonene, artemisia ketone, myrcene and sabinene) and their subtractive bioassays is given in Table 4.16.

Table 4.16: Percentage inhibition of *F. verticillioides*, *A. niger* and *A. flavus* in media treated with different compounds and blends of essential oils of *L. javanica* and their subtractive bioassays

		Percentage inhibition (mean±sd) N=5		
Treatment	**Compound**	***F.* verticillioides**	***A. niger***	***A. flavus***
1	L+A+M+S	45.07 ± 2.26^{aG}	49.06 ± 1.34^{bG}	46.95 ± 1.53^{aG}
2	L+A+M	42.25 ± 1.93^{bF}	43.66 ± 1.44^{bF}	40.38 ± 2.26^{aF}
3	A+M+S	40.85 ± 1.96^{bEF}	38.26 ± 1.57^{aDE}	37.09 ± 2.43^{aE}
4	L+M+S	38.50 ± 2.43^{bE}	38.03 ± 1.53^{bD}	35.45 ± 1.86^{aDE}
5	L+A+S	37.79 ± 1.17^{bE}	36.85 ± 3.04^{bD}	34.04 ± 1.74^{aD}
6	L+A	35.21 ± 1.53^{bD}	35.21 ± 0.98^{bCD}	32.63 ± 1.57^{aC}
7	A+M	33.80 ± 1.96^{bC}	34.51 ± 0.98^{bC}	31.22 ± 1.78^{aC}
8	M+L	31.46 ± 1.9^{aC}	33.57 ± 2.57^{bC}	30.05 ± 1.34^{aC}
9	M+S	28.87 ± 1.57^{bB}	30.52 ± 1.29^{cB}	25.82 ± 1.29^{aB}
10	Limonene (L)	25.59 ± 1.78^{bA}	26.76 ± 1.05^{bA}	23.24 ± 1.57^{aA}
11	Artemisia ketone (A)	25.35 ± 0.64^{bA}	26.53 ± 1.78^{bA}	23.00 ± 0.64^{aA}
12	Myrcene (M)	25.12 ± 1.53^{bA}	26.29 ± 0.98^{bA}	22.77 ± 1.29^{aA}
13	Sabinene (S)	24.88 ± 1.17^{bA}	26.06 ± 1.17^{cA}	22.54 ± 2.35^{aA}

Mean values± SD with the same small letter in each row and with the same capital letter in each column are not significantly different $P < 0.05$ (SNK).

From table 4.16 above, the level of fungal growth inhibition varied depending on the number of compounds blended in the treatment. The percentage inhibition increased with increased number

of compounds in the blend. A 1:1:1:1 blend of the four major compounds of *T. asiatica* (treatment 1) demonstrated the highest inhibition activity against the fungus growth. The inhibition activity exhibited by treatment 1 on the three fungus differed signifintly with *F. verticillioides* (45.07 %) showing the highest percentage inhibition in comparison to *A. niger* (49.06 %), and *A. flavus* (46.95 %).

Fungal assays (treatment 10,11,12 and 13) using individual compounds of essential oils of *L. javanica* demonstrated the least inhibition activities in comparison to the blends. The mean percentage inhibition of limonene (treatment 10), artemisia ketone (treatment 11), myrcene (treatment 12) and sabinene (treatment 13) against the growth of *F. verticillioides*, *A. niger* and *A. flavus* did not differ significantly. When the compounds were blended in pairs (treatment 6, 7, 8 and 9), the percentage inhibitions were higher than those exhibited by individual compounds (treatment 10, 11, 12 and 13).

Among the blends, limonene and artemisia ketone (treatment 6), had the highest percentage inhibition of *F. verticillioides* (35.21 %), *A. niger* (35.21 %) and *A. flavus* (32.63 %). A blend of myrcene and sabinene (treatment 9) had the lowest percentage inhibition of *F. verticillioides* (28.87 %), *A. niger* (30.52 %) and *A. flavus* (25.82 %). Limonene and artemisia ketone accounted for a higher percentage composition in essential oil of *L. javanica* than myrcene and sabinene. A compound's percentage composition in the essential oil significantly impacted the fungus percentage inhibition.

Blends of three compounds had greater inhibition activity than the blends of two and treatments containing individual compounds. Synergistic effects of the constituents of *L. javanica* essential oils are demonstrated in experimental treatments where different number of constituents was incorporated to form blends. The results of the percentage inhibition of fungal growth by major compounds of essential oils of *L. javanica* and their subtractive bioassays is comparable to a review study by Langeveld *et al*. (2014) on synergy between essential oil components.

4.7.3 Percentage fungal growth inhibition in media treated with the individual and different blends of major compounds of essential oils of *O. gratissimum*

The percentage inhibition of *F. verticillioides*, *A. niger* and *A. flavus* in media treated with a blend of major compounds of essential oils of *L. javanica* and their subtractive bioassays is given in Table 4.17.

Table 4.17: Percentage inhibition of *F. verticillioides*, *A. niger* and *A. flavus* in media treated with different compounds and blends of essential oils of *O. gratissimum* and their subtractive bioassays

		Percentage inhibition (mean±sd) N=5		
Treatment	**Compound**	***F. verticillioides***	***A. niger***	***A. flavus***
1	E1+T+E2+M	54.23 ± 2.62^{cI}	56.81 ± 2.26^{bH}	44.37 ± 1.34^{aF}
2	E1+ T+E2	50.23 ± 1.78^{bH}	51.17 ± 1.34^{bG}	42.25 ± 1.53^{aE}
3	E1+T+M	48.36 ± 1.44^{bG}	49.30 ± 1.53^{bF}	39.67 ± 1.78^{aD}
4	E1+E2+M	43.43 ± 1.53^{cF}	45.31 ± 1.96^{bE}	39.20 ± 1.74^{aD}
5	E2+T+M	40.38 ± 0.98^{cE}	44.84 ± 2.35^{bE}	38.50 ± 2.43^{aD}
6	E1+T	37.79 ± 1.66^{aD}	42.25 ± 1.53^{bD}	37.79 ± 1.66^{aCD}
7	E1+E2	37.56 ± 1.93^{aD}	42.02 ± 1.05^{bD}	36.15 ± 1.34^{aC}
8	T+E2	34.04 ± 1.74^{aC}	37.79 ± 1.17^{bC}	34.27 ± 1.86^{aB}
9	E2+M	31.92 ± 0.83^{aB}	34.27 ± 0.83^{bB}	32.39 ± 1.05^{aB}
10	Eugenol (E1)	30.75 ± 1.17^{bA}	29.34 ± 1.29^{bA}	25.35 ± 1.96^{aA}
11	Terpinen-4-ol (T)	30.52 ± 1.74^{cA}	28.87 ± 1.57^{bA}	25.12 ± 1.74^{aA}
12	*β*-Elemene (E2)	30.28 ± 1.05^{cA}	28.64 ± 0.98^{bA}	24.41 ± 1.05^{aA}
13	Methyl eugenol (M)	29.81 ± 1.53^{bA}	28.17 ± 0.98^{bA}	24.18 ± 1.57^{aA}

Mean values± SD with the same small letter in each row and with the same capital letter in each column are not significantly different $P < 0.05$ (SNK).

From table 4.17, the level of fungal growth inhibition varied depending on the number of compounds blended in the treatment. The percentage inhibition increased with increased number of compounds in the blend. A combination of all the four major constituents (treatment 1) yielded the highest percentage inhibition against the growth of *F. verticillioides* (54.23 %), *A. niger* (56.81 %), and *A. flavus* (44.37 %).

When the major constituents of *O. gratissimum* essential oils (eugenol, terpinen-4-ol, *β*-elemene and methyl eugenol) were assayed individually as fungal growth inhibitors (treatment 10, 11, 12 and 13) there was no significant difference ($P<0.05$) in their inhibition activities. The percentage

inhibitions increased when when the individual compounds were paired (treatment 6, 7, 8 and 9). Blends of three compounds (treatment 2, 3, 4 and 5) exhibited a higher inhibition activity than the blends of two (treatment 6, 7, 8 and 9) and the individual compounds (treatment 10, 11, 12 and 13). The major constituents of *O. gratissimum* demonstrated increased fungal growth inhibition due to the additive effect achieved by combining the individual compounds. Fungal inhibition attributed to additive effects of individual compounds in essential oils has been reported in previous studies (Langeveld *et al*., 2014; Nikkhah *et al*., 2017)

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSIONS

This study confirmed fumonisin B1 (FB1), aflatoxin B1 (AFB1) and ochratoxin A (OTA) contamination in chicken feeds from various agro-ecological zones of Kenya. Contamination levels of OTA, AFB1 and FB1 differed significantly within the study regions and in different chicken feeds and products. Chicken feed and products from Kakamega County had the highest levels of OTA and AFB1 contamination. The mean levels of OTA in samples from Kakamega were 165.33±0.42 and 7.12±1.25 ng/g for chicken feeds and chicken products respectively. Kakamega had average levels of AFB1 of 32.44±1.54 ng/g in chicken feed and 4.10±1.33 ng/g in chicken products. Makueni County had the highest level of FB1 contamination in chicken feed (27700 ng/g). FB1 was not detected in all the chicken products. The level of mycotoxins detected in the chicken feeds exceeded the allowed limits by the EU.

The mycotoxins, OTA, AFB1 and FB1, in chicken feed and products are as a result of specific fungi metabolites. OTA can be produced by more than one fungus; in this study, only *A. niger* was identified and isolated as the only OTA producing fungi. The OTA contamination in chicken feed and products from various agro-ecological zones of Kenya are produced by *A. niger*. *A. flavus* was present in chicken feed and is considered to be responsible for the production of AFB1 contamination when exposed to condusive environment for fungal growth. *F.*

verticilloides was identified and isolated from chicken feed and products and thus responsible for FB1 contamination in chicken feed and products from various agro-ecological zones of Kenya.

From the study, the three plant essential oils (*T. asiatica*, *O. gratissimum* and *L. javanica*) were found to inhibit fungal growth. The inhibition efficacy increased with increase in essential oil concentrations. *T. asiatica* demonstrated the highest inhibition activity against fungal growth while *L. javanica* had the least inhibition activity (appendices 15, 16 and 17). The essential oils had higher inhibition activity in blends than when tested individually probably due to synergistic effects. A blend of the three essential oils had the highest fungal growth inhibition activity followed by blends of two essential oils (appendices 15, 16 and 17). At a concentration of 32µL/ mL, a blend of the three oils had 100 % fungal growth inhibition.

Major constituents of the essential oils had low inhibition activity when applied individually. The inhibition activity of the major constituents increases when applied as blends where blends with the highest number of compounds had a higher inhibition activity. Blends of compounds that accounted for high percentage compositions in the essential oils constituents had a higher inhibition activity than those of lower percentage composition. The compounds worked best as blends probably due to additive/synergistic effect. Essential oils from various medicinal plants contain phytochemicals which can be explored for their ability to inhibit the growth of various mycotoxins in chicken feeds.

5.2 RECOMMENDATIONS

5.1.1 Recommendations from this study

i. A blend of *L. javanica, O. gratissimum* and *T. asiatica* at 32uL/mL showed promising antifungal activity and toxicity studies should be carried out to determine its efficacy.

ii. Ethno medicinal plants such as *L. javanica, O. gratissimum* and *T. asiatica* in addition to other species should be grown in botanical gardens for the purpose of conservation of these species and sustanaibility.

iii. The quantification of FB1 and OTA was below the detection limit in some samples, it is recommended that some more sensitive techniques like enzyme-linked immunosorbent assay (ELISA) and Ultra high-performance liquid chromatography–tandem mass spectrometry method for the quantification of OTA and FB1 be employed to confirm their presence.

iv. Based on the results obtained from OTA, AFB1 and FB1 quantification, there is need for Kenya to set up OTA, AFB1 and FB1 monitoring and control units within the existing standards bodies such as the Kenya Bureau of Standards and set maximum allowed levels in feeds and in chicken products. The set maximum levels would serve as a reference point in monitoring OTA, AFB1 and FB1 levels in chicken feeds and which would lay down the bases of putting in place practices and interventions that would reduce levels of these mycotoxins.

v. Poultry farmers in Kenya need to practice mycotoxins control in chicken feed so as to improve poultry health, productivity, performance and profits.

5.1.2 Recommendations for further work

i. Further studies should be carried out on the growth inhibition activities of the essential oils on other mycotoxin causing fungi such as *A. nidulans* ansd *A. versicolor* that produce sterigmatocystin and *F. graminearum* that produces zearalenone that can be identified in chicken feeds.

ii. Screening of other medicinal plants for their phytochemicals whose combination with the identified compounds will increase the fungal inhibition efficacy

iii. Quantification of mycotoxins in other animal feeds and animal products should be carried out in order to ensure safety of consumed animal products.

iv. Further studies on subtraction bioassay should be carried out using all the compounds in the essential oils so as to establish their role in fungi inhibition activities of each essential oil.

REFERENCES

Alakonya, A. E., Monda, E. O., and Ajanga, S. (2009). S Hort C Ommunication Fumonisin B 1 and Aflatoxin B 1 Levels in Kenyan Maize. *Journal of Plant Pathology*, *91*, 459–464.

Andreasson, U., Perret-Liaudet, A., van Waalwijk van Doorn, L. J. C., Blennow, K., Chiasserini, D., Engelborghs, S., and Teunissen, C. E. (2015). A practical guide to immunoassay method validation. *Frontiers in Neurology*, *6*(Aug), 1–8. https://doi.org/10.3389/fneur.2015.00179

Anjarwalla, P., Belmain, S., Koech, G., Jamnadass, R., and Stevenson, P. (2015). Pesticidal plant leaflet. In University of Greenwich (Ed.) (p. 2). South Africa: World Agroferestry Center.

Ariyo, A. L., Anthony, M. H., and Lami, M. H. (2013). Survey of Mycotoxigenic Fungi in Concentrated Poultry Feed in Niger State, Nigeria. *Journal of Food Research*, *2*(2), 128. https://doi.org/10.5539/jfr.v2n2p128

Atela, J., Tuitoek, J., Onjoro, P., Obonyo, M., and Judith, C. (2016). Occurrence of Aflatoxins in Feed Ingredients Used For Feeding Indigenous Chicken in Baringo and Kisumu Counties , Kenya . *IOSR Journal of Agriculture and Veterinary Science*, *9*(1), 40–44. https://doi.org/10.9790/2380-09114044

Banks, J., Holmes, S., and Scudamore, K. (2004). Project Report 332 Ochratoxin a (Ota) in Cereals : Development of a Rapid Test ; Species and Conditions Favouring Development Project Report 332 Ochratoxin a (Ota) in Cereals : Development of a Rapid Test ; Species and Conditions Favouring Development, (February).

Bekele, J., and Hassanali, A. (2001). Blend effects in the toxicity of the essential oil constituents of Ocimum kilimandscharicum and Ocimum kenyense (Labiateae) on two post-harvest insect pests. *Journal of Phytochemistry*, *57*(3), 385–391.

Bezencon C., Cavin T., Delatour M., Marin-Kuan D., Holzhauser L., H. C. (2007). Reduction in antioxidant defences may contribute to ochratoxin A toxicity and carcinogenicity. *Toxicological Sciences*, *96*, 30–39. https://doi.org/doi:10.1093/toxsci/kfl169

Carballo, D., Font, G., Ferrer, E., and Berrada, H. (2018). Evaluation of mycotoxin residues on ready-to-eat food by chromatographic methods coupled to mass spectrometry in tandem. *Toxins*, *10*(6). https://doi.org/10.3390/toxins10060243

Cicoňová, P., Laciaková, A., and Máté, D. (2010). Prevention of Ochratoxin A Contamination of Food and Ochratoxin A Detoxification by Microorganisms. *Czech Journal of Food Sciences* A Review, *28*(6), 465–474.

De Santis, B., Debegnach, F., Gregori, E., Russo, S., Marchegiani, F., Moracci, G., and Brera, C. (2017). Development of a LC-MS/MS method for the multi-mycotoxin determination in composite cereal-based samples. *Toxins*, *9*(5). https://doi.org/10.3390/toxins9050169

el Khoury, A. E., and Atoui, A. (2010). Ochratoxin A: General overview and actual molecular status. *Toxins*, *2*(4), 461–493. https://doi.org/10.3390/toxins2040461

Faruk, B. (2009). Different Mycotoxin Inactivation Applications and Their Inactivation Mechanisms. *Technical University Ankara, Turkey*, (117), 27–35. https://doi.org/10.2298/ZMSPN0917027B

Filazi, A., Yurdakok-Dikmen, B., Sireli, Ozgur, K., and Ufuk, T. (2018). Mycotoxins in poultry. In *Mycotoxins in poultry* (4th ed., pp. 12–18). London: IntechOpen. Retrieved from https://www.intechopen.com/books

Food and Agriculture Organization (FAO) of the United Nations. (2004). Mycotoxin regulations in 2003 and current developments. *Worldwide Regulations for Mycotoxins in Food and Feed in 2003*, 9–28. Retrieved from ftp://ftp.fao.org/

Fuchs, R., and Peraica, M. (2005). Ochratoxin A in human kidney diseases. *Food Additives and Contaminants*, *22*(Suppl 1), 53–57. https://doi.org/10.1080/02652030500309368

Gakuubi, M. M. (2016). Steam distillation extraction and chemical composition of essential oils of Toddalia asiatica L . and Eucalyptus. *Journal of Pharmacognosy and Phytochemistry*, *5*(2), 99–104.

Gathumbi, K. (1993). A survey of mycotoxigenic fungi and mycotoxins in poultry feed. *UoN Digital Repository*. Retrieved from http://erepository.uonbi.ac.ke/handle/11295/21051

Ghaemmaghami, S. S., Modirsaneii, M., Khosravi, A. R., and Razzaghi-Abyaneh, M. (2016). Study on mycoflora of poultry feed ingredients and finished feed in Iran. *Iranian Journal of Microbiology*, *8*(1), 47–54.

Government of Hong Kong. (2006). Ochratoxin A in Food. *Risk Assessment Studies, Report No. 23*, (23), 1–36.

Greco, M. V., Franchi, M. L., Rico Golba, S. L., Pardo, A. G., and Pose, G. N. (2014). Mycotoxins and mycotoxigenic fungi in poultry feed for food-producing animals. *Scientific World Journal*, *2014*. https://doi.org/10.1155/2014/968215

Grío, S. J. L., Frenich, A. G., Vidal, J. L. M., and Romero-González, R. (2010). Determination of aflatoxins B1, B2, G1, G2 and ochratoxin a in animal feed by ultra high-performance liquid

chromatography-tandem mass spectrometry. *Journal of Separation Science*. https://doi.org/10.1002/jssc.200900663

Henry, M. H., and Wyatt, R. D. (2001). The Toxicity of Fumonisin B 1 , B 2 , and B 3 , Individually and in Combination , in Chicken Embryos 1.

Herzallah, S. M. (2013). Aflatoxin B1 residues in eggs and flesh of laying hens fed aflatoxin B1 contaminated diet. *American Journal of Agricultural and Biological Science*, *8*(2), 156–161. https://doi.org/10.3844/ajabssp.2013.156.161

Ibrahim, M. J., Kabir, J., Kwanashie, C. N., Salawudeen, M. T., and Joshua, Z. (2017). Occurrence of mycotoxigenic fungi in poultry feeds at live-bird markets, Zaria, Nigeria. *Sokoto Journal of Veterinary Sciences*, *15*(4), 53. https://doi.org/10.4314/sokjvs.v15i4.8

Iqbal, S. Z., Nisar, S., Asi, M. R., and Jinap, S. (2014). Natural incidence of aflatoxins, ochratoxin A and zearalenone in chicken meat and eggs. *Food Control*, *43*, 98–103. https://doi.org/10.1016/j.foodcont.2014.02.046

ISO 5725-5, I. (1998). Iso 5725-5, *1998*.

Jemal, A., Bray, F., Forman, D., Brien, M. O., and Ferlay, J. (2012). Cancer Burden in Africa and Opportunities for Prevention. https://doi.org/10.1002/cncr.27410

Klich, M. (2002). Identification of common Aspergillus Species. *Central Bureau Voor Schimmecuktures, Utrecht, Netherlands.*, *17*(3).

KMT. (2016). Animal feed study: mapping amimal feed manufacturers and ingradient suppliers in Kenya. *Kenya Market Trust*, 1–74. Retrieved from http://www.kenyamarkets.org/wp-content/uploads/2017/10/Study-Report-on-Animal-Feed-Millers-and-Ingredient-Suppliers-in-Kenya-2017.pdf%0Ahttps://www.kenyamarkets.org/wp-content/uploads/2017/10/Summary-Report-on-Animal-Feed-Millers-and-Ingredient-Suppliers-

KNBS. (2017). statistical abstract 2013. *Kenya National Bureau of Statistics*.

Kokwaro, J. (2009). *Medicinal Plants of East Africa* (Third). Nairobi, Kenya: University of Nairobi Press. Retrieved from www.africanbookscollective.com

Kosgei, C. J., Matasyoh, J. C., and Mwendia, C. M. (2017). Chemical Composition And Larvicidal Activity Of Essential Oil From Lippia javanica against Rhipicephalus appendiculatus larvae. *Journal of Phamarcy and Biological Sciences*, *12*(6), 46–49. https://doi.org/10.9790/3008-1206064649

Kothari, C. R. (2004). *Research Methodology—Methods and Techniques.* (Vol. 2). New Age, New Delhi.

Kunio, D., and Uetsuka, K. (2011). Mechanisms of Mycotoxin-Induced Neurotoxicity through Oxidative Stress-Associated Pathways. *International Journal of Molecular Sciences.*, *12*(6), 5213–5327. https://doi.org/doi:10.3390/ijms12085213

Langeveld, W. T., Veldhuizen, E. J. A., and Burt, S. A. (2014). Synergy between essential oil components and antibiotics: A review. *Critical Reviews in Microbiology*, *40*(1), 76–94. https://doi.org/10.3109/1040841X.2013.763219

Lee, M., Seo, D. J., Jeon, S. B., Ok, H. E., Jung, H., Choi, C., and Chun, H. S. (2016). Detection of foodborne pathogens and mycotoxins in eggs and chicken feeds from farms to retail markets. *Korean Journal for Food Science of Animal Resources*, *36*(4), 463–468. https://doi.org/10.5851/kosfa.2016.36.4.463

Leslie, J. F., and Summerell, B. A. (2006). The Fusarium laboratory manual.

Marasas, W. F. O., Miller, J. D., RIley, R. T., and Visconti, A. (2000). Fumonisin B1. *WHO*, 174.

Matasyoh, L., Matasyoh, J., Wachira, F., Kinyua, M., Muigai, A., and Mukiama, T. (2007). Chemical composition and antimicrobial activity of the essential oil of Ocimum gratissimum L. growing in Eastern Kenya. *African Journal of Biotechnology*, *6*(6), 760–765. Retrieved from http://www.ajol.info/index.php/ajb/article/view/56899

Mi-jeong, J., Dongwon, B., Hanhong, B., Soo, L. I., Kim, J. A., Sung, C. S., … Park, S.-C. (2013). Inhibition of Botrytis cinerea Spore Germination and Mycelia Growth by Inhibition of Botrytis cinerea Spore Germination and Mycelia Growth by Frequency-specific Sound, (May 2015). https://doi.org/10.1007/s13765-013-3088-7

Ministry of Agriculture Livestock and Fisheries. (2015). Economic Review of Agriculture [ERA] 2015. In Central Planning and Project Monitoring Unit (Ed.) (pp. 3–39). Kenya: Kenya government press.

Miriam, K. N., Gowland, M. J., and Nkurumwa, A. (2014). Indigenous Chicken Rearing Systems and Their Influence on Household Income Among Small-Scale Farmers in Mau-Narok Division of Njoro District , Nakuru County , Kenya. *International Journal of Advanced Research*, *2*(5), 638–650. Retrieved from www.journalijar.com/uploads/881_IJAR-3293.pdf

Missmer, S. A., Suarez, L., Felkner, M., Wang, E., Merrill, A. H., Rothman, K. J., and Hendricks, K. A. (2006). Exposure to fumonisins and the occurence of neutral tube defects

along the Texas-Mexico border. *Environmental Health Perspectives*, *114*(2), 237–241. https://doi.org/10.1289/ehp.8221

Mokubedi, S. M., Phoku, J. Z., Changwa, R. N., Gbashi, S., and Njobeh, P. B. (2019). Analysis of Mycotoxins Contamination in Poultry Feeds Manufactured in Selected Provinces of South Africa Using UHPLC-MS/MS. *Toxins*, *11*(8), 452. https://doi.org/10.3390/toxins11080452

Monda, E. O., and Alakonya, A. E. (2016). A REVIEW OF AGRICULTURAL AFLATOXIN MANAGEMENT. *African Journal of Food, Agriculture, Nutrition and Development*, *16*(3), 11126–11138.

Mutiga, S. K., Hoffmann, V., Harvey, J. W., Milgroom, M. G., and Nelson, R. J. (2015). Assessment of Aflatoxin and Fumonisin Contamination of Maize in Western Kenya. *Phytopathology*, *105*(9), 1250–1261. https://doi.org/10.1094/phyto-10-14-0269-r

Nattudurai, G., Paulraj, M. G., and Ignacimuthu, S. (2014). Toddalia Asiatica (L .) Lam . Essential Oil : a Potential Natural Fumigant and Repellent Against Three Coleopteran Pests of Stored Products, *2*(3), 246–255.

Netherlands-African Business Council. (2015). Factsheet Kenya Poultry , Meat and Processing Sector Contact information. In H. Duns and D. Willems (Eds.). Hague, Netherlands: Netherlands-African Business Council.

Ng'ang'a, M. M., Hussain, H., Chhabra, S., Langat-Thoruwa, C., Riaz, M., and Krohn, K. (2011). DrypetdNg'ang'a, M. M., Hussain, H., Chhabra, S., Langat-Thoruwa, C., Riaz, M., and Krohn, K. (2011). Drypetdimer A: A New Flavone Dimer from Drypetes gerrardii. Natural Product Communications, 6(8), 1115–1116. https://doi.org/10.1177/1934578x1100600816imer. *Natural Product Communications*, *6*(8), 1115–1116. https://doi.org/10.1177/1934578x1100600816

Nikkhah, M., Hashemi, M., Habibi Najafi, M. B., and Farhoosh, R. (2017). Synergistic effects of some essential oils against fungal spoilage on pear fruit. *International Journal of Food Microbiology*, *257*, 285–294. https://doi.org/10.1016/j.ijfoodmicro.2017.06.021

Nuhu, A. A. (2015). Occurrence, harmful effects and analytical determination of Ochratoxin A in coffee. *Journal of Applied Pharmaceutical Science*, *5*(1), 120–127. https://doi.org/10.7324/JAPS.2015.50121

Oliveira, H. F. de, Souto, C. N., Martins, P. C., Di Castro, I. C., and Mascarenhas, A. G. (2018). Micotoxinas na produção de frangos de corte. *Revista de Ciências Agroveterinárias*, *17*(2), 292–299. https://doi.org/10.5965/223811711722018292

Omiti, J. (2007). Overview of the Kenyan Poultry Sector and Its Hpai Status. In *poultry sector issues* (pp. 1–27). Kenya.

Orwa, J. A., Jondiko, I. J. O., Minja, R. J. A., and Bekunda, M. (2008). The use of Toddalia asiatica (L) Lam. (Rutaceae) in traditional medicine practice in East Africa. *Journal of Ethnopharmacology*, *115*(2), 257–262. https://doi.org/10.1016/j.jep.2007.09.024

Ostry, V., Malir, F., Toman, J., and Grosse, Y. (2017). Mycotoxins as human carcinogens—the IARC Monographs classification. *Mycotoxin Research*, *33*(1), 65–73. https://doi.org/10.1007/s12550-016-0265-7

Paíga, P., Morais, S., Oliva-Teles, T., Correia, M., Delerue-Matos, C., Duarte, S. C., … Lino, C. M. (2012). Extraction of ochratoxin A in bread samples by the QuEChERS methodology. *Food Chemistry*, *135*(4), 2522–2528. https://doi.org/10.1016/j.foodchem.2012.06.045

Pfohl-Leszkowicz, A., and Manderville, R. A. (2007). Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. *Molecular Nutrition and Food Research*, *51*(1), 61–99. https://doi.org/10.1002/mnfr.200600137

Pitt, J. I., Wild, C. P., Baan, R. A., Gelderblom, W. C. A., Miller, J. D., Riley, R. T., and WU, F. (2012). Improving Public Health through Mycotoxin Control. *World Health*, *33*(0), 165.

Prabhu, K. S., Lobo, R., Shirwaikar, A. A., and Shirwaikar, A. (2009). Ocimum gratissimum : A Review of its Chemical , Pharmacological and Ethnomedicinal Properties, 1–15.

Praveena, A., and Suriyavathana, M. (2013). Phytochemical Characterization of Toddalia asiatica . L Var . Floribunda STEM, *6*(4), 4–7.

Probst, C., Njapau, H., and Cotty, P. J. (2007). Outbreak of an acute aflatoxicosis in Kenya in 2004: Identification of the causal agent. *Applied and Environmental Microbiology*, *73*(8), 2762–2764. https://doi.org/10.1128/AEM.02370-06

Saleemi, M. K., Khan, M. Z., Ahrar, K., and Javed, I. (2010). Mycoflora of poultry feeds and mycotoxins producing potential of Aspergillus species. *Pakistan Journal of Botany*, *42*(1), 427–434.

Sarma, U. P., Bhetaria, P. J., Devi, P., and Varma, A. (2017). Aflatoxins: Implications on Health. *Indian Journal of Clinical Biochemistry*, *32*(2), 124–133. https://doi.org/10.1007/s12291-017-0649-2

Sherazi, S. T. H., Shar, Z. H., Sumbal, G. A., Tan, E. T., Bhanger, M. I., Kara, H., and Nizamani, S. M. (2015). Occurrence of ochratoxin A in poultry feeds and feed ingredients from

Pakistan. *Mycotoxin Research*, *31*(1), 1–7. https://doi.org/10.1007/s12550-014-0216-0

Spanjer, M. C., Rensen, P. M., and Scholten, J. M. (2008). LC–MS/MS multi-method for mycotoxins after single extraction, with validation data for peanut, pistachio, wheat, maize, cornflakes, raisins and figs. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, *25*(4), 472–489. https://doi.org/10.1080/02652030701552964

Standard digital media. (2018). *Jomo Kenyatta University launches a nixtamalization machine*. https://doi.org/https://www.standardmedia.co.ke/article/2001282285/launch-of-nixtamalization-machine-in-jkuat

Swamy, M. K., Akhtar, M. S., and Sinniah, U. R. (2016). Antimicrobial properties of plant essential oils against human pathogens and their mode of action: An updated review. *Evidence-Based Complementary and Alternative Medicine*, *2016*. https://doi.org/10.1155/2016/3012462

Terra, M. F., Prado, G., Pereira, G. E., Ematné, H. J., and Batista, L. R. (2013). Detection of ochratoxin A in tropical wine and grape juice from Brazil. *Journal of the Science of Food and Agriculture*, *93*(4), 890–894. https://doi.org/10.1002/jsfa.5817

The University of Adelaide. (2017). Fungal description and antifungal susceptibility. *Micology Online*. Retrieved from https://mycology.adelaide.edu.au/descriptions/hyphomycetes/aspergillus/

Thiem, B., Kikowska, M., Kurowska, A., and Kalemba, D. (2011). Essential oil composition of the different parts and in vitro shoot culture of eryngium planum L. *Molecules*, *16*(8), 7115–7124. https://doi.org/10.3390/molecules16087115

Thuita, F. N., Tuitoek, J. K., King'ori, A. M., and Obonyo, M. A. (2019). Prevalence of aflatoxins contamination in commercial broiler feeds in Kenya. *Livestock Research for Rural Development, 31*(3). Retrieved from http://www.lrrd.org/lrrd31/1/jtuit31003.htmL

Varga, J., Kocsube, S., Zsanett, P., Csaba, V., and Beata, T. (2010). Chemical , Physical and Biological Approaches to Prevent Ochratoxin Induced Toxicoses in Humans and Animals. *NCBI*, *2*, 1718–1750. https://doi.org/10.3390/toxins2071718

Viljoen, A., Subramoney, S., Vuuren, V., Baser, K., and Demirci, B. (2005). The composition , geographical variation and antimicrobial activity of Lippia javanica (Verbenaceae) leaf essential oils. *Journal of Ethnopharmacology*, *96*, 271–277. https://doi.org/10.1016/j.jep.2004.09.017

WHO. (2011). Improving Public Health Through Mycotoxin Control. In G. C. Miller J., Ronald T. , Felicia W., John I., Christopher P., Robert A., Baan W. (Ed.), *international Agency for Research on Cancer*. Switzerland: World Health Organization press.

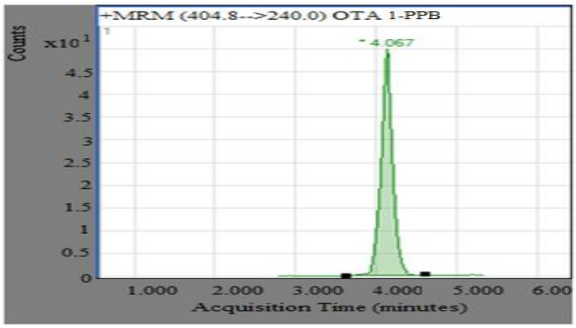
WHO. (2018). Food safety digest: department of Food Safety and Zoonoses, (February), 1–5. Retrieved from https://www.who.int/foodsafety/FSDigest_Fumonisins_EN.pdf

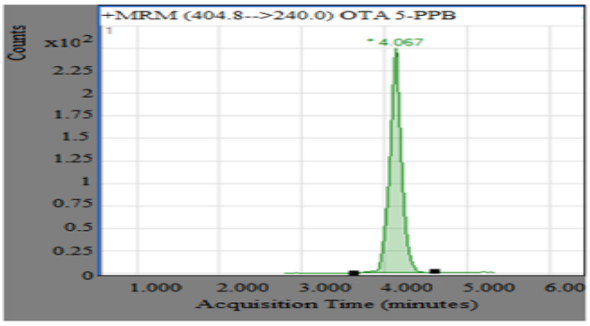
World Bank. (2011). Missing food : In *The Case of Postharvest Grain Losses in Sub-Saharan Africa* (pp. 27–40). Washington, DC: The World Bank.

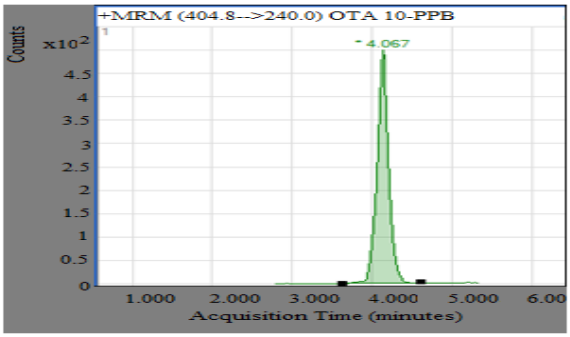
Yasushi, H., Yu, K., Masahiro, N., Hajime, M., and Chisato, Y. (2015). Influence of repeated ochratoxin A ingestion on milk production and its carry-over into the milk, blood and tissues of lactating cows. *Animal Science Journal*, *84*(4), 541–546. https://doi.org/10.1111/asj.12466

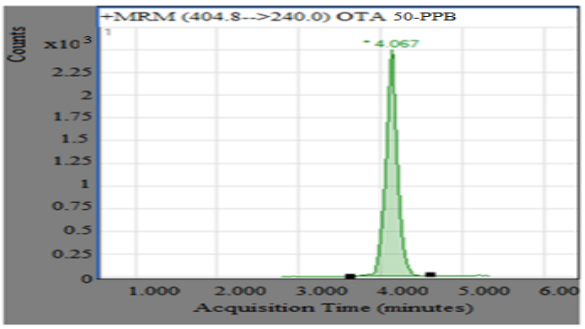
Yu, Z., Gonciarz, M. D., Sundquist, W. I., Hill, C. P., and Jensen, J. (2009). NIH Public Access, *377*(2), 364–377. https://doi.org/10.1002/mnfr.201300116.Dietary

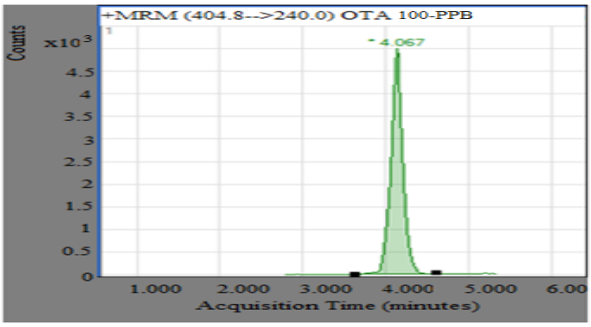
Appendix 1: The LC-MS chromatograms of OTA analysis

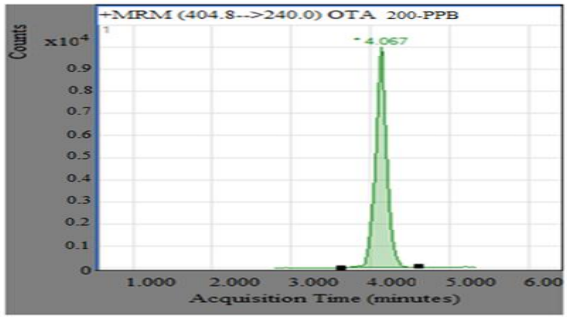


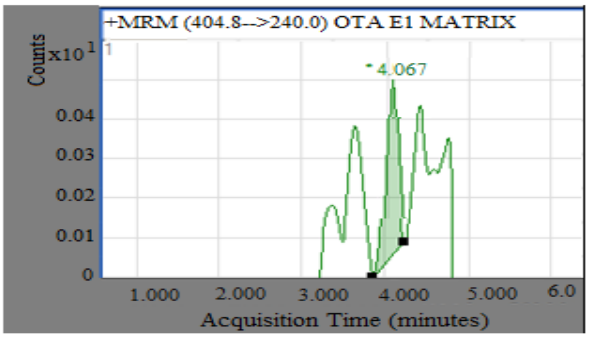


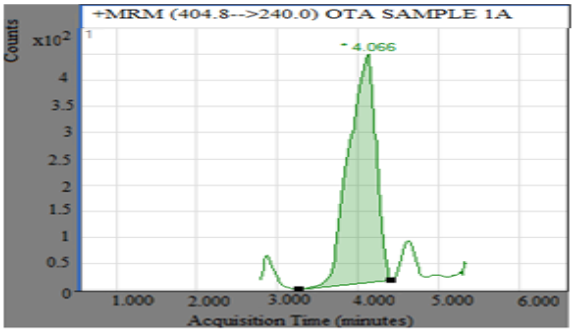


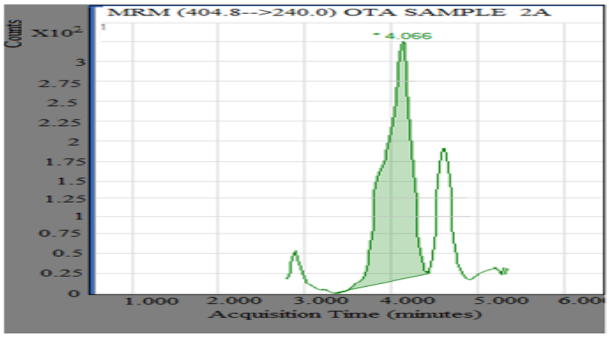


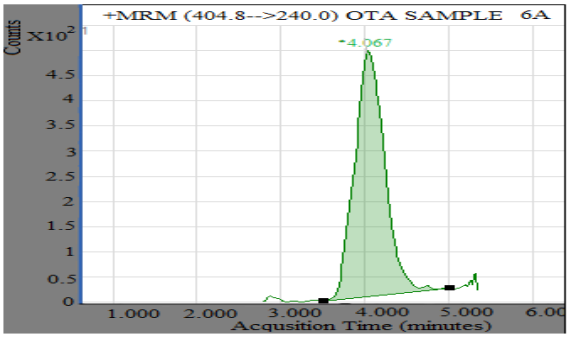


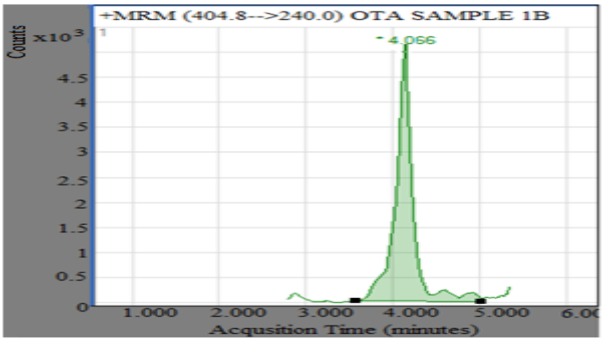


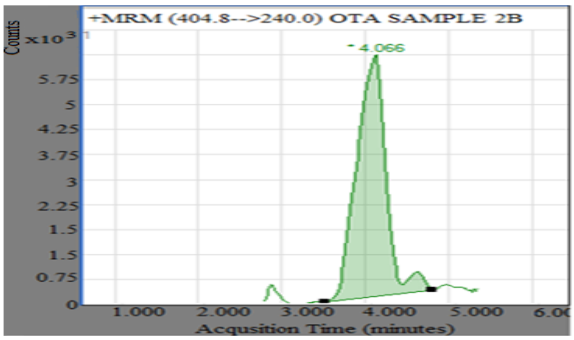


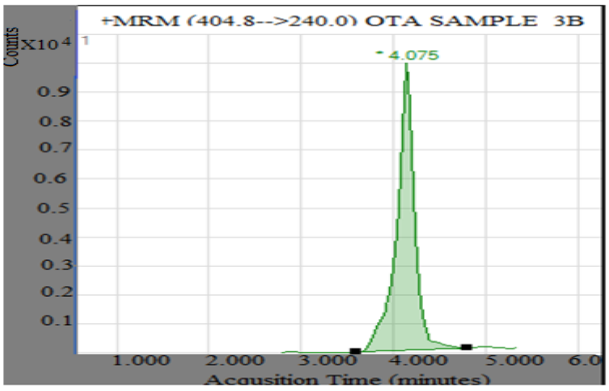


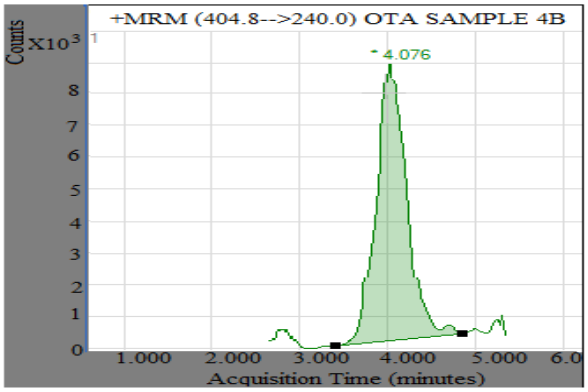

MRM (404.8-->240.0) OTA SAMPLE 2A
Counts
X10²
4.066
Acquisition Time (minutes)

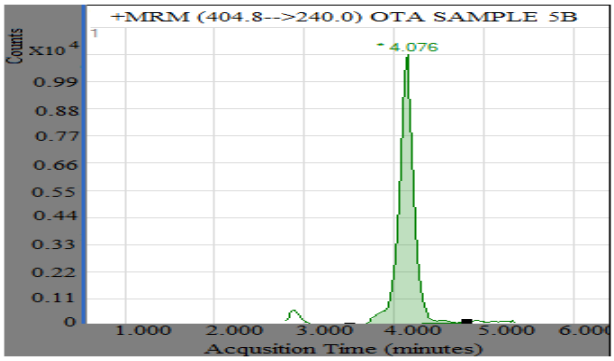

+MRM (404.8-->240.0) OTA SAMPLE 6A
Counts
X10²
4.067
Acquisition Time (minutes)

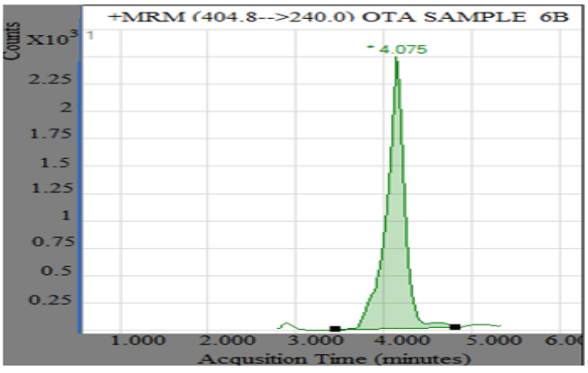

+MRM (404.8-->240.0) OTA SAMPLE 1B
Counts
X10³
4.066
Acquisition Time (minutes)

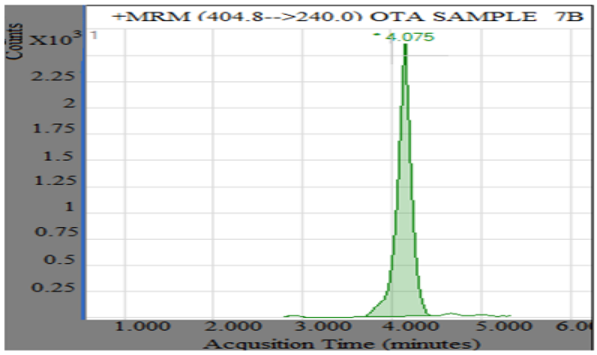

+MRM (404.8-->240.0) OTA SAMPLE 2B
Counts
X10³
4.066
Acquisition Time (minutes)

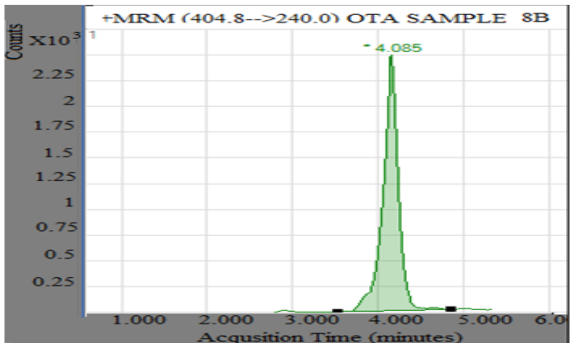

+MRM (404.8-->240.0) OTA SAMPLE 3B
Counts
X10⁴
4.075
Acquisition Time (minutes)

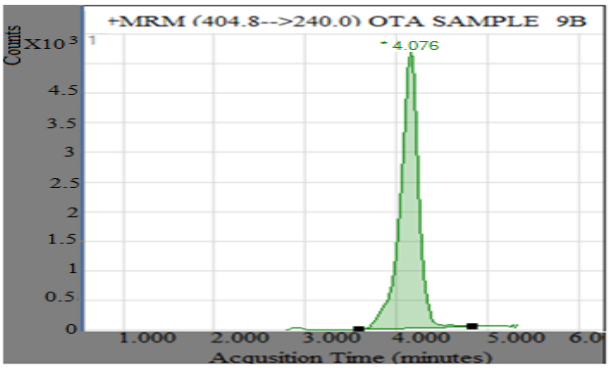

+MRM (404.8-->240.0) OTA SAMPLE 4B
Counts
X10³
4.076
Acquisition Time (minutes)

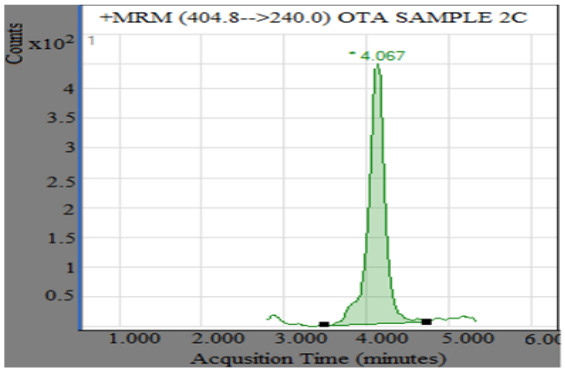

+MRM (404.8-->240.0) OTA SAMPLE 5B
Counts
X10⁴
4.076
Acquisition Time (minutes)

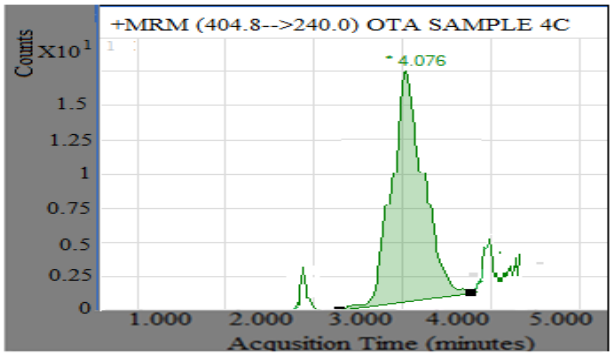

+MRM (404.8-->240.0) OTA SAMPLE 6B
Counts
X10³
4.075
Acquisition Time (minutes)


+MRM (404.8-->240.0) OTA SAMPLE 7B
Counts
X10 3
4.075
Acquisition Time (minutes)


+MRM (404.8-->240.0) OTA SAMPLE 8B
Counts
X10 3
4.085
Acquisition Time (minutes)

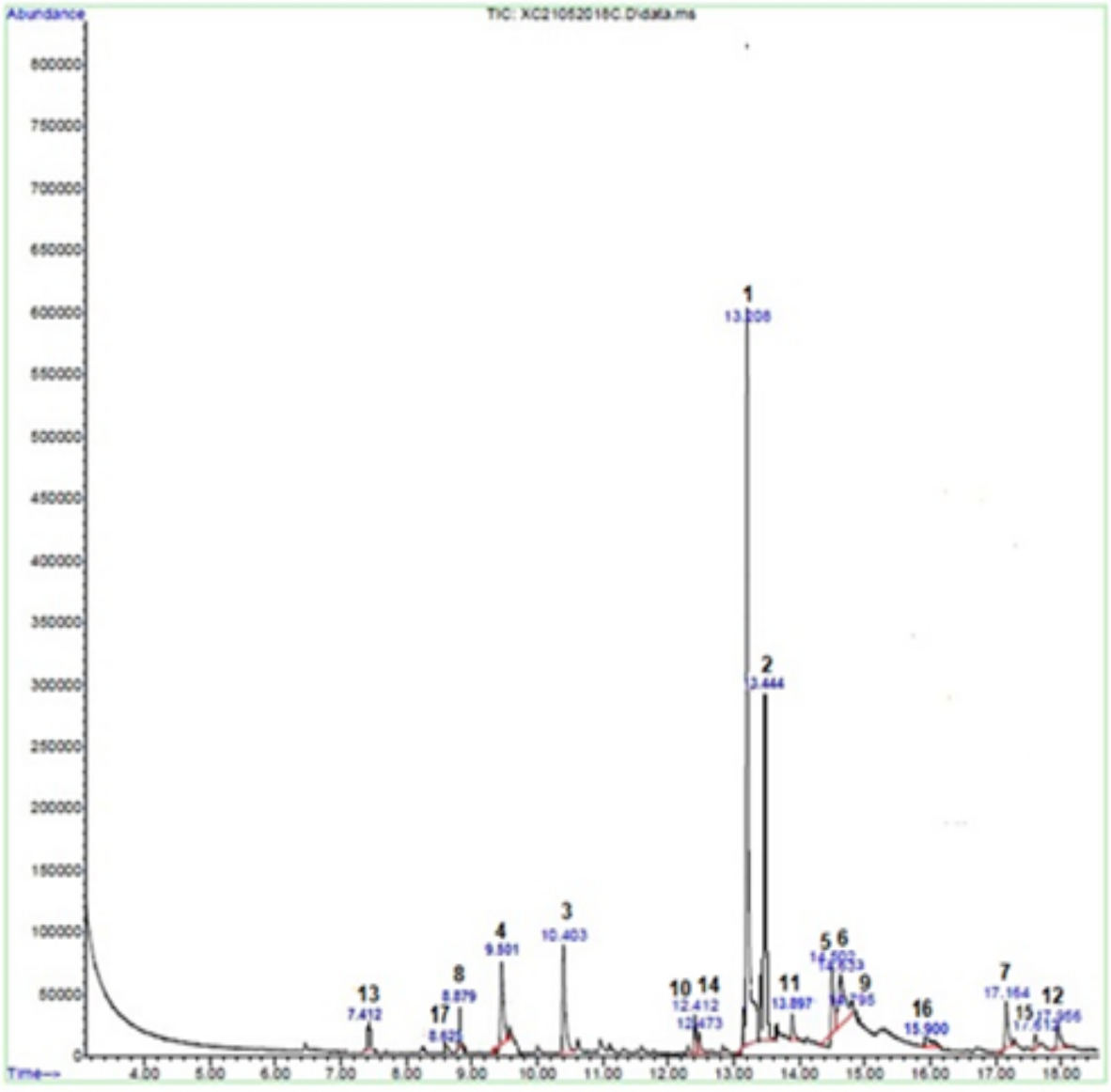

+MRM (404.8-->240.0) OTA SAMPLE 9B
Counts
X10 3
4.076
Acquisition Time (minutes)


+MRM (404.8-->240.0) OTA SAMPLE 2C
Counts
X10 2
4.067
Acquisition Time (minutes)


+MRM (404.8-->240.0) OTA SAMPLE 4C
Counts
X10 1
4.076
Acquisition Time (minutes)

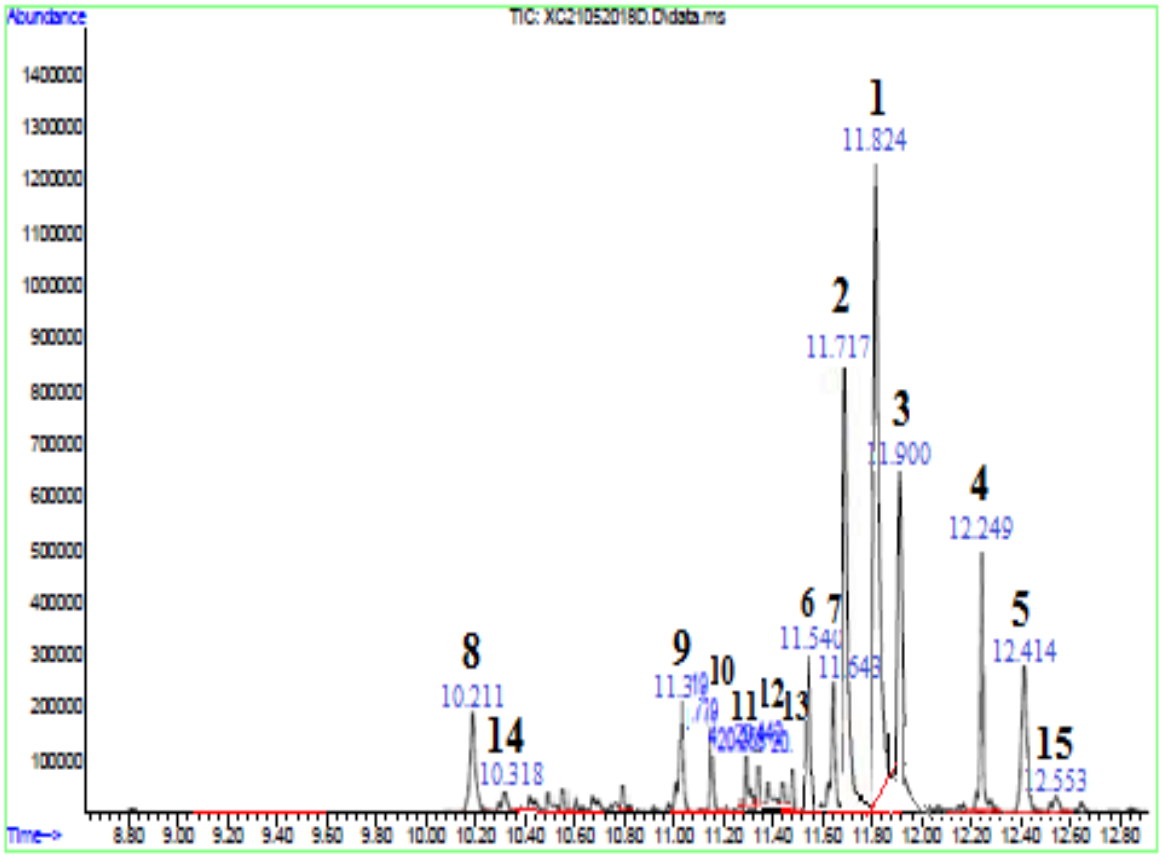
Appendix 2: The GC-MS chromatogram of *L. javanica* essential oils

File :C:\msdchem\1\DATA\21052018\XC21052018C.D
Operator : ICIPEBCED\SSR-PC\admin
Acquired : 21 May 2018 18:57 using AcqMethod DCM-HP5-MS Prog-35-280 temp-50min.M
Instrument : Icipe MSD2
Sample Name: Lippia javanica
Misc Info : Lippia javanica
Vial Number: 2



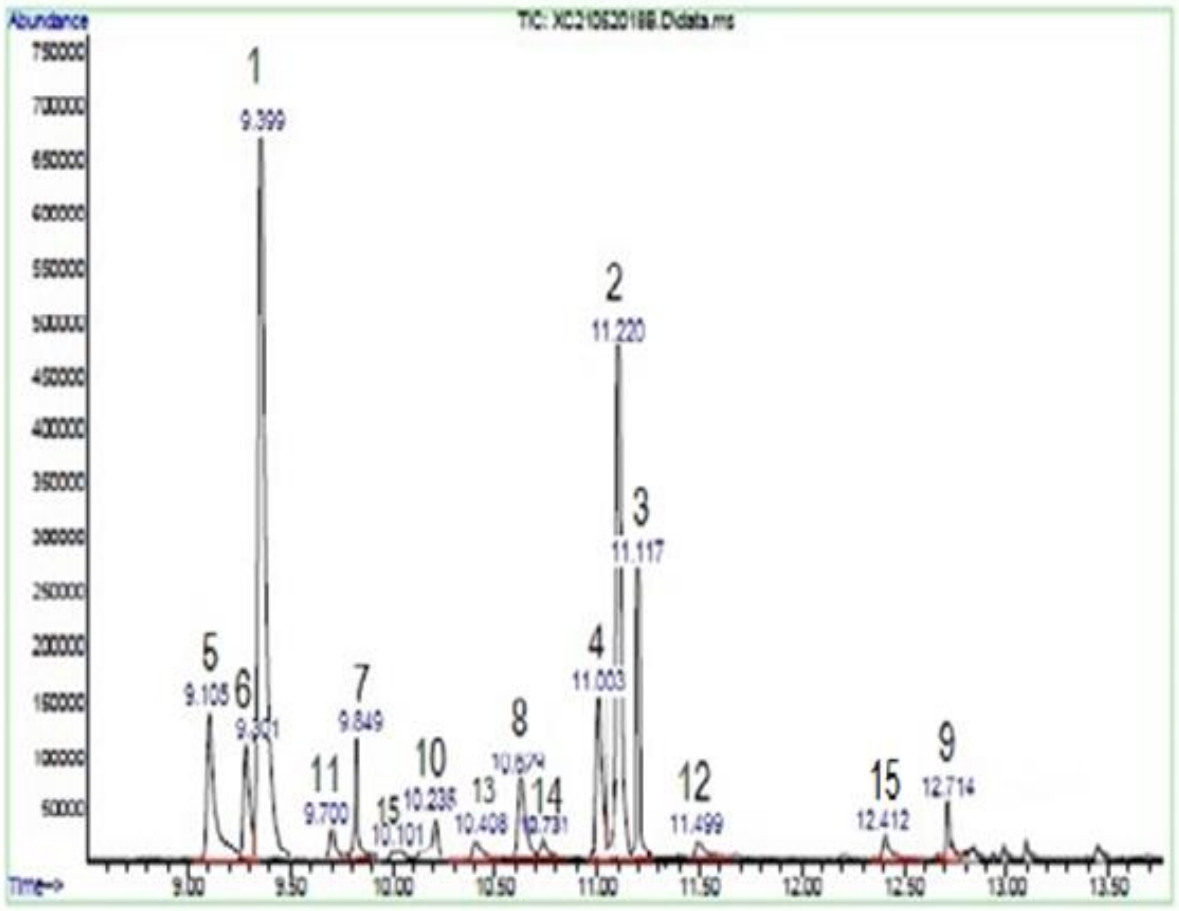
Appendix 3: The GC-MS chromatogram of *O. gratissimum* essential oils

File :C:\msdchem\1\DATA\21052018\XC21052018D.D
Operator : ICIPEBCEDSSR-PC\admin
Acquired : 21 May 2018 19:57 using AcqMethod DCM-HP5-MS Prog-35-280 temp-50min.M
Instrument : Icipe MSD2
Sample Name: Oscimum gratiscimum
Misc Info : Oscimum gratiscimum
Vial Number: 3



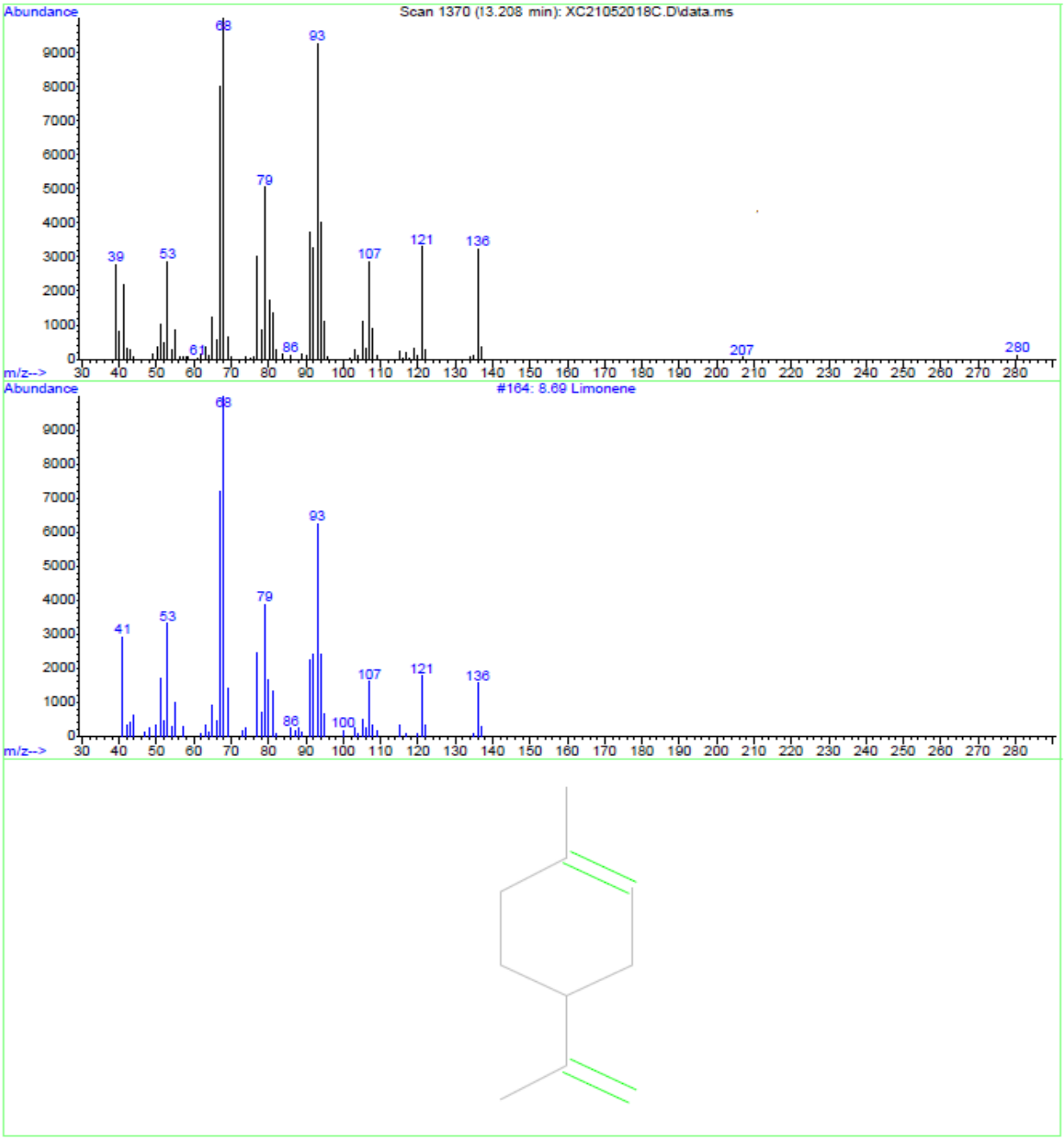
Appendix 4: The GC-MS chromatogram of *T. asiatica* essential oils

File :C:\msdchem\1\DATA\21052018\XC21052018B.D
Operator : ICIPEBCEDSSR-PC\admin
Acquired : 21 May 2018 17:57 using AcqMethod DCM-HP5-MS Prog-35-280 temp-50min.M
Instrument : Icipe MSD2
Sample Name: Toddalia asiatica
Misc Info : Toddalia asiatica
Vial Number: 1



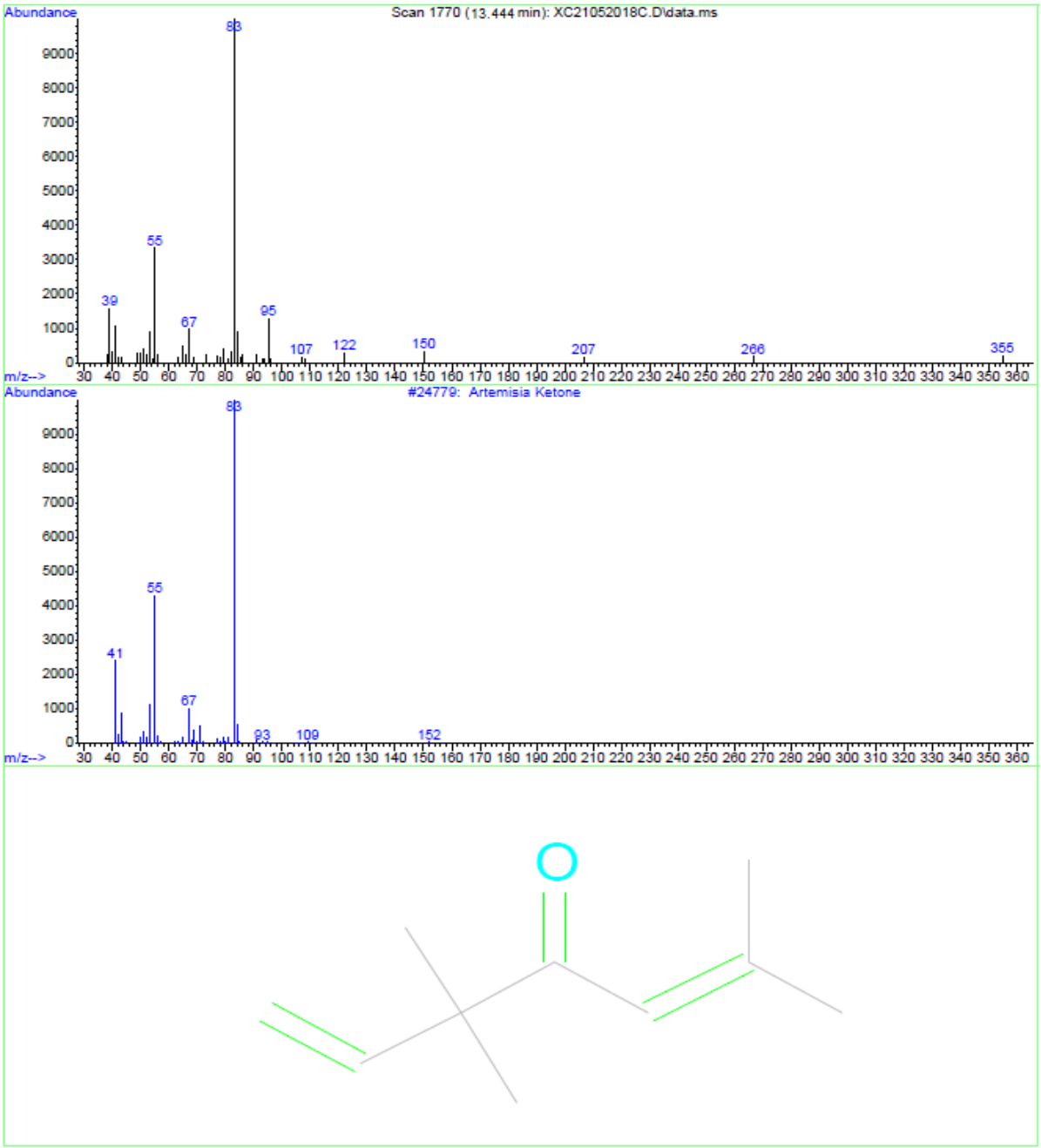
Appendix 5: Mass spectrum of limonene

Library Searched : C:\Database\Adams2.L
Quality : 98
ID : 8.69 Limonene



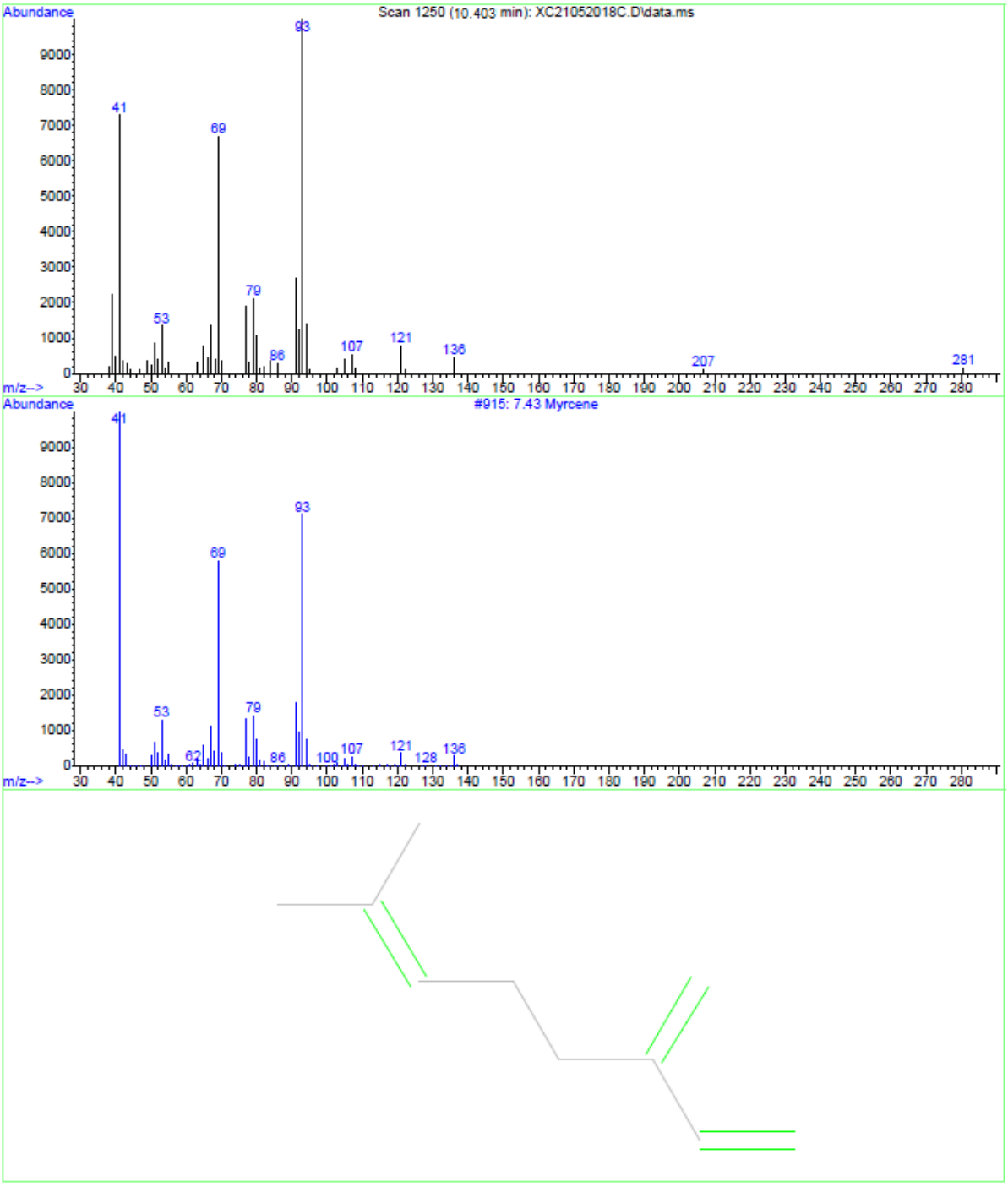
Appendix 6: Mass spectrum of artemisia ketone

Library Searched : C:\Database\NIST08.L
Quality : 94
ID : 9.12 Artemisia Ketone



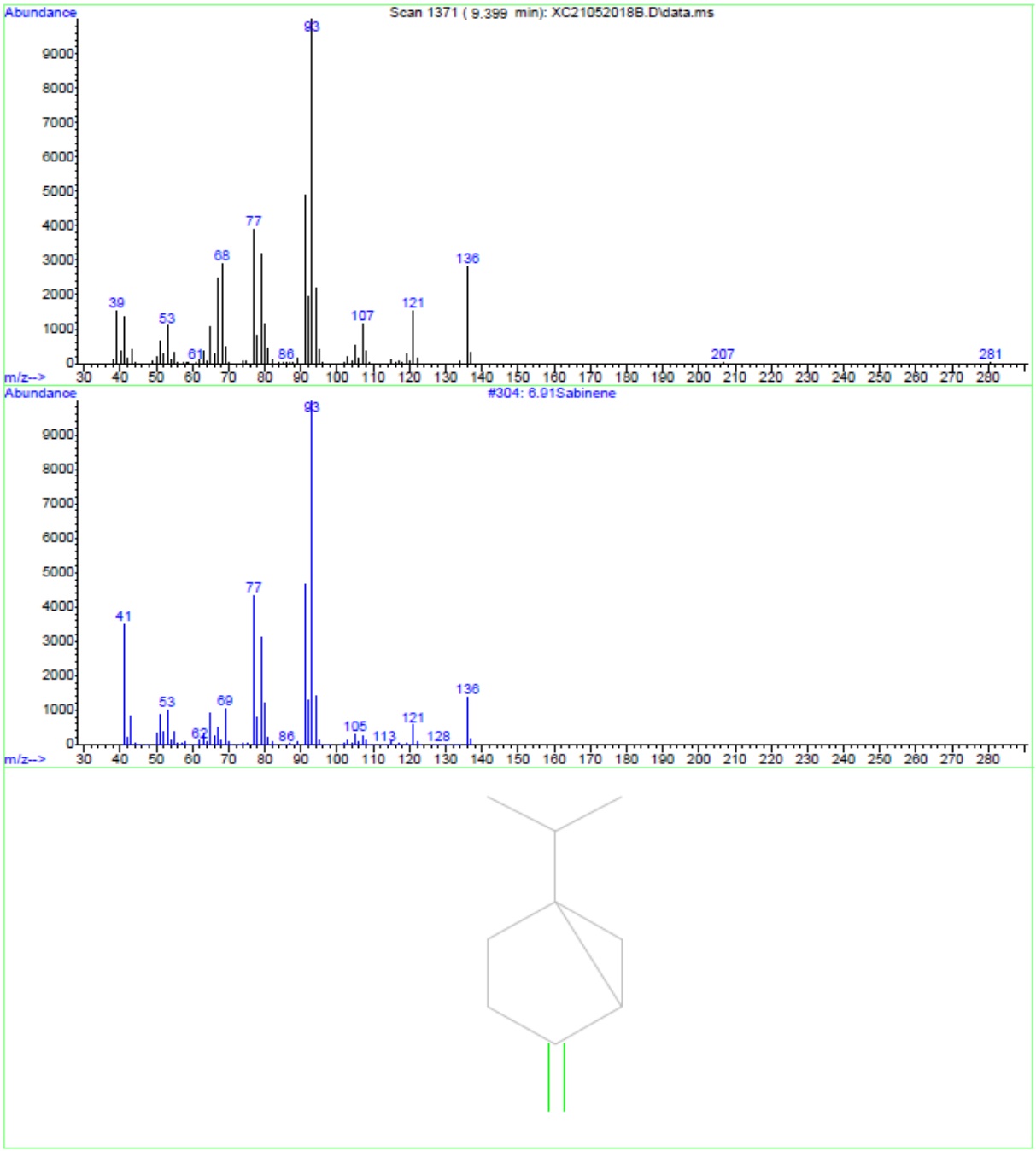
Appendix 7: Mass spectrum of myrcene

Library Searched : C:\Database\Adams2.L
Quality : 91
ID : 7.43 Myrcene



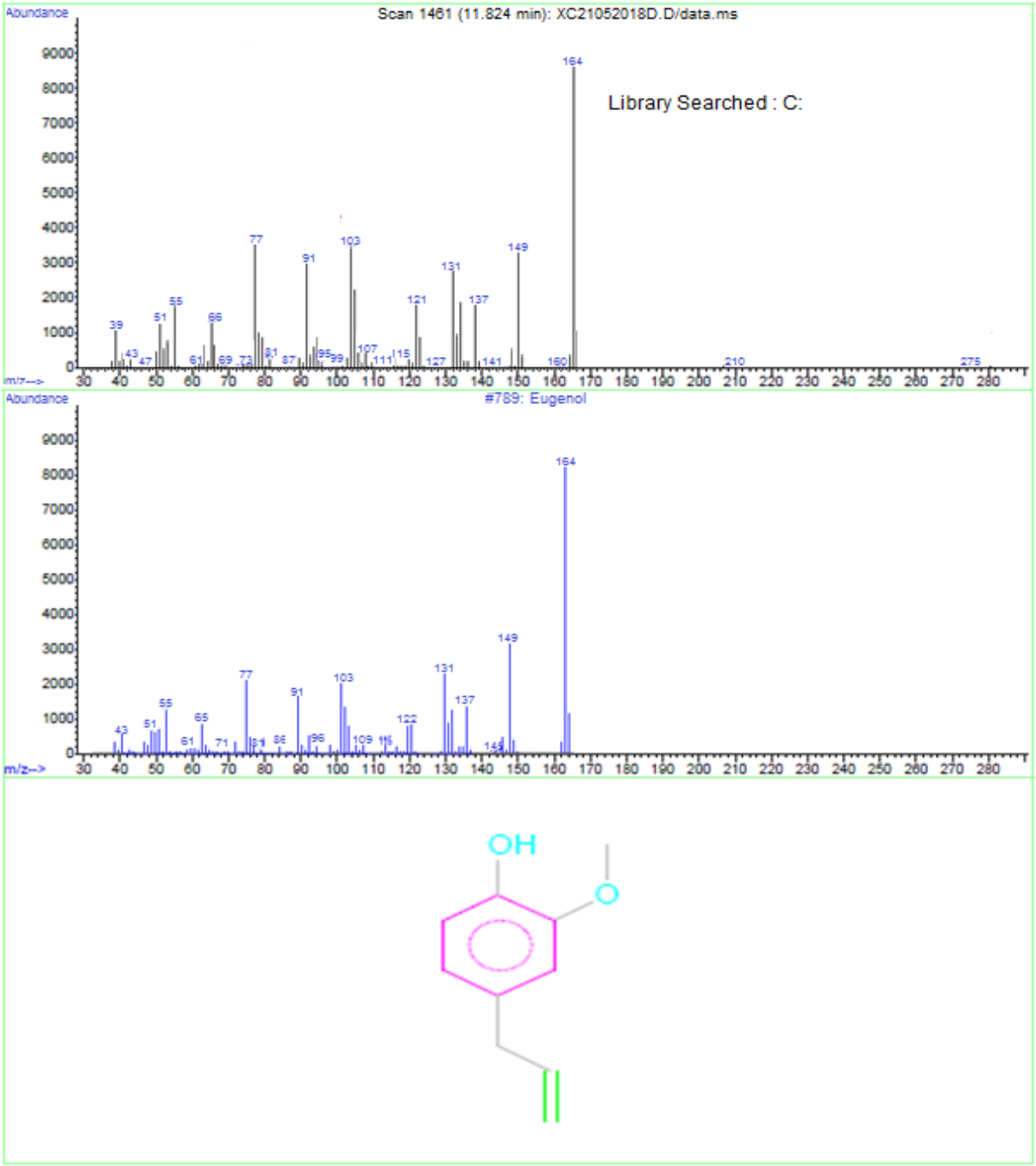
Appendix 8: Mass spectrum of sabinene

Library Searched : C:\Database\Adams2.L
Quality : 96
ID : 6.91Sabinene



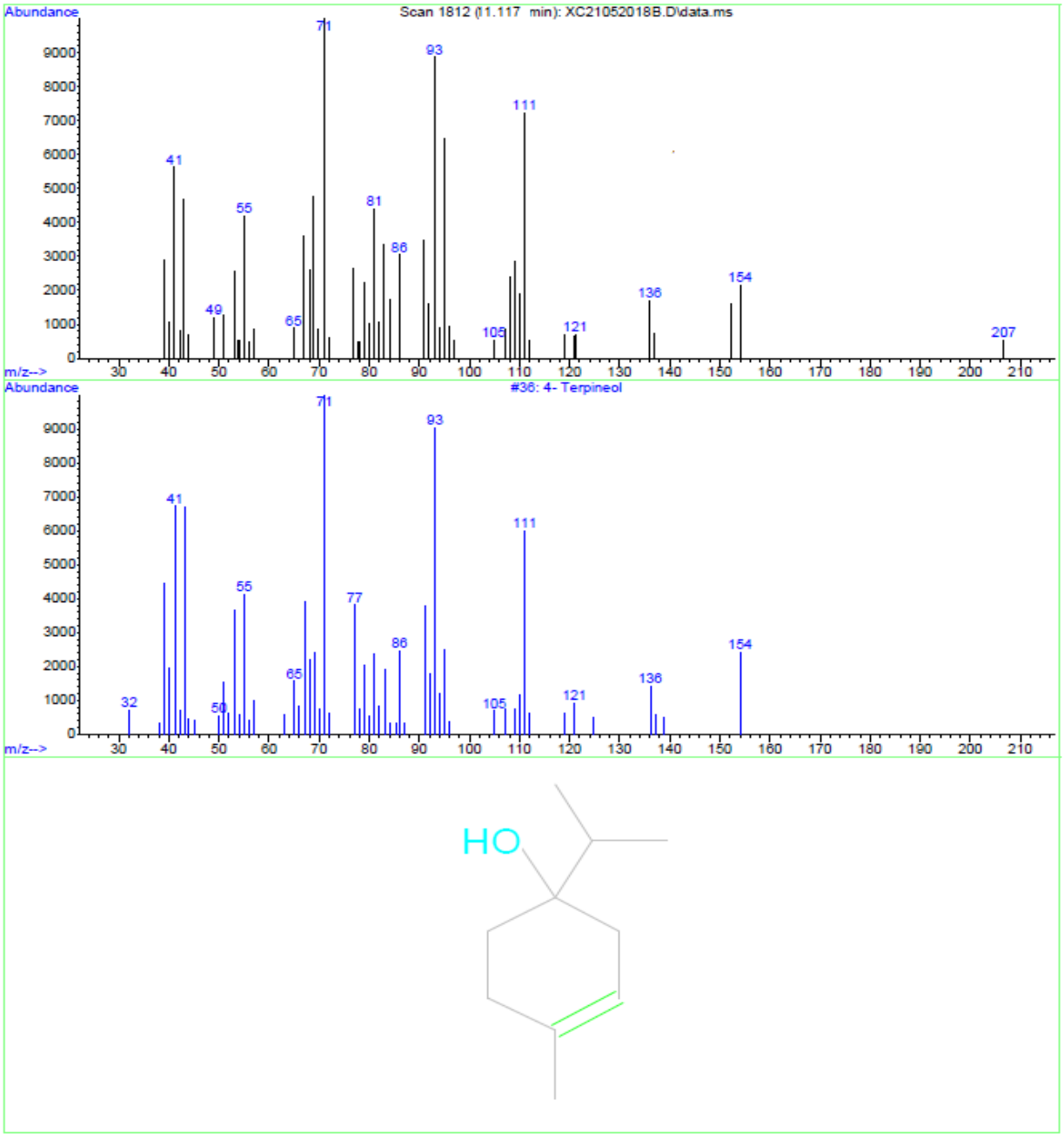
Appendix 9: Mass spectrum of eugenol

Library Searched : C:\Database\Adams2.L
Quality : 97
ID : Eugenol



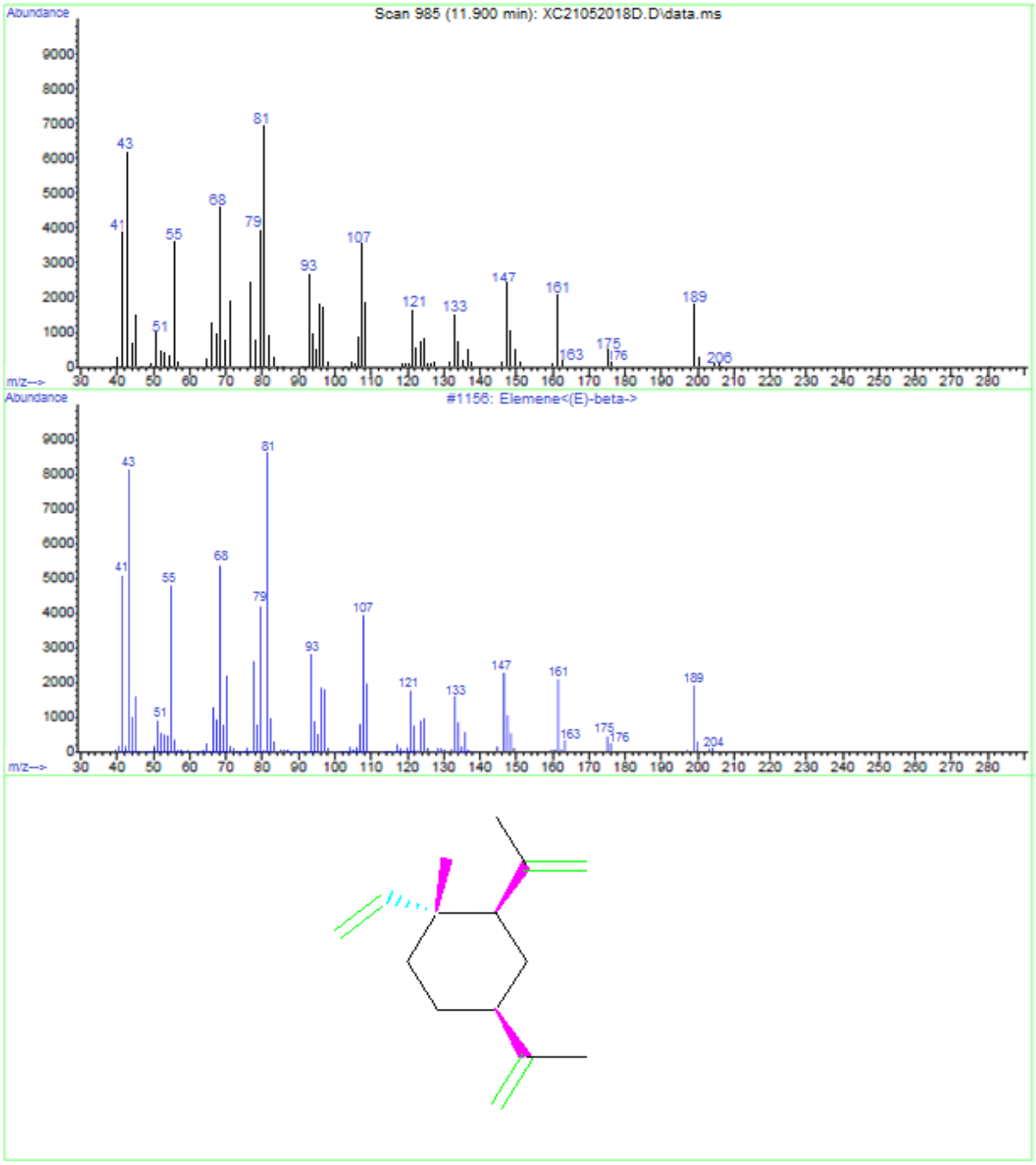
Appendix 10: Mass spectrum of terpinen-4-ol

Library Searched : C:\Database\chemecol.L
Quality : 96
ID : 4- Terpineol



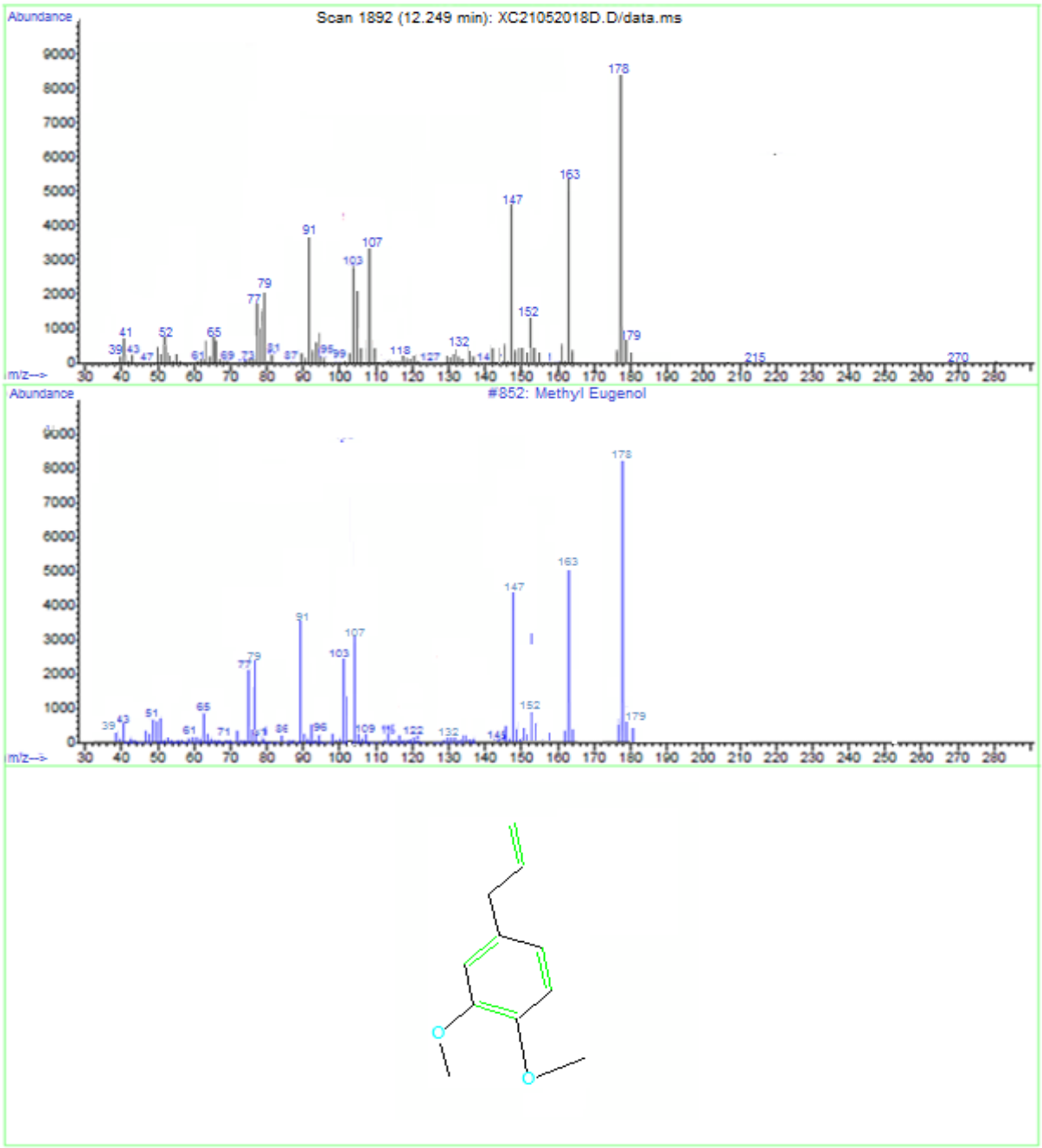
Appendix 11: Mass spectrum of beta-elemene

Library Searched : C:\Database\Adams2.L
Quality : 96
ID : Elemene<(E)-beta-->



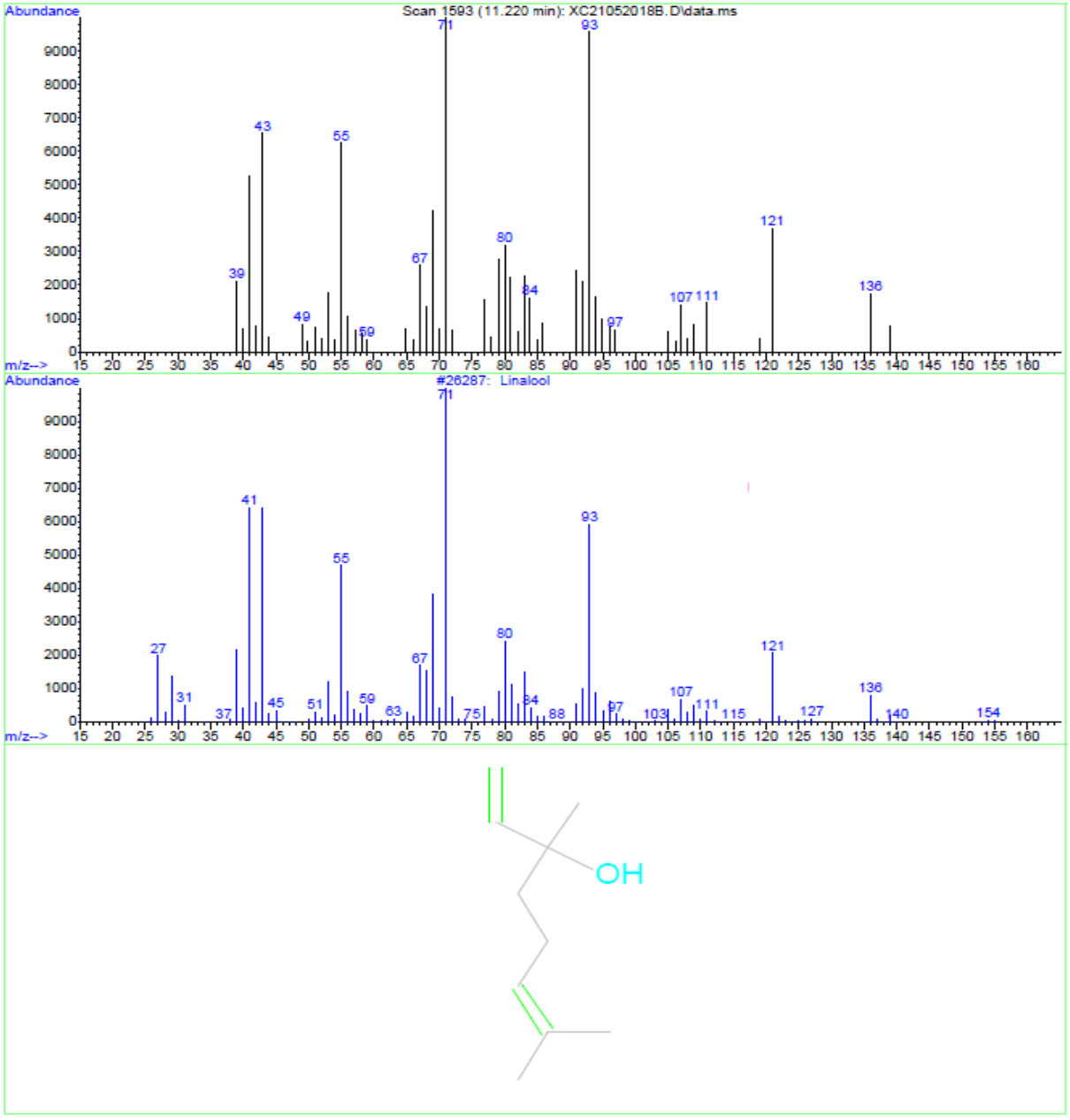
Appendix 12: Mass spectrum of methyl eugenol

Library Searched : C:\Database\Adams2.L
Quality : 97
ID : Methyl Eugenol



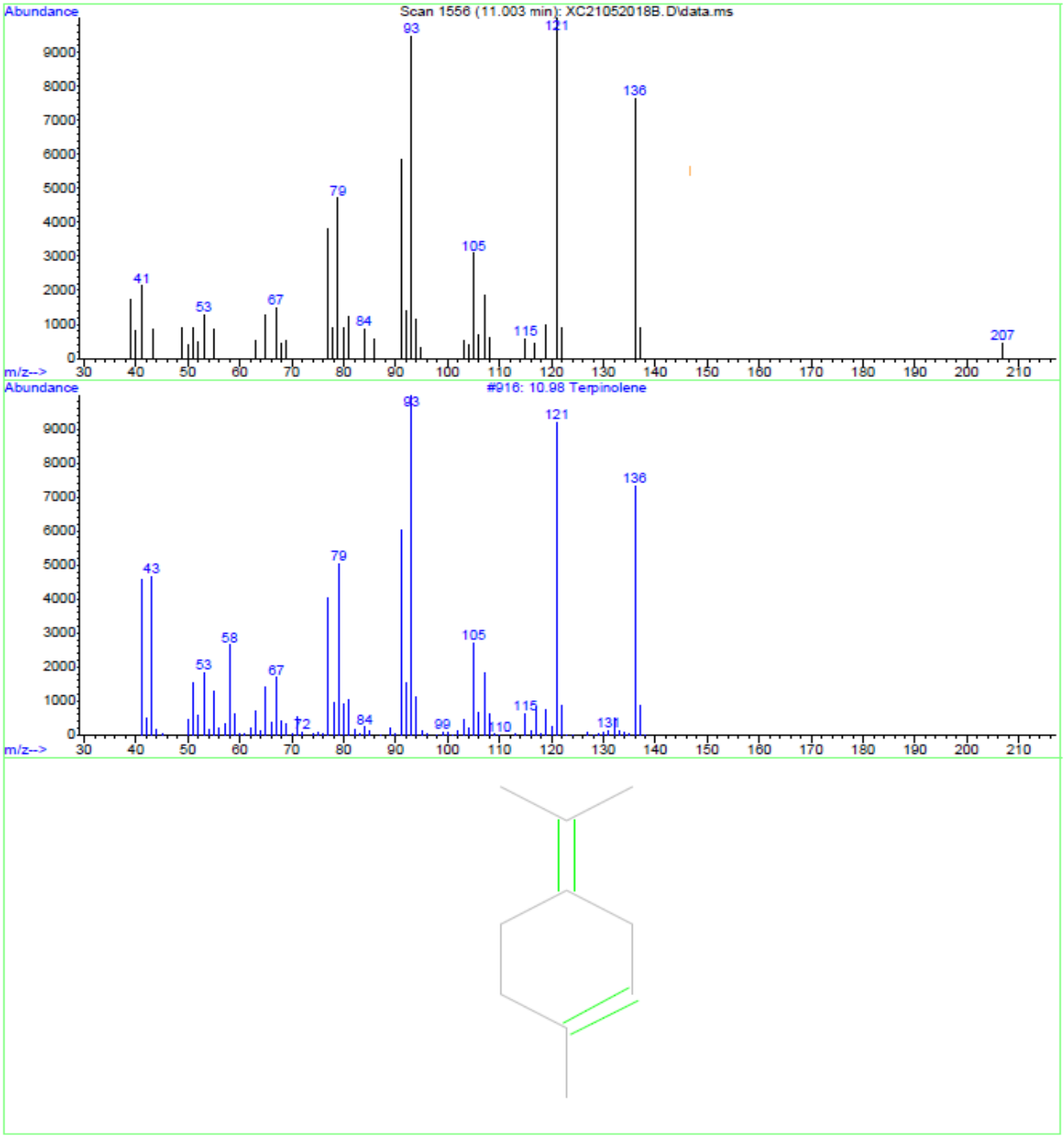
Appendix 13: Mass spectrum of linalool

Library Searched : C:\Database\NIST08.L
Quality : 92
ID : Linalool

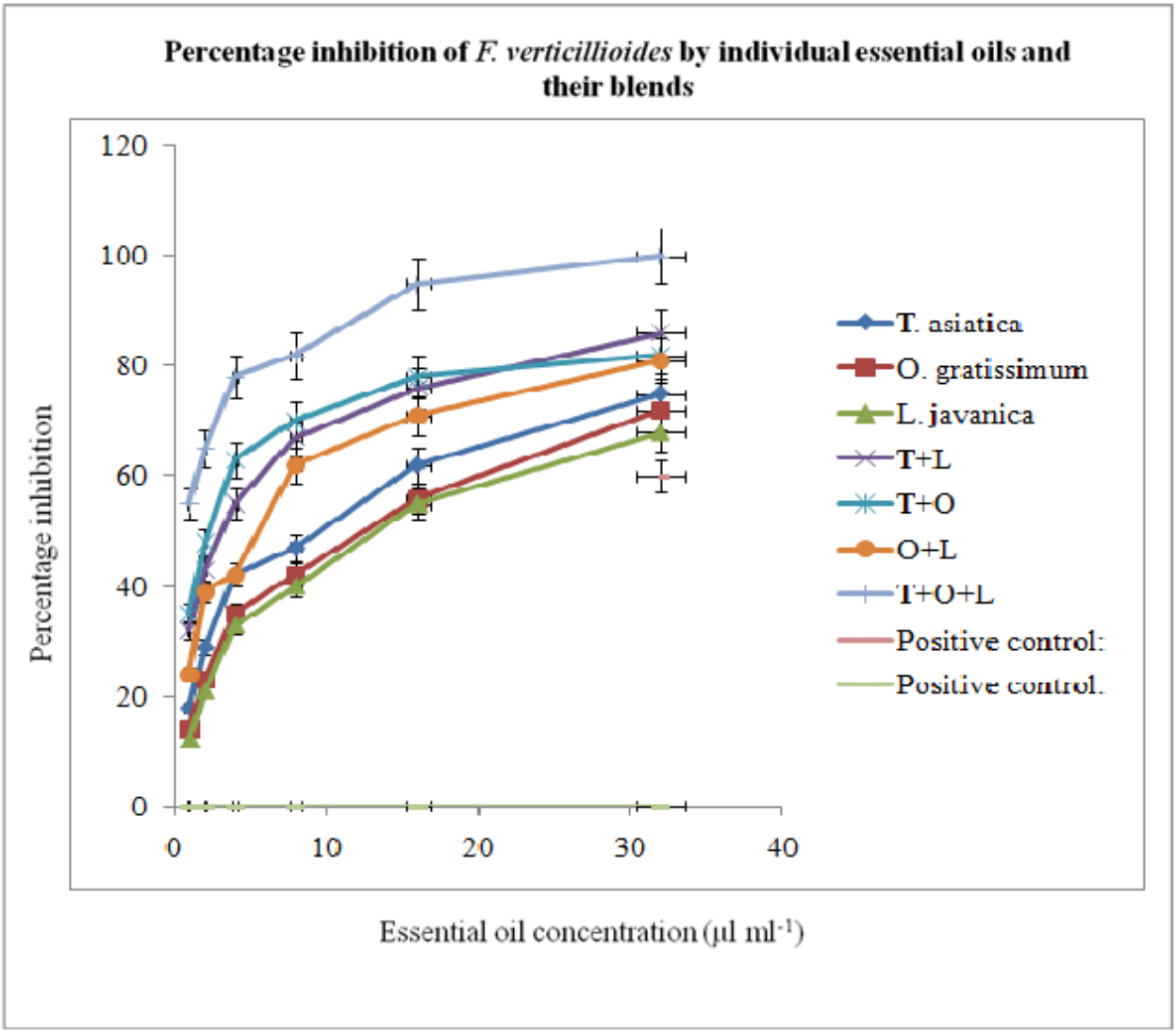


Appendix 14: Mass spectrum of terpinolene

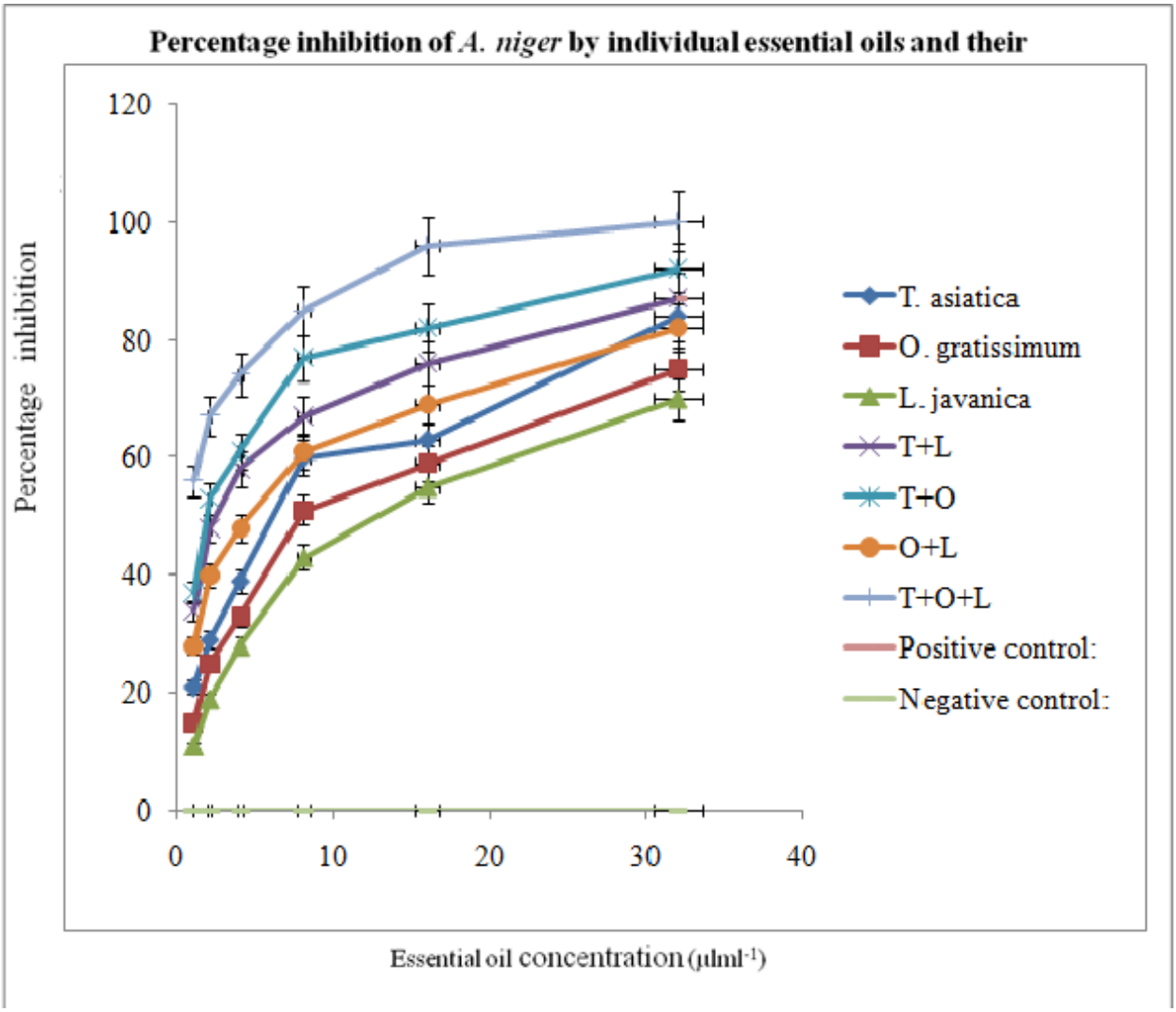
Library Searched : C:\Database\Adams2.L
Quality : 95
ID : 10.98 Terpinolene



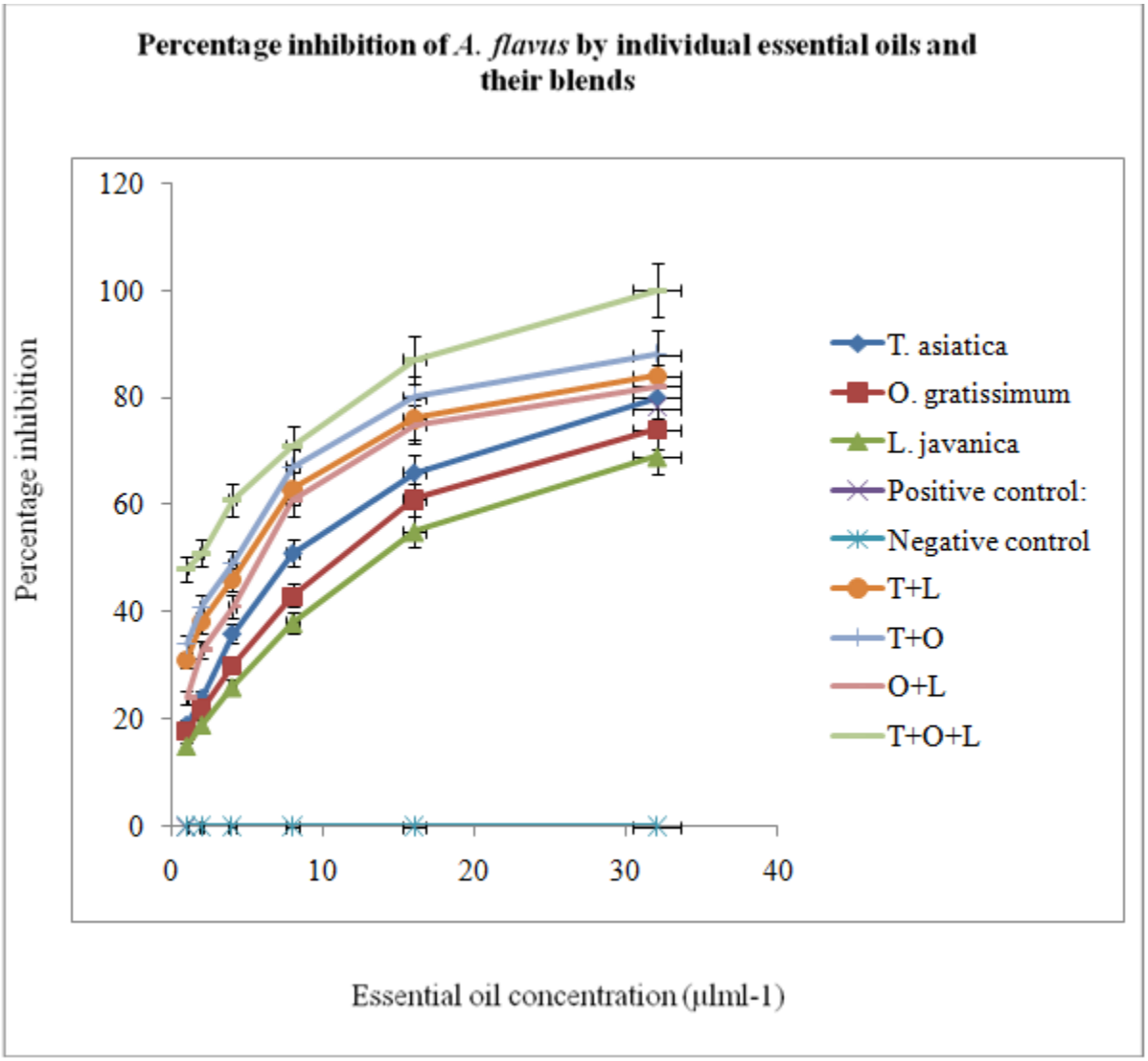
Appendix 15: Percentage inhibition of *F. verticillioides* in media treated with individual essential oils and their blends at different concentrations



Appendix 16: Percentage inhibition of *A. niger* in media treated with individual essential oils and their blends at different concentrations



Appendix 17: Percentage inhibition of *A. flavus* in media treated with individual essential oils and their blends at different concentrations



Appendix 18: Main ingredients in different types of chicken feed

Feed type	Ingredients	Weight/ 700Kg	% composition
Mash	Whole maize	335	47.86
	Wheat bran	229	32.71
	Wheat pollard	80	11.43
	Fish meal	33	4.71
	Additives (minerals and vitamins)	23	3.29
Crumb	Whole maize	340	48.57
	soya	130	18.57
	Maize bran	100	14.29
	Fish meal	88	12.57
	Additives (minerals and vitamins)	42	6
Pellet	Whole maize	400	57.15
	Soya bean meal	140	20
	Fish meal	120	17.14
	Additives (minerals and vitamins)	40	5.71