# EFFECT OF ESSENTIAL OILS ON *ASPERGILLUS* SPORE GERMINATION, GROWTH AND MYCOTOXIN PRODUCTION: A POTENTIAL SOURCE OF BOTANICAL FOOD PRESERVATIVE

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| **AF** | Aflatoxin |
| **AFB1** | Aflatoxin B1 |
| **ATCC** | American Type Culture collection |
| **CDC** | Center of Disease Control |
| **EC** | European commissions |
| **EHNRI** | Ethiopian Health and Nutrition Research Institute |
| **EOs** | Essential oils |
| **FDA** | Food and Drug Administration |
| **GC** | Gas Chromatography |
| **ICU** | Intensive Care Unit |
| **IFI** | Invasive Fungal Infection |
| **IPA** | Invasive Pulmonary Aspergillosis |
| **$LC_{50}$** | Lethal Concentration 50 |
| **MeOH** | Methanol |
| **MIC** | Minimum Inhibitory Concentration |
| **OTA** | Ochratoxin A |
| **PDA** | Potato Dextrose Agar |
| **SD** | Standard Deviation |
| **SE** | Standard Error |
| **SERO** | Scientific and Ethical Review Office |
| **SMKY** | Sucrose, MgSO4 7H2O, KNO3, Yeast extract |
| **TMMRD** | Traditional and Modern Medicine Research Directorate |
| **TLC** | Thin Layer Chromatography |
| **VS** | Vanillin-sulphuric acid reagent |
| **WHO** | World Health Organization |

**ABSTRACT**

**Background:** Contamination of food commodities with spoilage fungi presents a problem of global concern, since the growth and metabolism of these organisms can cause serious food-borne intoxications and a rapid spoilage of food products. *Aspergillus* species, a kind of opportunistic fungi linked to food spoilage are the leading cause of infection, mycotoxicosis and economic loss. Mycotoxins they produce are responsible for cancers and many diseases affecting the gastrointestinal, urogenital, vascular, kidney, and nervous systems. Many efforts have been exerted in attempting to control *Aspergillus* infection by using synthetic chemicals, however, its application has led to a number of environmental and health problems because they are themselves carcinogenic, teratogenic, and highly toxic with long degradation periods. As a result there should be continuous search for safe preservatives from natural products. For this purpose essential oils could be a possible source of new novel antimicrobial as they are rich in chemical constituents that have antifungal activity and seldom accumulate in the environment.

**Objective:** The aim of this study was to assess effect of essential oils on *Aspergillus* spore germination, growth and mycotoxin production of isolates from food commodities.

**Method:** *In vitro* antifungal assay and antiaflatoxigenic assay of essential oils that are obtained by hydrodistillation was carried out using poisoned food techniques, spore germination assay, agar dilution assay, aflatoxin arresting assay and mycelial weight inhibition assay on *Aspergillus flavus*, *Aspergillus fumigatus* and *Aspergillus niger*. *In vivo* animal model was also conducted to see the safety limit of essential oil.

**Result:** *A. flavus*, *A. fumigatus* and *A. niger* were isolated from a total of 36 food commodities. *Cymbopogon martinii, Foeniculum vulgare, Lippia adoensis Hochst. var. koseret, Rosmarinus officinalis, Ruta chalepensis* and *Trachyspermum ammi* essential oils were tested against seven isolates of aflatoxigenic *Aspergillus* species. *T. ammi* oil showed highest antifungal activity followed by *Lippia adoensis Hochst. var. koseret*. A hundred percent mycelial inhibition was recorded at 1µl/ml against *A. niger* and *A. flavus*; and

0.5µl/ml against *A. fumigatus* by *T. ammi* essential oils. Moreover, complete inhibition of spore germination of toxicogenic strains of *A. flavus*, *A. fumigatus* and *A. niger* was achieved with this essential oils at a concentration of 0.5µl/ml, 1 µl/ml and 1µl/ml, respectively. *T. ammi* oil also showed significant antiaflatoxigenic potency by totally inhibiting aflatoxin production from *A. niger* and *A. flavus* at 0.5 and 0.75µl/ml, respectively. *C. martini, F. vulgare, L. adoensis Hochst. var. koseret* and *T. ammi* essential oils as antifungal were found superior over chemical synthetic preservative. Moreover, a concentration of 5336.297µl/kg body weight was recorded for $LC_{50}$ on mice indicating the low mammalian toxicity and strengthening traditional use of this plant as safe food preservative.

**Conclusion:** The present study revealed that essential oils from *T. ammi* can be a potential source of safe natural food preservative for food commodities. For large scale utilization further studies are recommended to explore its activity on other toxigenic fungal species, mechanism of action and chronic toxicity of the oil.

**Key words:** *Aspergillus* species, Essential oils, food spoilage, mycotoxin, preservatives

# CHAPTER1. INTRODUCTION

## 1.1. General Introduction

Despite the advancement in medicine, food science and the technology of food production, diseases caused by foodborne fungal pathogens have continued to present a major problem of public health (DeWaal and Robert, 2005). Still, quarter of worlds` food commodities have been wasted due to the contamination by toxic fungi or by fungal metabolic products (Leslie *et al*., 2008). Among fungus, *Aspergillus* have potentials to contaminate food items by producing hydrolytic enzymes. In developing countries improper storage condition offer favorable environment for the growth and proliferation of *Aspergillus* spp (Leslie *et al*., 2008). Various mycotoxins have been reported in *Aspergillus* contaminated food commodities (Wild and Gong, 2010). Consumptions of such contaminated food leads to a serious cases of illness and mycotoxicoses in humans and livestock (Wild and Gong, 2010). Among these, aflatoxicosis have received significant public attention throughout the world because of their acute and chronic toxicological effect (DeWaal and Robert, 2005). In acute doses aflatoxin are characterized by hemorrhage, acute liver damage, edema, and death; while in chronic dose causes induction of cancer, mutagenicity, immune suppression, birth defects, estrogenic, gastrointenial, urogenital, vascular, kidney and nervous system disorder (Leslie *et al*., 2008, Zain, 2011). Within African region aflatoxin are threat of public health (DeWaal and Robert, 2005). In parts of sub-Saharan Africa about 250,000-hepatocarcinoma related deaths occur annually due to aflatoxin ingestion alone (Wagacha and Muthomi, 2008).

Management of food stuffs contaminations are required to ensure that food commodities remain safe and uncontaminated throughout the supply chain (from 'farm to plate') (Leslie *et al*., 2008). So far several synthetic preservatives have been effectively used in management of food commodity contamination by *Aspergillus* spp but their continuous application has led to the development of fungal resistance (Brul *et al*., 2002, Brul and Coote, 1999). Moreover, despite its efficacy, the application of synthetic preservatives has led to a number of environmental and health problems because they are themselves carcinogenic, teratogenic, and highly toxic with long degradation periods (Lingk, 1991,

Rubin, 2001). In addition its indiscriminate use has led to a number of ecological and medical problems due to residual toxicity, hormonal imbalance and spermatotoxicity, etc. (Nollet and Rathore, 2010). Another problem with the use of synthetic preservatives is that up on the consumption of food containing these substances some individuals produce allergic reactions to these substances (Wilson and Bahna, 2005).

However, natural products seldom accumulate in the ecosystem, less likely to develop resistance; they could potentially serve as effective alternatives of synthetic chemicals for the control of food contamination by *Aspergillus* spp. Some plant based preservative such as azadirachtin, carvone, allyl isothiocynate from *Azadirachta indica* and *Carum carvi*, have been in the market as safe antimicrobials and are used on large scale as food additives (Gopal *et al*., 2007). Among natural products, essential oils (EOs) of aromatic plant are gaining interest as food additives and widely accepted by consumers because of their relatively low toxicity, high volatility, transient nature and biodegradability (Burt, 2004). In United State of America, some essential oils have been classified as generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) as flavors or food additives. European Union also allowed the use of essential oils in food and aromatherapy (Ipsilantis *et al*., 2012). So, EOs with antimicrobial activity are possible candidates for the preservativation of food commodities against *Aspergillus* spp (Razzaghi-Abyaneh *et al*., 2009b).

*Cymbopogon martinii, Foeniculum vulgare*, *Lippia adoensis Hochst. var. koseret*, *Rosmarinus officinalis*, *Ruta chalepensis* and *Trachyspermum ammi* are medicinal aromatic plant of Ethiopia. They are used traditionally as food additives and also for the treatment of various diseases (Tadeg, 2004). However, there is no reliable evidence that indicated these plants essential oils have fungitoxic and antiaflatoxigenic potential against aflatoxigenic *Aspergillus* spp in Ethiopia. The aim of this study was to evaluate the effect of essential oils on growth, spore and mycotoxin production of *Aspergillus* spp; that could alternate synthetic chemical preservatives.

## 1.2. Literature Review

### 1.2.1. *Aspergillus* species

Human are frequently exposed to fungi, as they are ubiquitous in nature and cosmopolitan in distribution; estimated to comprise 25% of world's biomass (Pitt and Hocking, 2009). From more than 100,000 species of fungi identified; about 350 species was counted for *Aspergillus* (Pitt and Hocking, 2009). *Aspergillus* is a ubiquitous soil-dwelling organism that is found in humid areas, damp soil, decaying vegetation or mouldy hay, organic compost piles, leaf litter, seed, grain and the like (Bennett, 2010). Most species are known in degrading mainly complex plant polymers, but they can also degrade substrates as diverse as compost, human tissues, and old paper (Polacheck *et al.,* 1989). They produce and release millions of spores small enough to be found in air, water, soil, plant debris, rotten vegetation, manure, sawdust waste, bagasse waste, animal feed, on animals and indoor air environment. As *Aspergillus* spores swell with water and grow, they elongate, forming hyphae which secrete digestive enzyme and mycotoxins. *Aspergillus* spores can survive harsh environmental conditions, such as extreme dry conditions, that do not support normal mold growth (Caston-Osorio *et al*., 2008, Dantigny *et al*., 2005, Pagano *et al*., 2010, Soni and Wagstaff, 2005).

The genus *Aspergillus*, which includes about 350 species, are important in public health as human and animal pathogens, as toxin-producing food contaminants. Among the genus of *Aspergillus*, only a few well known species were recognized as important pathogens of humans or domestic animals. Of these, over 95% of all infections are caused by *Aspergillus fumigatus, Aspergillus flavus,* and *Aspergillus niger* (Bennett, 2010). Some other species of clinical importance include *A. nidulans*, *A. terreus*, *A. oryzae*, *A. ustus* and *A. versicolor* (Murphy et al., 2006, Pattron, 2006).

*Aspergillus* species compete with *Penicillium* and *Fusarium* species for dominance among the world's fungal flora (Bennett, 2010). *Aspergillus* lacks the total numbers and variety of *Penicillium* species but compensate by the capability to grow at higher temperatures or lower water activities or both (Pitt and Hocking, 2009). *Aspergillus* usually grows more

quickly than *Penicillium*, although they take longer to sporulate, and generally produce spores which are more resistant to light and chemicals. *Aspergillus* species dominate spoilage in the tropics; however, *Penicillium* species do in temperate zones. A small number of *Aspergillus* spp directly compete with *Fusarium* than *Penicillium* in plant colonization (Pitt and Hocking, 2009).

*Aspergillus* spp are common contaminant of indoor and outdoor environment and they can be major threat to human beings worldwide. We are frequently exposed to airborne spores of *Aspergillus*; in immunocompetent persons it rarely leads to disease. However, in infections underlying immunocompromised individuals *Aspergillus* spp were predominantly recovered; indicating a shift of fungal species since 1980s (Pfaller *et al*., 2006). *Candida albicans* used to be the overwhelming pathogen of fungal infections for many years. However, mold species, such as *Aspergillus*, *Mucor*, and *Fusarium*, have been causing a larger proportion of these infections in the late years. The most common causative species in the nonneonatal group was *Aspergillus* (41%), followed by *Mucor species* (23%) and *Fusarium* (13.5%). Specially in neonates, the most frequent causative species was *Aspergillus*, accounting for 64% of cases (Katta *et al*., 2005). When we come to invasive mold infections it is reported in children with a frequency ranging from 11% in acute leukemia to 22% in acute myelogenous leukemia. In their review they also caught magnitude of invasive mold infection 3 to 14% in patients after Allogeneic Hematopoietic Stem Cell Transplant (Castagnola *et al*., 2011).

As populations of immunocompromised and severely ill patients grown over the past several decades, so too have the frequencies and types of life-threatening, opportunistic fungal infections (Cuenca-Estrella *et al*., 2008a, Wanke *et al*., 2000). The majority of invasive fungal diseases in severely immunocompromised patients are attributed to moulds mainly; *Aspergillus* species, *Zygomycetes*, *Fusarium* species, *Scedosporium* species and *Acremonium* species in which invasive aspergillosis were the major infection. Various cofactors such as neutropenia, genetic predisposition, iron overload, patients undergoing treatment for haematological malignancies, and patients undergone allogeneic

haematopoietic stem cell transplantation, patients who have received a solid organ transplant and a variety of patients with other severe immunosuppressive condition (Pagano *et al*., 2011).

Picazo, Candel and Gonz´alez-Romo (2008) reported from the international update symposium on fungal infections took place in Madrid, Spain, 2008 that the significance of fungal infection in adults: the non-neutropenic critically ill patient and the immunecompromised patient (neutropenic or undergoing solid organ transplantation or hematopoietic stem cell transplantation) has increased greatly in recent years (Picazo *et al*., 2008). With the expanding populations of susceptible patients due to various cofactors; the burden of IFIs continues to increase. Whilst *Candida albicans* and *Aspergillus fumigatus* remain the most common causative pathogens, notable increases in the frequencies of infections caused by non-*albicans Candida*, non-*fumigatus Aspergillus* and moulds other than *Aspergillus fumigatus* have been reported (Shao *et al*., 2007).

*Aspergillus* species is a kind of fungi linked to infection, mycotoxicosis and food spoilage in public awareness. Aerosolized *Aspergillus* spores are found nearly everywhere so we are routinely and almost constantly exposed to them. *Aspergillus* causes animal disease in three major ways: through localized or systemic infections; through induction of allergenic responses; and through the production of mycotoxins. Moreover, it caused loss of economic expenditures worldwide for control and management.

### 1.2.2. *Aspergillus* Infections

Aspergillosis is a large spectrum of fungal diseases, which primarily affect the lungs and are caused by members of the genus *Aspergillus*. It is associated with high rates of morbidity and mortality in immunecompromised patients including transplant recipients and patients receiving chemotherapy for hematologic or other malignancies (Muñoz *et al*., 2006). Aspergillosis represent a wide range of clinical manifestations, including allergic aspergillosis, colonization of cavities with or without the formation of a fungus ball (mainly in the lungs, paranasal sinuses, bronchiectasis), acute to chronic necrotizing invasive forms,

ocular infections (keratitis), endocarditis, osteomyelitis, skin infections (Muñoz *et al*., 2006). Even though, many organs are affected by *Aspergillus* infection, lung is the most commonly involved one with the clinical manifestation of allergic bronchopulmonary aspergillosis, chronic necrotizing pulmonary aspergillosis, aspergilloma and invasive aspergillosis (Muñoz *et al*., 2006). The clinical manifestations and severity of aspergillosis depend upon the host immune status. In patients who are severely immunocompromised *Aspergillus* may be hematogenously disseminated beyond the lung, potentially causing endophthalmitis, osteomyelitis and abscesses in myocardium, kidney, liver, spleen and soft tissue (Dantigny *et al*., 2005). Other risks is also related to the degree of exposure to *Aspergillus* spores (Murphy *et al*., 2006).

As studies in Spain over 2 years indicated aspergillosis is a major public health problem by accounting 8.3% of the global burden of invasive fungal infections (Camps, 2008). In other study Cuenca-Estrella and his colleagues tried to update us with the studies of different authors on invasive fungal infection; they showed that aspergillosis is the most common invasive fungal infections in immunocompromised patients (Cuenca-Estrella *et al*., 2008b, Pattron, 2006). In their updates aspergillosis is still common in haematopoietic stem cell recipients, by citing the studies of Pagano and his colleague were they showed from 3000 recipients and found 91 cases of invasive aspergillosis (2.8%), with a 72% mortality rate. In their analysis they reported the risk of aspergillosis in solid organ transplant by pointing on the study of Gavalda and his colleague were they showed the global incidence of invasive aspergillosis (1.4%), which was to some extent elevated in lung transplant recipients (3%) and heart transplant recipients (2.4%). In this update Cuenca-Estrella and his colleague are also stressed the arrival of aspergillosis in Intensive Care Units by addressing the study of Meersseman and his collaborators were incidence ranges from 2.7 to 58 cases per 1000 admissions, with a mortality of 75–95% (Cuenca-Estrella *et al*., 2008b, Pattron, 2006).

The initial and most common site of infection for invasive aspergillosis is the respiratory tract (paranasal sinuses, lungs), followed by severely traumatized skin. From these sites, dissemination to other organs by haematogenous spread occurs frequently. *Aspergillus*

species, have the tendency to invade the blood vessels causing haemorrhagic infarction and thrombosis in these severely immunocompromised patients (Patterson *et al*., 2000). Many studies have shown that invasive aspergillosis is now more widely recognized, reflecting its main association with severe immunocompromised as a result of both neutropenia and compromised cell-mediated immunity. Invasive aspergillosis caused by *Aspergillus* spp remains one of the most challenging and exciting area of medicine, since they encompass rapidly developing diagnostic and therapeutic modalities (Patterson *et al*., 2000). From the study of Patterson and his coworker pulmonary disease was present in 56%, with disseminated infection in 19%. Bone marrow transplantation was major risk factors by accounting for 32% followed by hematologic malignancy (29%), solid organ transplants (9%), AIDS (8%), and pulmonary diseases (9%) (Patterson *et al*., 2000). From this aspergillosis were used as a marker of severe immune deficiency, usually associated with poor survival.

More recent analyses suggest that the epidemiology of invasive pulmonary aspergillosis (IPA) in the ICU may be shifting away from those traditionally considered at risk. Several recent case series have described IPA in nonimmunocompromised critically ill subjects, i.e. patients with chronic obstructive pulmonary disease (Pemana *et al*., 2011). Invasive aspergillosis in critically ill patients is associated with high in-hospital fatality rates (75.7%) (Khasawneh *et al*., 2006). However, as mortality in the control group was high as well (56.8%), it must be recognized that most of the deaths were due to severity of underlying disease and acute illness (Vandewoude *et al*., 2004). In other study mortality rates from *Aspergillus* spp infection were 50% in the colonization group and 80% in the invasive infection group (Garnacho-Montero *et al*., 2005).

### 1.2.3. *Aspergillus* and Food spoilage

From the time when primitive man began to cultivate crops and store food; spoilage fungi (*Aspergillus* and *Penicillium*) were the problems (Hocking, 2006). Fuzzes, powders and slimes of white or black, green, orange, red and brown have silently invaded: - acidifying, fermenting, discoloring and disintegrating, rendering nutritious commodities unpalatable or

unsafe. It has been more clear since first described 300 years ago the genus *Aspergillus* is an important genus in foods, from the point of view of spoilage or biodeterioration than other fungi (Hocking, 2006). Even currently, in the era of quite technological advancement in food industries, *Aspergillus* food spoilage are major problem of food and feedstuffs during storage (Dao and Dantigny, 2011). They are extremely common in stored commodities such as grains and nuts and occur more frequently in tropical and subtropical than in temperate climate (Hocking, 2006). Once their spores are produced in the environment, air can be a vector in the distribution of *Aspergillus* in food spoilage but packaging materials may also be source of contamination (Dao and Dantigny, 2011). It is troublesome that after germination of the spores, they spread rapidly by aerial mycelia along the fruits, cereals, and food (Dao and Dantigny, 2011).

Food contamination of food commodities occurs at almost all stages; starting from field level through storage of raw products, and subsequent transport and trade causing considerable economic losses annually for food manufacturers and consumers alike. Despite efforts to control food contamination by storage fungi, various foods are still remain susceptible to *Aspergillus* spp. *Aspergillus* are able to grow on almost all kinds of food: cereals, meat, milk, fruit, vegetables, nuts, fats and products of these (Pitt and Hocking, 2009). The growth may result in several kinds of food-spoilage: off-flavors, toxins, discoloration, rotting and formation of pathogenic or allergenic propagules (Filtenborg *et al*., 1996). Food spoilage cause great economic losses worldwide as Pitt and Hocking estimated that between 5 and 10% of the world's food production is wasted due to deterioration by storage fungi (Pitt and Hocking, 2009) and Murphy and his colleague have cited the estimate of Huisin't Veld that a 25% of the world's food supply is lost through microbial activity alone (Murphy *et al*., 2006).

*Aspergillus* infections of food commodities has been identified as a major contributing factors for the huge agro-economic losses in the world as Karabulut and his collaborator analyzed it from studies of different authors in different place (Karabulut *et al*., 2004). Estimate from one bakery in the US was 5% losses. Even assuming only 1% losses,

*Aspergillus* could be spoiling over 23,000 tons of bread worth nearly £20 million in the UK every year. Losses in damp tropical climate and countries with less developed technology remain staggering. In the fruit industry, postharvest losses are 50% or higher, an average of 52.8% (range 15-100%) of the fruit decayed during the ripening (Karabulut *et al*., 2004).

In spite of modern improvements in food production techniques, food safety is an increasingly important public health issue in the world without discriminating between developed and developing countries (Shephard, 2008). It has been estimated that as many as 30% of people in the world suffer from a food borne disease each year (WHO, 2002a). Despite global effort to develop and implement safe food handling for the entire food chain by all level of government and food industries; modern society are still suffering from food safety hazards and wastage of huge quantities of food every year because they are invaded by toxic fungi or contaminated by mycotoxins. An estimate of CDC food borne diseases are responsible for more than 76 million illnesses, 325 000 hospitalizations and 5000 deaths in the United States each year with the economic loss of $10 to $83 billion each year (Food and Drug Administration (FDA), 2004b ). In Netherlands there are an estimated 700,000 cases of food borne illness and 80 deaths per year with the economic loss of 65 million Euro per year (Havelaar *et al*., 2010). The figures are even estimated to be higher in tropical and sub-tropical countries, as improper and traditional storage conditions provide conducive conditions for the growth and proliferation of *Aspergillus* (Prakash *et al*., 2010).

### 1.2.4. Mycotoxicosis

Mycotoxicosis is a disease produced by coming in contact with mycotoxins. Eating contaminated food is the most common cause of mycotoxicosis; however, some mycotoxins cause disease upon dermal contact or inhalation. Mycotoxins are a structurally diverse set of typically small molecular weight natural compounds, produced mostly by the secondary metabolism of some filamentous fungi that are harmful to animals in low concentration. Pittet defined mycotoxins as fungal metabolites that when ingested, inhaled or absorbed through the skin cause illness or human and animal death (Pittet, 1998). They are secondary metabolites and not proteins or enteric toxins and they have no biochemical significance in

fungal growth and development; however, they vary from simple C4 compounds, e.g., moniliformin, to complex substances such as the phomopsins. Currently, more than 300 mycotoxins are known, scientific attention is focused mainly on those that have proven to be carcinogenic and/or toxic. Since filamentous fungi are common and opportunistic organism, mycotoxins are widespread. The most significant genera of mycotoxigenic fungi are: *Aspergillus*, *Alternaria*, *Claviceps*, *Fusarium*, *Penicillium* and *Stachybotrys* (Steyn, 1995).

During their lifecycle, over 40 species of *Aspergillus* have been listed as capable of producing a wide range of mycotoxins harmful to humans and animals that consume them (Zain, 2011), but the *Aspergillus* mycotoxins of greatest public health and agro-economic significance are aflatoxins (by *Aspergillus flavus* and *Aspergillus parasiticus*), ochratoxin A (by *Aspergillus ochraceus, Aspergillus carbonarius* and *Aspergillus niger*), sterigmatocystin (by *Aspergillus versicolor*), and cyclopiazonic acid (by *Aspergillus flavus* and *Aspergillus tamarii*). Citrinin, patulin and penicillic acid may also be produced by certain *Aspergillus* species, and tremorgenic toxins are produced by *Aspergillus terreus* (territrems), *Aspergillus fumigatus* (fumitremorgens) and *Aspergillus clavatus* (tryptoquivaline) (Zain, 2011).

The mycotoxins produced constitutes a major risk to human and animal health *(Dantigny et al*., 2007). Aflatoxin has carcinogenic, teratogenic, hepatotoxic, mutagenic, and immunosuppressive properties and can inhibit several metabolic systems, mainly found on several fruits, peanuts, pistachio nuts, Brazil nuts and figs. The principal classes of mycotoxins include aflatoxin B1 (AFB1), the most potent hepatocarcinogenic substance known, which has been proven to also be genotoxic (Zain, 2011). Ochratoxin A (OTA), which is a nephrotoxin, teratogen, and carcinogen, has mainly been found in cereals as well as in other products like coffee, wine, dried fruits, beer, grape juice and meats (Zain, 2011). Citrinin affect kidney function. Cyclopiazonic acid has a wide range of effects, and tremorgenic toxins such as territrems affect the central nervous system (Zain, 2011).

Human exposure to mycotoxins may result from consumption of foods commodities that are contaminated with toxins. Food commodities can be contaminated with mycotoxins at

various stages in the food chain; before they are harvested, as well as between harvesting and drying, and in storage and at processing (Miller, 1995). The presence of mycotoxins in food products is a chemical hazard of biological origin of increasing concern due to the wide range of food types where they can be found (Garcia *et al*., 2009). Mycotoxin contaminations of foods and feeds are significant problems worldwide. Consumption of foods containing mycotoxins causes mycotoxicosis (Zain, 2011). Mycotoxins can cause acute or chronic intoxication and damage to humans and animals after ingestion of contaminated food and feed. Furthermore, mycotoxins are responsible for generating huge economic losses in the producing countries (Bhat and Vasanthi, 2003 ). A few of them have been implicated as chemical warfare agents. Mycotoxins are responsible for cancers as well as many different disorders affecting the gastrointestinal, urogenital, vascular, kidney, and nervous systems. Some mycotoxins are immunocompromising, thereby reducing resistance to infectious disease (Zain, 2011).

Mycotoxin is a major public health and agroeconomic problems in both developing and developed countries. Pittet reported that 25–40% of cereals consumed in the world are contaminated by these toxic compounds (Pittet, 1998). In Yugoslavia, studies on mycotoxigenic fungi in raw milk have indicated 91% contamination. In the USA, a study reported mycotoxins in 24.7% of the samples and also studies showed aflatoxin contamination in corn leaded to 97 million dollars losses with additional 100 million dollars in production losses (Zain, 2011). Even India economy was affected with the estimate of 10 million dollars losses due to groundnut contamination with mycotoxins (Zain, 2011). In developing countries aflatoxicosis alone ranked $6^{th}$ among the 10 most important health risk by the magnitude of 4.5 billion people are affected (Williams *et al*., 2004).

In Africa, certain aflatoxin productions are associated with hot, dry agroecozones with latitudinal shifts in climate influencing fungal community structure (Cotty and Jaime-Garcia, 2007). Aflatoxin producing fungi are native to warm arid, semi-arid, and tropical regions with changes in climate resulting in large fluctuations in the quantity of aflatoxin producers. Most of the mycotoxin contamination problems occur in the sub-Saharan Africa in which

about 250,000-hepatocellular carcinoma related deaths occur annually in parts of sub-Saharan Africa due to aflatoxin ingestion alone. About 5.2 million cancer deaths occur each year, 55% of which occur in developing countries. In sub-Saharan Africa the aflatoxin, fumonisin and ochratoxin mycotoxins are common (Sibanda *et al*., 1997).

Recently researchers have shown the impact of climate change on increasing fungal infection, growth and mycotoxin productions in which the temperature and humidity's in place not familiar for fungal growth are now changed in fever of fungal growth and mycotoxin productions (Tirado *et al*., 2010). Whereas there are many factors involved in mycotoxin contamination, climate is the most important. There may be an increase of "high temperature fungi and mycotoxins" such as *Aspergillus flavus* and aflatoxin. An "up shift" in regions will be experienced, e.g. sub-tropical regions become tropical, with associated changes in mycotoxin contamination patterns (Paterson and Lima, 2011). Mycotoxins are climate dependent, plant- and storage-associated problems, also influenced by non-infectious factors, which are in turn driven by climatic conditions. Climate represents the key agro-ecosystem driving force of fungal colonization and mycotoxin production. Environmental conditions (climate) strongly influence fungal growth. In general there will be more mycotoxins in the new climate change era (Paterson and Lima, 2010).

On a global basis, the mycotoxins of main significance include aflatoxins in tree nuts, dry fruits, and spices, *Fusarium* toxins in maize, wheat, and barley, and ochratoxin A in cereals and coffee. On a regional scale, mycotoxins of importance include primarily patulin in fruits, ochratoxin A in grapes and dried vine fruits, cacao and coffee. Mycotoxins may occur in raw agricultural products pre- and post-harvest (Agrios, 2000). Aflatoxins (AFs), on several fruits, like peanuts, pistachio nuts, Brazil nuts and figs, signaled at high risk. On the other hand, almonds, pecans, walnuts and raisins are considered at lower AF risk (Jelinek *et al*., 1989).

Mycotoxins are ''unavoidably" consumed or ingested by animals or humans, but many countries regulate the limit of mycotoxin in food commodities (Murphy *et al*., 2006).

Regulations minimizing human exposure to mycotoxins result in high economic loss to handlers, producers, processors, and marketers of food commodities. It account for millions of dollars annually in losses worldwide in human health, animal health, and condemned agricultural products. The concern about the cited mycotoxins is confirmed by EC regulation No. 1881/2006, where all contaminants in food were included and limits were fixed for OTA presence in unprocessed and processed cereals and coffee, dried vine fruits, grape juice and wine, for AFs presence in groundnuts, nuts, dried fruits, cereals, maize, several spices and milk, and for PAT in fruit juices, apple juices, spirit and fermented drinks derived from apple juice and solid apple products.

The impact of mycotoxin in Ethiopia date back to 1978 where the gangrenous ergotism epidemic occurred from the consumption of grains contaminated with *Claviceps purpureu* that resulted with 34% mortality from 140 affected peoples (King, 1979). Three years later aflatoxins were reported from the study conducted by Besrat and Gebre (1981) on some selected Ethiopian food. Later on outbreak of Gangrenous Ergotism was reported by Urga and his coworker which was found to be associated with consumption of contaminated barley in Arsi, Ethiopia (Urga *et al*., 2002). All of the samples were contaminated with ergot alkaloids and 55% mortality was reported on mice upon consuming of this contaminated barley. Another study conducted in Addis Ababa indicated aflatoxin contamination in Shiro and ground red pepper (Fufa and Urga, 1996). Ochratoxin contaminations are not exceptional in Ethiopia as the study of Ayalew and his colleague reported 23.4% of the 107 wheat sample (Ayalew *et al*., 2006). A recent study reminded us how serous is the mycotoxin problem is in Ethiopia by reporting the presence of aflatoxin and *A. flavus* in all 38 peanut samples collected from three administrative region of the country (Legesse, 2010).

### 1.2.5. Preservatives

Preservatives are used in food for two main reasons: 1) to prevent/control growth of fungi, including *Aspergillus* species, and 2) to control natural spoilage processes (food preservation). Food preservatives play a vital role in maintaining a tasty, nutritious and safe

supply of food year-round to our growing population. It prevents food from deteriorating due to age or mold growth. Without preservatives, a great amount of food on shop shelves would "go off" before being bought and/or consumed. For example, bread would last only about two days before becoming stale. Preservatives also make possible an array of convenience foods that we have come to accept as part of modern life (Brul and Coote, 1999, Maier *et al*., 2010).

Food preservation is carried out during food processing in an attempt to maintain raw material quality, physicochemical properties and functionality whilst providing safe products that have a low spoilage potential. Modern food processing is dependent on a range of preservative technologies to ensure that food is maintained at an acceptable level of quality from the time of manufacture through to the time of consumption. The most common classical preservative agents are synthetic preservatives including acetic, lactic, benzoic, sorbic acid, benzimidazole, diphenylamine, phenyl mercuric acetate, zinc dimethyl dithiocarbamate, carbendazim, sulphur. These molecules inhibit the outgrowth of both bacterial and fungal cells and sorbic acid is also reported to inhibit the germination and outgrowth of bacterial spores (Brul *et al*., 2002).

However, the application of synthetic preservatives has led to a number of environmental and health problems because they are themselves carcinogenic, teratogenic, and highly toxic with long degradation periods (Lingk, 1991). In addition its indiscriminate use has led to a number of ecological and medical problems due to residual toxicity, hormonal imbalance and spermatotoxicity, etc. (Lingk, 1991).

Another problem with the use of synthetic preservatives is that up on the consumption of food containing these substances some individuals produce allergic reactions to these substances may result in adverse reactions (Wilson and Bahna, 2005). For example, reactions to sulphur dioxide and sodium benzoate occur more commonly in asthmatics than reactions to the colourant tartrazine. Reactions to Food Additives and Preservatives include

vomiting, rashes, hives, a tight chest, headaches, worsening of eczema, and many other symptoms (Lingk, 1991).

### 1.2.6. Essential oils

Current consumer opinion suggests a desire for high quality foods that are more natural, minimally processed and preservative free, while remaining safe and with an extended shelf life. Many approaches have been proposed for controlling microbial food spoilage, including use of chemical preservatives (Pitt and Hocking, 2009). Major disadvantage of this approach is their environmental and health problems and the development of resistance. This raises considerable challenges, particularly since there is increasing unease regarding the use of chemical preservatives and artificial antimicrobials to inactivate or inhibit growth of spoilage and pathogenic micro-organisms (Pitt and Hocking, 2009).

Since natural products seldom accumulate in the ecosystem, they do not lead to development of resistance as a result it could potentially serve as effective alternatives to control foods from spoiling by microorganisms. Natural product is getting a good deal of attention for a number of microorganism control issues: reducing the need for antibiotics, controlling microbial contamination in food, improving shelf-life extension technologies to eliminate undesirable pathogens and/or delay microbial spoilage, decreasing the development of antibiotic resistance by pathogenic microorganisms or strengthening immune cells in humans are some of the benefits (Tajkarimi *et al*., 2010). Amongst different groups of plant products, essential oils (EOs) have recently become a focal point of research in botanical preservations of food ( Gupta *et al*., 2011, Kumar *et al*., 2008). Being volatile in nature, the essential oils are being recommended as fumigants for preservation of food commodities (Kumar *et al*., 2008, Tajkarimi *et al*., 2010).

Essential oils (EOs) are volatile, natural, complex compounds characterized by a strong odour and are formed by aromatic plants as secondary metabolites. Known for their bactericidal, virucidal and fungicidal, and medicinal properties and their fragrance, they are used in the preservation of foods and as antimicrobial, analgesic, sedative, anti-

inflammatory, spasmolytic and locally anesthesic remedies. At present, approximately 3000 essential oils are known, 300 of which are commercially important especially for the pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries. EOs are rich sources of small terpenoids and phenolic compounds that appear to be responsible for much of their antimicrobial activity, although there is evidence that minor components also play an important role, mainly through synergistic effects (Rodr´ıguez *et al*., 2007).

Essential oils is an increasingly important area of natural product in search for agents that control microorganisms (Razzaghi-Abyaneh *et al*., 2009b). In European Union for example essential oils are allowed in food (as flavorings), perfumes (fragrances and aftershaves), pharmaceuticals (for their functional properties) and aromatherapy constitutes little more than 2% of the total market. Even in the United State some essential oils have been classified as generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) as flavors or food additives. So, EOs with antimicrobial activity are possible candidates for using as natural antimicrobial preservatives to controlling microbial food contaminations, which might replace synthetic fungicide (Razzaghi-Abyaneh *et al*., 2009b).

### 1.3. Significance of the Study

As shown in many countries by various researchers; molds especially *Aspergillus* is a major public threat by causing *Aspergillus* infection, mycotoxicosis and loss of food commodities. The consequences are severe in developing countries (Wagacha and Muthomi, 2008). When food supply is limited, the mycotoxin hazard is exacerbated in at least two ways. First, more fungus-damaged, potentially mycotoxin-containing foodstuffs are consumed rather than discarded, and second, malnutrition enhances the susceptibility to lower levels of food borne mycotoxins. The situation is worse in Ethiopia, where the pre and post-harvest treatment of crops is not stringent enough to discourage fungal infection, growth and the subsequent production of mycotoxins.

Synthetic fungicides have been used in the Ethiopia with varying magnitude for controlling pathogenic and toxigenic fungi but their treatment can be often problematic due to their residual nature and high toxicity to mammals, environmental and health concerns, and development of resistance. With this practice there are high numbers of patients suffering from residual toxicity, carcinogenicity, teratogenicity, hormonal imbalance and spermatotoxicity, etc (Nollet and Rathore, 2010).

As a result there should be continuous search mechanisms for safe and effective preservatives from natural products that have antifungal and antimycotoxigenic activity. One possible source of new drugs is traditional medicine especially essential oils. Essential oils and/or their constituents have a broad spectrum of activity against *Aspergillus* species (Isman, 2000). As such, they have considerable potential as food spoilage protectants and for infection management in other situations (Gonzalez-Coloma *et al*., 2010). Present information indicates that they are safe to the user and the environment (Ujváry, 2010).

One of the traditional practices that kept till today in Ethiopia is the use of aromatic plants as aroma-treatment of food commodities and skin diseases (Tadeg, 2004). Taking in to accounts the availability and acceptability of the practices in the Ethiopian society, this study tries to examine fungitoxic and antimycotoxigenic potentials of selected essential oils used in agrarian community of the country.

## 1.4. Objective of the Study

### 1.4.1. General Objective

To assess effect of essential oils on *Aspergillus* spore germination, growth and mycotoxin production.

### 1.4.2. Specific Objective

- To isolate *A. flavus, A. fumigatus* and *A. niger* from some selected food commodities
- To evaluate aflatoxin producing potentials of the *Aspergillus* spp
- To evaluate growth and spore germination inhibition by EOs
- To determine MIC of essential oils
- To determine aflatoxin arresting potential of EOs
- To determine safety limits of EOs

# CHAPTER II: MATERIAL AND METHOD

## 2.1. Study design and Period

Experimental study was conducted to see fungitoxic and anti aflatoxigenic property of essential oils *in vitro* and to see the safety limits of essential oils *in vivo*. The study was conducted from November 2011 to April 2012.

## 2.2. Sample Collection Site

### a. Food commodities

Processed, semi processed and unprocessed food commodities of six kinds: Peanut, Pea flour (shiro), Barley & Emmer flour (besso & mittin aja), Cookies, Maize and roasted Barley (kolo and senef kolo) was collected from Addis Ababa. A total of 36 food commodities, six samples from each kind of food commodities were collected from six open markets around Teklehaimanot, Merkato, Messalemia, Medhanialem, Addisu Mikael and Enkulal Fabrika.

### b. Plant material

Plants for study were collected from the wild and some of them are also collected from the local markets and botanical garden of Traditional and Modern Medicine Research Directorate (TMMRD) in EHNRI from November– December, 2011. Different parts of the test plants of *Cymbopogon martinii* (aerial part) and *Rosmarinus officinalis* (aerial part) were collected from the botanical garden of TMMRD, EHNRI, Ethiopia, *Foeniculum vulgare* (leaf lamina and leaf sheath) were collected from Shashamane, Ethiopia, *Lippia adoensis Hochst. var. koseret* (leaves) were collected from Sibu-Sire district, East Wellega, Ethiopia, *Ruta chalepensis* (fruits) were bought from local market of Addis Ababa from the merchant of Alemgena, Ethiopia and *Trachyspermum ammi* (fruits) were collected from Tepi, Ethiopia.

### 2.3. Sample Processing

#### I. Plants

#### a. Collection procedure

Clean areas were selected to pick plants away from the pollution (traffic and dusts). Aerial parts of *Cymbopogon martinii* L. (Poaceae) and *Rosmarinus officinalis* L. (Lamiaceae) were harvested leaving 5cm and 15cm stubble from ground level, respectively. Leaf lamina and leaf sheath of *Foeniculum vulgare* Miller (Apiaceae) and leaves of *Lippia adoensis* Hochst. var. koseret (Verbenaceae) were harvested. Mature fruits of *Ruta chalepensis* L. (Rutceae) and *Trachyspermum ammi* L. Sprague ex Turrill (Apiaceae) fruits were collected (bought). The plants were collected into paper bags as this allows the plant to breath. The samples were labeled (write plant name, plant part, date, name of the collector, place). All the plant materials were transported to the TMMRD, EHNRI laboratory for the processing and extraction as soon as possible.

#### b. Taxonomic Identification of Plants

The identities of collected plant materials were confirmed by Taxonomist and Botanist in Traditional and Modern Drug Research Department of the Ethiopian Health and Nutrition Research Institute (EHNRI).

#### c. Plant processing and Preparation of crude extracts

Fresh areal part of *Cymbopogon martinii* and *Rosmarinus officinalis*; leaf lamina and leaf sheath of *Foeniculum vulgare*; and leaves of *Lippia adoensis Hochst. var. koseret* were chopped into small pieces in order to get the maximum yield of essential oils. Fresh fruits of *Ruta chalepensis* and dried fruits of *Trachyspermum ammi* were used for the extraction of EOs. Essential oils were extracted by hydro-distillation of the plant materials. Plant materials (250g) were placed in a 5L round-bottom distillation flask and 3L distilled water was added. The essential oils were obtained by hydro-distillation for 3h using Clevenger-type apparatus. The isolated fractions of plant parts exhibited two distinct layers: an upper oily layer and the lower aqueous layer. Dichloromethane was used to remove the essential oil from the water layer. The organic layer was dried with anhydrous sodium sulfate.

Filtration and concentration on a rotary evaporator gave the crude essential oil. The essential oils were stored in clean, dark brown (amber glass) bottles. The essential oil yields (%) in the plant tissue samples were calculated:

$$\text{Essential oil yield (\%)} = \frac{\text{ammount oil extracted(ml)}}{\text{amount of plant biomass distilled (g)}} \text{X}100$$

Figure 2.1: Essential oil extracts of plant material using Clevenger-type apparatus

### c. Phytochemical test of the Essential oils

#### i. TLC Analysis

Preliminary qualitative phytochemical screenings of the most active essential oils for various secondary metabolites that have antimicrobial activity were conducted. For this purpose a silica-gel thin-layer chromatography (TLC) finger print analyses were conducted.

**Preparation and application of samples:** Prior to testing, 1 part of the plants essential oils was diluted with 9 part of toluene. Five micro liter of the prepared solution were transferred to TLC plates (pre coated Silica gel G60 $F_{254}$) by micropipette in the form of spot having a diameter of around 4mm into the base line. Reference compound (Thymol) 3 µl (≈ 100 µg) were used for control.

**Saturation of the chromatographic chamber:** To achieve saturation, the inside wall of the TLC chamber was lined with filter paper and 10 ml of the mobile phase (Toluene-ethyl acetate (93:7)) was poured into it. This system is suitable for the analysis and comparison of all six essential oils. The chamber was then closed and allowed to equilibrate for half an hour at room temperature.

**Development of chromatograms:** The applied bands of the essential oil solutions were allowed to dry and the plates were placed vertically into the chamber, ensuring that the points of application are above the surface of the mobile phase. The chamber was closed and the chromatogram was allowed to develop at room temperature. The mobile phase was allowed to ascend 9 cm from the starting line and the plates were then removed and dried.

**Observation and interpretation of the chromatograms**: After development, when the solvent front reached the top of the marked plate (about 25-35 minute) the plates were removed from the tank and dried after marking the solvent front. The migrated spots were then detected using Vanillin-sulphuric acid reagent (VS) (solution I: 1% ethanolic vanillin; solution II: 10% ethanolic sulphuric acid) color-forming reagents. The plate is sprayed with 10 ml solution I, followed immediately by 10 ml solution II. After heating the plate at $110^{O}C$ for 5-10min under observation, the plate is evaluated in visual. After treatment with the VS reagent the monoterpene alcohols and their esters, cineole, the aldehyde citral and citronella; show blue or blue-violet color in visual. The phenylpropane derivatives safrole, anethole, rnyristicin, apiol and eugenol are brown-red/ violet, while thymol and carven are red to red-violet: pipcritonc shows a typical orange color, Anethole & methyl chavicol (Red violet – brown violet). Identification of each spots was estimated by calculating the $R_f$ value of the major components of each essential oils beside the color of spots developed.

**ii. GC-analysis**

Gas chromatographic analysis of the oil of *Trachyspermum ammi* was performed on a Shimadzu GC-2010 system, with split mode. The column used was a ZB-1MS equivalent to OV-1, fused silica capillary column 30 m × 0.25 mm i.d., film thickness 0.25μm, coated

with 5% diphenyl-95% polydimethylsiloxane, operated with the following oven temperature programme: 50 °C, held for 2 min, rising at 3 °C/min to 210 °C. Injection temperature and volume, 250 °C and 1.0μl, respectively; injection mode, split; split ratio, 10:1; carrier gas, nitrogen at 65.2cm/s linear velocity and inlet pressure 100KPa; detector temperature, 270 °C; nitrogen flow rate, 52.1 ml/min; air flow rate, 400 ml/min; make-up 32, ($H_2$/air) flow rate 40 ml/min; sampling rate, 40ms.

## II. Food commodities

### a. Collection Procedure

A total of 36 samples, six samples from six different kinds of food commodities: Peanut, Pea flour (shiro), Barley & Emmer Wheat flour (besso & mittin aja), Cookies, Maize and roasted Barley (kolo and senef kolo) was collected from Addis Ababa. About 1kg of each sample was bought from the market and aseptically taken with paper bag covered with plastic bag. Codes was given to each sample and transported to the laboratory. Samples were kept in the refrigerator with its temperature sated between 4 to $8^{O}C$ to minimize the organism growth or death till the processing and mycological analysis of the food samples to be analyzed.

Table 2.1: Sample of food commodities from Addis Ababa open market

| Kind of food commodity sample and corresponding codes | | | | | |
|---|---|---|---|---|---|
| peanut | pea ‘shiro’ | ‘besso & ajja' | cookies | maize | kolo & senef kolo |
| Mr-001 | Th-007 | Mr-013 | Am-019 | Ms-037 | Ms-043 |
| Mr-002 | Mr-008 | Am-014 | Mr-032 | Ms-038 | Ms-044 |
| Md-003 | Am-009 | Ef-015 | Mr-033 | Ms-039 | Ms-045 |
| Am-004 | Ef-010 | Mr-025 | Am-034 | Ms-040 | Ms-046 |
| Ms-005 | Mr-011 | Ef-026 | Ef-035 | Mr-041 | Ms-047 |
| Ms-006 | Mr-012 | Md-027 | Md-036 | Mr-042 | Am-048 |

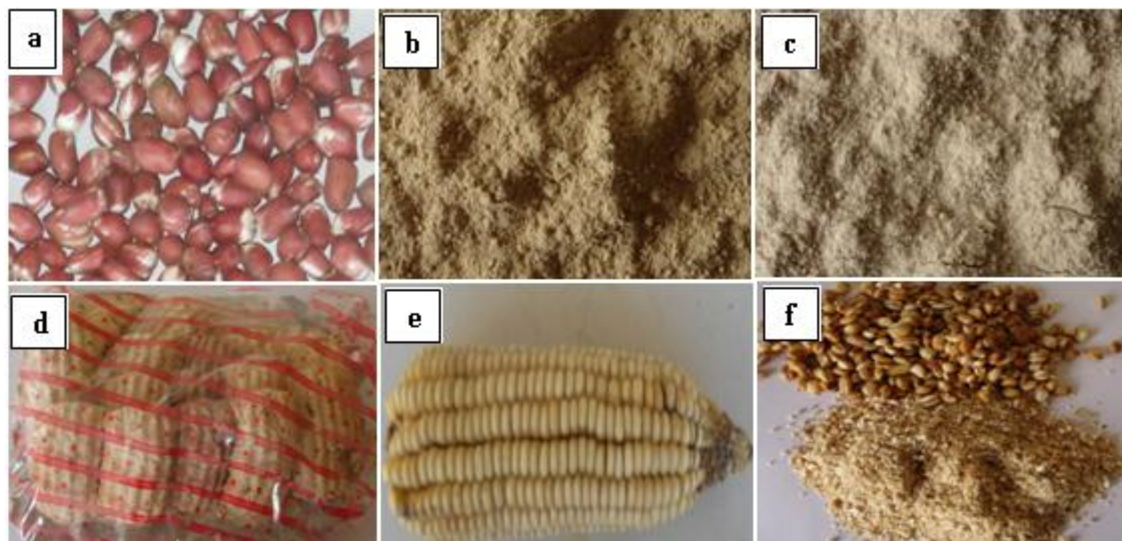


Figure 2.2: Food commodities used in study (a= Peanut, b= Pea flour, c= Barley, d=cookies, e=maize and f= roasted Barley)

**b. Culture and Identification of *Aspergillus* species**

Mycological analysis of the collected food commodities was carried out according to Annex I. Each sample was aseptically sub-sampled (25gm) for mycological analysis. A serial dilution of food samples were prepared using 1:10 dilution (i.e., 1gm of food in 9ml of sterile distilled water) and then homogenized by vortex mixer. Two milliliter of the samples from each dilution were added into 18ml of PDA in a test tube and homogenized by vortex mixer and poured into 90mm Petri dish. After incubation the plates were observed for the presence of growth of visible fungal colonies.

## 2.4. Characterization of *Aspergillus* species for production of aflatoxin

*Aspergillus flavus*, *Aspergillus niger* and *Aspergillus fumigatus* isolates from each food commodities were screened for the production of aflatoxin following Kumar and his collogues protocol (Kumar *et al*., 2008). The isolates were cultured separately in 25ml SMKY broth (sucrose 200g; MgSO4·7H2O, 0.5g; KNO3, 0.3g and yeast extract, 7g; 1000ml distilled water) in 100ml flask for 10days. The content of each flask was filtered and extracted with 20ml chloroform in a separating funnel. The extract was evaporated to dryness on water bath and redissolved in 1ml chloroform. Aflatoxin production was detected by thin layer chromatography. Fifty micro liter chloroform extract was spotted on TLC

plates and developed in the solvent system comprising chloroform: acetone (9:1V/V). The plate was air dried the intensity of aflatoxin observed in UV-366.

### 2.5. Antifungal and antiaflatoxigenic activity of essential oils on *Aspergillus* species

Antifungal activity of the essential oils was conducted using different techniques. Food poisoning techniques were used for screening program to select four essential oils that have better antifungal activity. Agar dilution assay to determine MIC and spore germination inhibition assay was used to choose one essential oils on which mycelial dry weight inhibition and antiaflatoxigenic activity was performed.

#### a. Poison food technique

Generally antifungal activity was determined by poisoned food technique (Das *et al*., 2010). Five-day old fungal cultures were punched aseptically with a sterile cork borer of generally 5mm diameter. The fungal discs were then put on the gelled agar plate. The agar plates were prepared by impregnating desired concentration of essential oils (2µl/ml) at a temperature of 45 - 50°C. The plates are then incubated at temperature 26 ± 2°C for fungi. Colony diameter was recorded by measuring the two opposite circumference of the colony growth. Percentage inhibition of mycelial growth is evaluated by comparing the colony diameter of poisoned plate (with plant essential oils) and non-poisoned plate (with distilled water) and calculated using the formula given below (Das *et al*., 2010):

$$\%\ \text{Mycellial Inhibition} = \frac{\text{Mycellial growth (control)} - \text{Mycellial growth (treatment)}}{\text{Mycellial growth (control)}}$$

#### b. Spore germination assay

Sporicidal activity was conducted using spore germination assay on four essential oils (*Cymbopogon martinii, Foeniculum vulgare*, *Lippia adoensis Hochst. var. koseret*, and *Trachyspermum ammi*) that have showed better fungicidal activity by using the above screening program (Das *et al*., 2010). Desired concentration and volume of the essential oil were tested for their potential of spore germination inhibition on tested *Aspergillus* spp. The test organisms were grown on PDA medium for sporulation and spores were harvested when the cultures were fully sporulated; which was achieved after 10 days of incubation. Spores

were collected by adding 5 ml of sterile water containing 0.1% (V/V) Tween 80 (for better spore separation) to each Petri dish and rubbing the surface with a sterile L-shaped spreader (3 times). The suspension was collected and then centrifuged at room temperature at 2000 rpm for 5 min. The supernatant was discarded and re-centrifuged until 1ml of highly concentrated spore solution remained. A haemocytometer slide was used to count spore production to have approximately $10^8$spore/ml (Uldahl and Knutsen, 2009).

Various concentrations: 0.25µl/ml, 0.5µl/ml, 1µl/ml, 2µl/ml, 4µl/ml and 8µl/ml of *Cymbopogon martinii, Foeniculum vulgare*, *Lippia adoensis Hochst. var. koseret*, and *Trachyspermum ammi* essential oils were prepared in 5ml of sabouraud dextrose broth in 100ml flask and then 1ml of the spore suspension were added to each flask. The flasks were then incubated for 24 h at 25°C on a rotary shaker (60 rpm) as to evenly disperse the oil throughout the broth. At the end of the incubation period, germinated spores were observed using a light microscope at 400X magnification. Experiment was performed in triplicate and the extent of spore germination was assessed by looking for the presence of germ tubes. Results were expressed in terms of the percentage of spores germinated as compared to the control from the average of the triplicates. Percentage spore germination inhibition is calculated according to the following formula:

$$\%\ \text{Spore Germination ihnibition} = \frac{(\text{sc} - \text{st})}{(\text{sc})} \text{X } 100$$

Where: sc, average number of spore germinated in control set; st, average number of spore germinated in test set.

### c. MIC determination of essential oils

The minimum inhibitory concentrations (MIC) were evaluated by the agar dilution methods for *Cymbopogon martinii, Foeniculum vulgare*, *Lippia adoensis Hochst. var. koseret*, and *Trachyspermum ammi* essential oils (Balows, 1991, Das *et al*., 2010). Twofold serial dilution of each EOs in Sabouraud dextrose agar was made by adding two milliliter of each dilution of the desired concentrations of EOs into each 18 milliliter of agar in a test tube which was well mixed and poured in to 90mm Petri dish. As the concentration of essential oils was diluted 1:10 in media the pre stock solutions of EOs should be made in ten times

the required final concentration to be tested. The experiments were performed in triplicates. The agar was permitted to solidify in the plates on a level surface at room temperature. Control plates, containing no essential oils were run simultaneously. The agar surface of the plates containing the dilution of EOs and the control plate are inoculated five millimeter discs of the test fungi taken from advancing edge of 7-day-old cultures. The plate containing the lowest concentration of EOs was seeded first. Control plates were seeded last to insure that viable organisms were present throughout the procedure. Incubate the inoculated plates at 26 ± 2$^{O}$C for seven days before being read. End-points for each EOs are best determined by placing plates on a dark background and observing for the lowest concentration that inhibits visible growth, which is recorded as the MIC. The MIC of each antimicrobial agent is usually recorded in micro liter per milliliter.

**d. Determination of mycelial dry weight**

The mycelial dry weight for the plant essential oils were evaluated in sabouraud dextrose broth. Twofold serial dilution of each *Trachyspermum ammi* essential oils sabouraud dextrose broth was made by adding one part of the desired concentrations of essential oils into each nine part of sabouraud dextrose broth in a 100ml flask. As the concentration of essential oils was diluted 1:10 in the broth the pre stock solutions of EOs should be made in ten times the required final concentration to be tested. The experiments were performed in triplicates. Control sets, containing no essential oils were run simultaneously. The flasks were aseptically inoculated with 0.36ml spore suspensions (≈$10^{6}$spore/ml). The flask containing the lowest concentration of EOs was seeded first. Control plates were seeded last to insure that viable organisms were present throughout the procedure. Incubate the inoculated plates at 26 ± 2$^{O}$C for seven days before being read. Flasks containing mycelia were be filtered through Whatman filter No. 1 and then were washed with distilled water. The mycelia were placed on pre weighed Petri plates and were allowed to dry at 60$^{O}$C for 6h and then at 40$^{O}$C overnight. The flasks containing dry mycelia were weighed. Percent growth inhibition on the basis of dry weight was calculated as (Rasooli and Owlia, 2005):

$$\%\ \text{mycelial dry weight} = \frac{\text{control weight} - \text{sample weight}}{\text{control weight}} \text{X } 100$$

**e. Efficacy of *Trachyspermum ammi* essential oil in arresting aflatoxin elaboration**

The methods that has been adopted by Kumar and his collogues (Kumar *et al*., 2008) were followed to determine antiaflatoxigenic efficacy of EOs with lowest MIC on *A. flavus* using SMKY broth medium (Sucrose, 200g; MgSO4 7H2O, 0.5g; KNO3, 0.3g; Yeast extract, 7.0 g; Distilled water, 1000 ml; pH, 5.6±0.2). Different concentrations of the oil (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0µl/ml) were prepared separately by dissolving their requisite amount in 0.5 ml 5% tween-20 and then mixing it with 24.5ml of SMKY medium in 100 ml Erlenmeyer flask. The control sets were kept parallel to the treatment sets without essential oil. Then flasks were inoculated aseptically with 1 ml spore suspension (≈106 spores/ml) prepared in 0.1% tween-80 and incubated at 27±2 °C for 10 days. The content of each flask was filtered (Whatman filter paper no. 1). The filtrate was extracted with 20 ml chloroform in a separating funnel and the extracts were passed through anhydrous sodium sulphate kept in Whatman filter paper no. 42. The extracts were evaporated till dryness on water bath at 70°C. Dry residues were dissolved in 1 ml chloroform and 50µl of chloroform extract spotted on TLC plate (20×20 cm2 of Silica gel-G60 $F_{254}$) then developed in chloroform: acetone (9:1v/v). The intensity of aflatoxin was observed in Ultra Violate Fluorescence Analysis Cabinet at an excitation wavelength of 366nm (Kumar *et al*., 2008).

## 2.6. Animal trials to determine safety limit of the oils

The safety limit of the best fungitoxic essential oils were determined by recording $LC_{50}$ value on mice following the protocol of Kumar and his collaborator (2008). Mice with an average weight and age (35 g, 3months) were selected as test animal for the mammalian toxicity experiments. Requisite amount of essential oils were mixed properly with Tween 80 to prepare different solutions containing desired dose of essential oils. The mice were administered 0.5ml of each solution of essential oils orally separately through a gavage syringe to each set containing 10 mice (equal proportion for gender). In control sets equal volume of Tween 80 was given to mice. After 72 h, the mortality of the animals was recorded and $LC_{50}$ was calculated in terms of per kg body weight of mice using SPSS version 20.0 and Minitab version 16 computer software's by probit analysis.

## 2.7. Statistical analysis

All the measurements were replicated three times for each treatment and data were entered into excel spreadsheet and are presented as mean ± SE/SD. Significant differences between strains aflatoxin producer and non producer were analyzed using statistical software (SPSS 20.0; Chicago, IL, USA) at 95% level of confidence by Chi-square analysis. Significant differences between treatment and sensitive strains; data were first tested for normality and then subjected to one-way analysis of variance (ANOVA) using statistical software (SPSS 20.0; Chicago, IL, USA and Minitab 16.0, England). Significant differences between mean values were determined using Tukey's and Dunken's multiple range tests following one-way ANOVA and $P$ values $< 0.05$ were considered as significant.

## 2.8. Ethical considerations

The M.Sc. research project proposal were ethically cleared by the Department Research and Ethical Review Committee (DREC) and approved by Department of Microbiology, Immunology and Parasitology, School of Medicine, Addis Ababa University. Permission from the study site was obtained. Written informed consent was not needed as the study project did not include human participant.

## CHAPTER III: RESULTS

### 3.1. Study Design and Period

*In vitro* experimental studies of fungitoxic and antiaflatoxigenic activity of essential oils were conducted from November 2011 to April 2012 on aflatoxigenic *Aspergillus* spp isolated from food commodities collected from Addis Ababa open market. Moreover, safety limits of *Trachyspermum ammi* essential oils were conducted by *in vivo* experimental study.

### 3.2. Sample Processing

**a. Yields of crude essential oils extracts**

Different parts of six species of plants belonging to five different families were extracted by hydrodistillation for 3h using a Clevenger type apparatus. The hydrodistillation of *Trachyspermum ammi* fruits yielded pale yellow colored oils with the highest percentage yield of essential oils (5%), followed by the light green colored essential oils extracted from fruits of *Ruta chalepensis L.*(3%), among the aerial parts *Rosmarinus officinalis* L. provided maximum yield (2.5%) light green essential oil followed by colorless *Foeniculum vulgare* Mill oil which gave (2.3%) and *Cymbopogon martinii* (0.82) while *Lippia adoensis Hochst. var. koseret* leaves yielded the minimum amount of yellow colored essential oil when compared to the other five plants used in the study (0.7) (Table 3.1).

**Table 3.1:** Essential oil yield of aromatic plants used in the study

| Plant Species | Family | Part Extracted | Percentage Yield (W/W) | Color of EOs |
|---|---|---|---|---|
| *Ruta chalepensis L.* | Rutaceae | Fruit | 3.0 ± 0.058 | Pale yellow |
| *Cymbopogon martinii* | Poaceae | Leaf | 0.82 ± 0.017 | Pale yellow |
| *Foeniculum vulgare* Mill | Apiaceae | Leaf | 2.3 ± 0.088 | Color less |
| *Lippia adoensis Hochst. var. koseret* | Verbenaceae | Leaf | 0.7 ± 0.116 | Yellow |
| *Rosmarinus officinalis* L. | Lamiaceae | Leaf | 2.5 ± 0.115 | Light green |
| *Trachyspermum ammi* L. | Apiaceae | Fruit | 5 ± 0.289 | Pale yellow |

Values are mean ± SD

**b. TLC profile of essential oils**

TLC fingerprint was used for screening of essential oils as bioactive compounds were separated in a sequence of different zones and characterized by the value of Retention Factors ($R_f$) in Toluene: Ethyl acetate (9.3:0.7) solvent system and the color of zone they produce after being treated with detection reagent (Vanillin-sulfuric acid). Each plant essential oils have shown quite different thin layer chromatography finger print (Table 3.2 and Figure 3.1). TLC screening indicated the presence of many terpenoids in the essential oil tested which was confirmed by the presence of different colored spots. Highest number of spots was obtained in the chromatogram of *Cymbopogon martini* and *Lippia adoensis Hochst. var. koseret* essential oils that showed distinctive 8 spot/bands, *Rosmarinus officinalis* were observed visually having 7 spot/bands, while *Trachyspermum ammi*, *Foeniculum vulgare* and *Ruta chalepensis* essential oil have separated in four different spot/bands when visually observed after treatment with Vanillin-sulphuric acid reagent (VS).

**Table 3.2:** TLC fingerprint of essential oils of aromatic plants

| plant | Spot (bands) $R_f$ value and corresponding colors | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1st | 2nd | 3rd | 4th | 5th | 6th | 7th | 8th |
| Thy | 0.51 (violet re) | - | - | - | - | - | - | - |
| *Ta* | 0.52 (violet red) | 0.45 (brown) | 0.35 (gray) | 0.23 (gray) | - | - | - | - |
| *La* | 0.96 (blue violet) | 0.85 (brown) | 0.71 (gray) | 0.63 (brown) | 0.54 (violet) | 0.45 (green) | 0.44 (blue) | 0.36 (blue) |
| *Rc* | 0.94 (blue violet) | 0.73 (violet) | 0.59 (gray) | 0.25 (brown) | - | - | - | - |
| *Cm* | 0.96 (t violet) | 0.78 (blue) | 0.73 (gray) | 0.65 (blue) | 0.54 (violet) | 0.49 (blue) | 0.39 (blue) | 0.3 (blue) |
| *Ro* | 0.96 (violet) | 0.65 (blue) | 0.56 (violet) | 0.48 (blue) | 0.39 (blue) | 0.3 (blue) | 0.23 (blue) | - |
| *Fv* | 0.95 (blue violet) | 0.88 (violet) | 0.69 (gray) | 0.56 (violet) | - | - | - | - |

Thy, Thymol standard; Cm, *Cymbopogon martinii*; Fv, *Foeniculum vulgare*; La, *Lippia adoensis Hochst. var. koseret*; Ro, *Rosmarinus officinalis*; Rc, *Ruta chalepensis*; Ta, *Trachyspermum ammi*

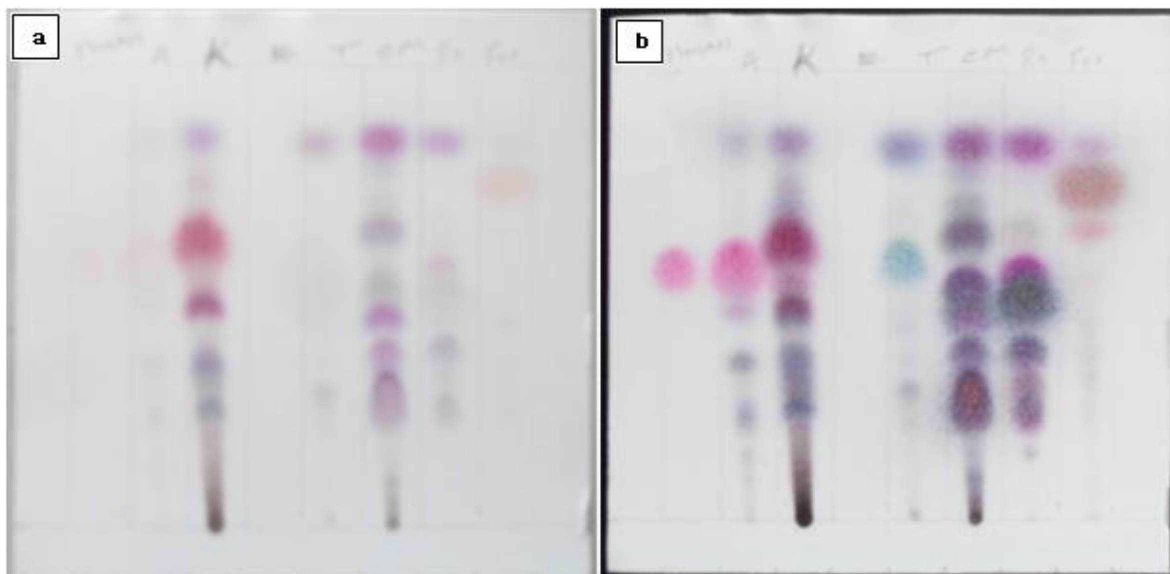


**Figure 3.1:** TLC fingerprint (a; b= before and after heating at 110OC for 5 minute respectively) of thymol standard and essential oils of *Trachyspermum ammi; Lippia adoensis Hochst. var. koseret; Ruta chalepensis; Cymbopogon martinii; Rosmarinus officinal* and *Foeniculum vulgare* respectively

### c. GC profile of *Trachyspermum ammi* essential oils

GC analysis of *Trachyspermum ammi* essential oil showed the presence of 14 components accounting for 100% of the total amount (Figure 3.2). It is clearly seen from the chromatogram that essential oils have two major peaks. Thymol (51.5%) was found as a major component of *Trachyspermum ammi* essential oil. Moreover, the GC analyses of *Trachyspermum ammi* essential oil the presence of other 12 minor components.

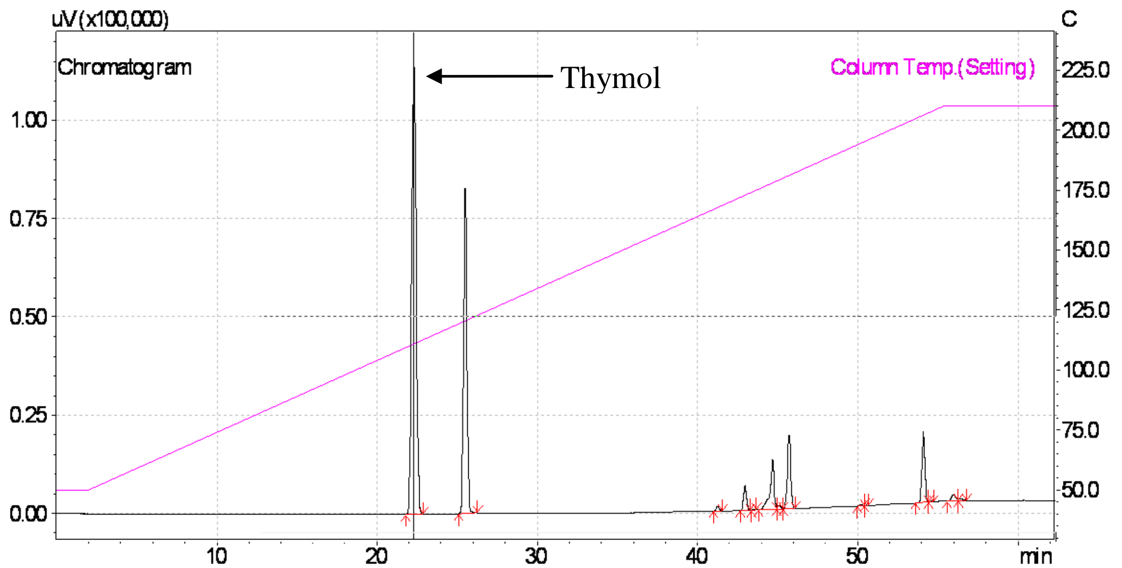


**Figure 3.2:** GC chromatograms of *Trachyspermum ammi* fruit essential oil

**d. Isolation of *Aspergillus* species from food commodities**

To better isolate the various fungal species from the samples, simple growth analyses made on mycological culture were carried out. Several fungal species became visible from the growth on PDA media after seven days of incubation. Only *Aspergillus* species (*Aspergillus flavus*, *Aspergillus niger* and *Aspergillus fumigatus*) were sub-cultured for further study since these species are believed as storage molds. Table 3.3 shows isolated *Aspergillus* spp, percentage frequencies from total samples, and total number of *Aspergillus* spp isolates from all samples using agar plate methods. Of the three *Aspergillus* spp isolated from the food commodities, *Aspergillus flavus were* the most predominant with 36.6% (Table 3.3) isolated from 11 samples, followed by *Aspergillus fumigatus* collected from 10 samples about 33.3% while *Aspergillus niger* were recovered from 9 samples about 30% of the samples collected for the study.

As the microscopy and macroscopy (culture) characteristics of fungal isolates that are presented in Fig 3.3 and Table 3.3 showed; a total of 30 *Aspergillus* spp (*A. flavus*, *A. niger* and *A. fumigatus*) were isolated from 36 samples of food commodities. As can be seen from the Table 3.3 the least number (2 isolate) of *Aspergillus* spp isolates were counted from the sample cookies while the highest (7 isolate) were counted from both peanut and besso & ajja

sample. From 11 *Aspergillus flavus* isolated 3 isolates (27.3%) were isolated from peanut sample, while the least isolated from shiro and cookies sample 1 (9.1%). Besso and ajja samples seemed to be highly infected by *Aspergillus fumigatus* with the frequency of 3 (30%). Two out of six samples of peanut, besso and ajja, maize and senef kolo were infected by *Aspergillus niger* which accounts for 22.2%

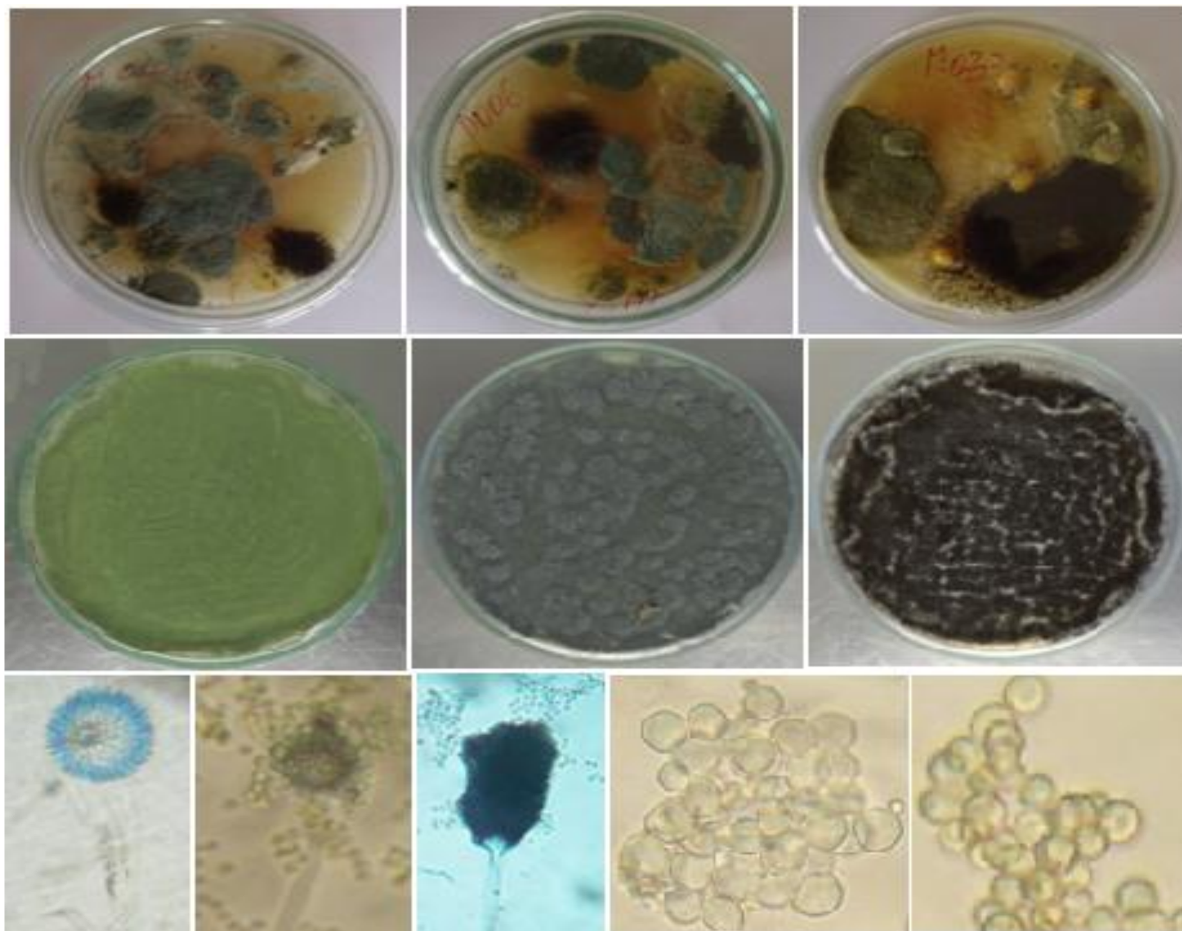

**Figure 3.3:** Culture and microscopy of *Aspergillus* species isolated from food commodities.

**Table 3.3:** *Aspergillus* species isolated from food commodities

| species | | peanut | pea 'shiro' | besso & ajja | cookies | maize | senef kolo | Total |
|---|---|---|---|---|---|---|---|---|
| *A.flavus* | Count | $3_a$ | $1_a$ | $2_a$ | $1_a$ | $2_a$ | $2_a$ | 11 |
| | % within *A. flavus* | 27.3% | 9.1% | 18.2% | 9.1% | 18.2% | 18.2% | 100.0% |
| | % within name of the sample | 42.9% | 33.3% | 28.6% | 50% | 40% | 33.3% | 36.6% |
| *A. fumigatus* | Count | $2_a$ | $1_a$ | $3_a$ | $1_a$ | $1_a$ | $2_a$ | 10 |
| | % within *A. fumigatus* | 20.0% | 10.0% | 30.0% | 10.0% | 10.0% | 20.0% | 100.0% |
| | % within name of the sample | 28.6% | 33.3% | 42.9% | 50% | 20% | 33.3% | 33.3% |
| *A. niger* | Count | $2_a$ | $1_a$ | $2_a$ | $0_a$ | $2_a$ | $2_a$ | 9 |
| | % within *A.s niger* | 22.2% | 11.1% | 22.2% | 0.0% | 22.2% | 22.2% | 100.0% |
| | % within name of the sample | 28.6% | 33.3% | 28.6% | 0.0% | 40% | 33.3% | 30% |
| Total | Count | 7 | 3 | 7 | 2 | 5 | 6 | 30 |

Each subscript letter denotes a subset of name of the sample categories whose column proportions do not differ significantly from each other at the 0.05 level.

### 3.3. Characterization of *Aspergillus* species for the production of aflatoxin

A total of 7 (23.3%) isolates of *Aspergillus* spp produced aflatoxin in vitro in SMKY broth culture out of 30 different *Aspergillus* spp isolated from food commodities. Among the aflatoxigenic species isolated *Aspergillus flavus* were responsible for the majority of cases by accounting for 6 (85.7%) followed by *Aspergillus niger* 1 (14.3%). None of the ten *Aspergillus fumigatus* were found to produce aflatoxin in vitro on SMKY broth medium. Within species, fifty five percent of *Aspergillus flavus* isolates found to produce aflatoxin *in vitro*, while 11% and none of *Aspergillus niger* and *Aspergillus fumigatus* isolates respectively produced aflatoxin in vitro on culture media (Table 3.4).

**Table 3.4:** *Aspergillus* species ability to produce aflatoxin

| *Aspergillus* species | | Aflatoxin production | | Total |
|---|---|---|---|---|
| | | no | yes | |
| *A. flavus* | Count | $5_a$ | $6_b$ | 11 |
| | % within *Aspergillus* species | 45.5% | 54.5% | 100.0% |
| | % within in vitro aflatoxin production | 21.7% | 85.7% | 36.7% |
| | % of Total | 16.7% | 20.0% | 36.7% |
| *A. niger* | Count | $8_a$ | $1_a$ | 9 |
| | % within *Aspergillus* species | 88.9% | 11.1% | 100.0% |
| | % within in vitro aflatoxin production | 34.8% | 14.3% | 30.0% |
| | % of Total | 26.7% | 3.3% | 30.0% |
| *A. fumigatus* | Count | $10_a$ | $0_b$ | 10 |
| | % within *Aspergillus* species | 100.0% | 0.0% | 100.0% |
| | % within in vitro aflatoxin production | 43.5% | 0.0% | 33.3% |
| | % of Total | 33.3% | 0.0% | 33.3% |
| Total | Count | 23 | 7 | 30 |
| | % within *Aspergillus* species | 76.7% | 23.3% | 100.0% |
| | % within in vitro aflatoxin production | 100.0% | 100.0% | 100.0% |
| | % of Total | 76.7% | 23.3% | 100.0% |

Each subscript letter denotes a subset of in vitro aflatoxin production categories whose column proportions do not differ significantly from each other at the .05 level.

As shown in Table 3.4 from *A. flavus* isolated AF001 isolated from peanut sample and AF037 isolated from maize sample were produced both B1 and B2 aflatoxin, while the other produce B1 aflatoxin. In addition, from the 9 *A. niger* isolated only AF002 isolated from the peanut sample produced aflatoxin B1 and B2. No isolates of *A. fumigatus* produced any kind of aflatoxin as detected by TLC. Standard strains of *A. flavus* and *A. niger* produced B2 and B1 aflatoxin.

## 3.4. Antifungal activity of essential oils on *Aspergillus* species

### 3.4.1. Antifungal screening of plant essential oils

Table 6 shows the fungitoxic activity screening program of essential oils from *Cymbopogon martinii, Foeniculum vulgare, Lippia adoensis Hochst. var. koseret, Rosmarinus officinalis, Ruta chalepensis* and *Trachyspermum ammi* at a concentration of 2µl/ml against toxigenic *Aspergillus flavus*, *Aspergillus fumigatus* and *Aspergillus niger* strains isolated from food commodities. In order to access the antifungal activity of the six essential oils, food poisoning techniques were used; and their average scores were compared. It can be clearly seen that all tested essential oils affected the growth of *Aspergillus* spp (Table 3.5).

**Table 3.5:** Antifungal screening of some plant essential oils against the toxigenic strain of *Aspergillus* spp using food poisoning technique

| *Aspergillus* species | Antifungal activity | | | | | |
|---|---|---|---|---|---|---|
| | Cm | Fv | La | Ro | Rc | Ta |
| AFST | 14.7 ± 0.3 | 15.0 ± 0.0 | 0.0 ± 0.0 | 21.0 ± 0.0 | 16.3 ± 0.8 | 0.0 ± 0.0 |
| AF001 | 16.0 ± 0.6 | 16.7 ± 0.3 | 0.0 ± 0.0 | 18.3 ± 0.3 | 18.7 ± 0.8 | 0.0 ± 0.0 |
| AF006' | 19.0 ± 1.0 | 18.7 ± 0.3 | 0.0 ± 0.0 | 19.7 ± 0.3 | 17.0 ± 1.2 | 0.0 ± 0.0 |
| AF009 | 17.0 ± 0.0 | 17.0 ± 0.0 | 0.0 ± 0.0 | 19.3 ± 1.2 | 18.0 ± 0.0 | 0.0 ± 0.0 |
| AF019 | 17.7 ± 0.8 | 18.0 ± 0.6 | 0.0 ± 0.0 | 19.0 ± 0.0 | 18.7 ± 1.2 | 0.0 ± 0.0 |
| AF027 | 15.3 ± 0.8 | 15.7 ± 0.3 | 0.0 ± 0.0 | 19.3 ± 0.3 | 16.7 ± 1.5 | 0.0 ± 0.0 |
| AF037 | 17.7 ± 0.7 | 18.3 ± 0.3 | 0.0 ± 0.0 | 19.7 ± 0.7 | 19.3 ± 0.8 | 0.0 ± 0.0 |
| ANST | 0.0 ± 0.0 | 0.7 ± 0.3 | 0.0 ± 0.0 | 16.3 ± 0.3 | 15.3 ± 0.8 | 0.0 ± 0.0 |
| AN002 | 0.0 ± 0.0 | 0.7 ± 0.3 | 0.0 ± 0.0 | 15.7 ± 0.3 | 15.0 ± 0.0 | 0.0 ± 0.0 |
| AFU037 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 6.0 ± 0.6 | 3.3 ±0.3 | 0.0 ± 0.0 |

AFST, *Aspergillus flavus* (ATCC 13697); AF, *Aspergillus flavus*; ANST, *Aspergillus niger* (ATCC 10535); AN, *Aspergillus niger*; AFU, *Aspergillus fumigatus*; Cm, *Cymbopogon martinii*; Fv, *Foeniculum vulgare*; La, *Lippia adoensis Hochst. var. koseret*; Ro, *Rosmarinus officinalis*; Rc, *Ruta chalepensis*; Ta, *Trachyspermum ammi,* Values are mean (n=3)±standard error.

Absolute fungitoxicity (zero mycelial expansion) against all tested organisms were shown by the essential oils of *Lippia adoensis Hochst. var. koseret* and *Trachyspermum ammi*. This potency was reduced against strains *Aspergillus flavus* (16.76 average mycelial expansions) by essential oils of *Cymbopogon martinii*, however, complete inhibition against toxigenic *Aspergillus niger* and *Aspergillus fumigatus* was recorded. *Aspergillus fumigatus* was highly susceptible to the essential oils of *Foeniculum vulgare* when compared to the other *Aspergillus* spp tested. The highest number of mycelial expansion meaning the lowest to moderate antifungal activity were seen in the essential oils of both *Rosmarinus officinalis* and *Ruta chalepensis* at the tested concentration. Hence, all except *Rosmarinus officinalis* and *Ruta chalepensis* plants were selected for further investigations of minimum inhibitory concentration and Aspergillus spore germination inhibition.

### 3.4.2. Effect of essential oils on spore germination of toxigenic *Aspergillus* species

Each tested concentration of *Cymbopogon martinii* essential oils showed notable inhibition of *Aspergillus* species spore germination (Table 3.6). Statistical results (one-way ANOVA) revealed that amount of essential oils tested have significant effect ($P < 0.05$) when compared with equivalent control plates that have no essential oil. In addition, spore production was completely inhibited at the highest concentration of *Cymbopogon martinii* essential oil (1µl/ml for both *Aspergillus niger* and *Aspergillus fumigatus*; 2µl/ml for *Aspergillus flavus*). The greater inhibition of spore was observed in *Aspergillus fumigatus* and the least in strains *Aspergillus flavus*.

**Table 3.6:** Percent of Spore Germination Inhibition of toxigenic *Aspergillus* spp by *Cymbopogon martinii* essential oil, using fungal spore germination assay

| *Aspergillus* species | Antifungal activity | | | | | |
|---|---|---|---|---|---|---|
| | 0.25µl/ml | 0.5µl/ml | 1µl/ml | 2µl/ml | 4µl/ml | 8µl/ml |
| AFST | 42.5 ±2.5 | 68.0 ±1.7 | 98.3 ±1.5 | 100.0 ±0.0 | 100.0 ± 0.0 | 100.0 ±0.0 |
| AF001 | 45.3 ±0.8 | 73.3 ±4.5 | 99.0 ±0.9 | 100.0 ±0.0 | 100.0 ± 0.0 | 100.0 ±0.0 |
| AF006' | 48.2 ±2.8 | 76.0 ±0.9 | 98.0 ±1.7 | 100.0 ±0.0 | 100.0 ± 0.0 | 100.0 ±0.0 |
| AF009 | 50.8 ±0.6 | 77.2 ±2.1 | 98.5 ±1.3 | 100.0 ±0.0 | 100.0 ± 0.0 | 100.0 ±0.0 |
| AF019 | 52.0 ±2.2 | 75.2 ±0.6 | 98.5 ±1.3 | 100.0 ±0.0 | 100.0 ± 0.0 | 100.0 ±0.0 |
| AF027 | 52.8 ±0.8 | 76.5 ±3.0 | 98.5 ±1.3 | 100.0 ±0.0 | 100.0 ± 0.0 | 100.0 ±0.0 |
| AF037 | 55.0 ±0.9 | 80.3 ±0.3 | 98.0 ±1.7 | 100.0 ±0.0 | 100.0 ± 0.0 | 100.0 ±0.0 |
| ANST | 61.7 ±1.1 | 95.0 ±0.5 | 100.0±0.0 | 100.0 ±0.0 | 100.0 ± 0.0 | 100.0 ±0.0 |
| AN002 | 65.5 ±0.0 | 94.3 ±0.3 | 100.0±0.0 | 100.0 ±0.0 | 100.0 ± 0.0 | 100.0 ±0.0 |
| AFU037 | 80.7 ±0.3 | 99.7 ±0.2 | 100.0±0.0 | 100.0 ±0.0 | 100.0 ± 0.0 | 100.0 ±0.0 |

AFST, *Aspergillus flavus* (ATCC 13697); AF, *Aspergillus flavus*; ANST, *Aspergillus niger* (ATCC 10535); AN, *Aspergillus niger*; AFU, *Aspergillus fumigatus;* Values are mean (n=3) ± standard deviation.

The effect of *Foeniculum vulgare* essential oil on the spore germination of aflatoxigenic *Aspergillus* spp at six different concentrations was represented by Table 3.7. It can be clearly seen that essential oils of this plant have inhibited the spore germination of the tested. Moreover, there was a significant difference of inhibition of fungal spore germination by different concentrations of *Foeniculum vulgare* essential oil in comparison to control group after 24 hour incubation ($P$<0.05). However, a 100% inhibition of spore germination was observed at 2µl/ml for *Aspergillus niger* and *Aspergillus fumigatus* and at 4µl/ml for *Aspergillus flavus*. It has also been observed under the microscope that those spores which germinated in presence of low concentrations of essential oil produced small germ tubes as compared to the control.

**Table 3.7:** Percent of Spore Germination Inhibition of toxigenic *Aspergillus* spp by *Foeniculum vulgare* essential oil, using fungal spore germination assay

| *Aspergillus* species | Antifungal activity | | | | | |
|---|---|---|---|---|---|---|
| | 0.25µl/ml | 0.5µl/ml | 1µl/ml | 2µl/ml | 4µl/ml | 8µl/ml |
| AFST | 27.8 ±0.8 | 42.5 ±2.0 | 74.5 ±0.5 | 99.5 ±0.5 | 100.0 ±0.0 | 100.0 ±0.0 |
| AF001 | 31.7 ±2.5 | 45.0 ±0.5 | 78.5 ±2.3 | 99.8 ±0.3 | 100.0 ±0.0 | 100.0 ±0.0 |
| AF006' | 31.8 ±1.5 | 45.0 ±0.9 | 82.8 ±1.9 | 99.7 ±0.3 | 100.0 ±0.0 | 100.0 ±0.0 |
| AF009 | 27.0 ±0.9 | 39.3 ±1.2 | 81.2±1.8 | 99.7 ±0.3 | 100.0 ±0.0 | 100.0 ±0.0 |
| AF019 | 36.3 ±1.1 | 50.5 ±0.9 | 84.7 ±0.8 | 99.7 ±0.3 | 100.0 ±0.0 | 100.0 ±0.0 |
| AF027 | 40.5 ±1.0 | 53.5 ±1.0 | 85.8 ±0.8 | 99.8 ±0.3 | 100.0 ±0.0 | 100.0 ±0.0 |
| AF037 | 32.3 ±0.8 | 42.7 ±1.3 | 79.0 ±0.0 | 99.7 ±0.3 | 100.0 ±0.0 | 100.0 ±0.0 |
| ANST | 48.8 ±2.5 | 74.0 ±1.3 | 96.5 ±0.0 | 100.0 ±0.0 | 100.0 ±0.0 | 100.0 ±0.0 |
| AN002 | 46.8 ±1.6 | 75.5 ±0.5 | 98.0 ±0.5 | 100.0 ±0.0 | 100.0 ±0.0 | 100.0 ±0.0 |
| AFU037 | 56.5 ±.0 | 92.5 ±1.8 | 99.7 ±0.3 | 100.0 ±0.0 | 100.0 ±0.0 | 100.0 ±0.0 |

AFST, *Aspergillus flavus* (ATCC 13697); AF, *Aspergillus flavus*; ANST, *Aspergillus niger* (ATCC 10535); AN, *Aspergillus niger*; AFU, *Aspergillus fumigatus;* Values are mean (n=3) ± standard deviation.

Table 3.8 shows the inhibition effect of *Lippia adoensis Hochst. var. koseret* essential oils on spore germination of aflatoxigenic *Aspergillus* spp. Tween20 (5%v/v) used as a control did not inhibited the spore germination of all *Aspergillus* spp used in the study. It can be clearly seen that this plants essential oils have remarkable spore germination inhibition activity. Even at the lowest concentration of the oil tested (0.25µl/ml), inhibited more than 69% of spore germination. Moreover, there was a significant inhibition of fungal spore germination by each concentration of *Lippia adoensis Hochst. var. koseret* essential oil in comparison to control plates after 24 hour incubation ($P<0.05$). A 100% inhibition was observed at 2µl/ml for all tested strains of *Aspergillus* spp. It has also been microscopically observed that those spores which germinated in presence of low concentrations of essential oil produced small and/or short germ tubes as compared to the control.

**Table 3.8:** Percent of Spore Germination Inhibition of toxigenic *Aspergillus* spp by *Lippia adoensis Hochst. var. koseret* essential oil, using fungal spore germination assay

| *Aspergillus* species | Antifungal activity | | | | | |
|---|---|---|---|---|---|---|
| | 0.25µl/ml | 0.5µl/ml | 1µl/ml | 2µl/ml | 4µl/ml | 8µl/ml |
| AFST | 71.0 ±1.1 | 83.3 ±1.6 | 93.1 ±0.6 | 100.0 ±0.0 | 100.0 ±0.0 | 100.0 ±0.0 |
| AF001 | 76.2 ±0.6 | 87.8 ±0.3 | 94.5 ±0.0 | 100.0 ±0.0 | 100.0 ±0.0 | 100.0 ±0.0 |
| AF006' | 69.0 ±0.5 | 80.0 ±1.3 | 94.0 ±0.9 | 100.0 ±0.0 | 100.0 ±0.0 | 100.0 ±0.0 |
| AF009 | 70.8 ±1.1 | 83.5 ±3.1 | 95.2 ±0.8 | 100.0 ±0.0 | 100.0 ±0.0 | 100.0 ±0.0 |
| AF019 | 74.8 ±0.6 | 88.0 ±1.5 | 96.5 ±0.0 | 100.0 ±0.0 | 100.0 ±0.0 | 100.0 ±0.0 |
| AF027 | 79.7 ±0.6 | 87.0 ±3.3 | 95.7 ±0.8 | 100.0 ±0.0 | 100.0 ±0.0 | 100.0 ±0.0 |
| AF037 | 75.5 ±0.0 | 88.3 ±1.1 | 95.5 ±0.0 | 100.0 ±0.0 | 100.0 ±0.0 | 100.0 ±0.0 |
| ANST | 82.2 ±1.4 | 91.2 ±1.2 | 97.5 ±1.0 | 100.0 ±0.0 | 100.0 ±0.0 | 100.0 ±0.0 |
| AN002 | 84.3 ±0.7 | 92.8 ±1.2 | 98.2 ±0.3 | 100.0 ±0.0 | 100.0 ±0.0 | 100.0 ±0.0 |
| AFU037 | 91.2 ±0.7 | 99.3 ±0.2 | 99.8 ±0.3 | 100.0 ±0.0 | 100.0 ±0.0 | 100.0 ±0.0 |

AFST, *Aspergillus flavus* (ATCC 13697); AF, *Aspergillus flavus*; ANST, *Aspergillus niger* (ATCC 10535); AN, *Aspergillus niger*; AFU, *Aspergillus fumigatus;* Values are mean (n=3) ± standard deviation.

Table 3.9 shows the inhibitory effect of *Trachyspermum ammi* essential oils on spore germination of aflatoxigenic *Aspergillus* spp. *Trachyspermum ammi* essential oils have pronounced spore germination inhibition effect on all tested organisms. Statistical results showed that the amount of *Trachyspermum ammi* essential oils have significant ($P<0.05$) inhibitory effect on spore germination, compared to control sets (Tween20 5%v/v). The six different concentrations of essential oil efficiently caused different degrees of inhibition in terms of spore germination. A hundred percent inhibition was recorded from 0.5µl/ml upward for *Aspergillus fumigatus* and at 1µl/ml for *Aspergillus niger*, while a complete inhibition of spore germination of *Aspergillus flavus* was recorded at 2µl/ml. In addition, this result can be revealed in several ways, first, unlike other essential oils; *Trachyspermum ammi* essential oil have pronounced efficacy on the spore germination of all *Aspergillus* spp tested. Second results showed that inhibition of spore production was significantly inhibited even, at 0.25µl/ml, with spore production reduced by more than 80% ($P<0.05$).

**Table 3.9:** Percent of Spore Germination Inhibition of toxicogenic *Aspergillus* spp by *Trachyspermum ammi* essential oil, using fungal spore germination assay

| *Aspergillus* species | Antifungal activity | | | | | |
|---|---|---|---|---|---|---|
| | 0.25µl/ml | 0.5µl/ml | 1µl/ml | 2µl/ml | 4µl/ml | 8µl/ml |
| AFST | 88.0 ± 0.5 | 94.3 ± 0.6 | 99.8 ± 0.3 | 100.0 ± 0.0 | 100.0±0.0 | 100.0±0.0 |
| AF001 | 88.5 ± 0.0 | 95.3 ± 0.7 | 99.8 ± 0.3 | 100.0 ± 0.0 | 100.00±0.0 | 100.0±0.0 |
| AF006' | 87.0 ± 0.9 | 93.7 ± 0.6 | 99.7 ± 0.3 | 100.0 ± 0.0 | 100.00±0.0 | 100.0±0.0 |
| AF009 | 86.7 ± 0.3 | 95.0 ±0.0 | 99.7 ± 0.3 | 100.0 ± 0.0 | 100.00±0.0 | 100.0±0.0 |
| AF019 | 88.5 ± 0.0 | 95.7 ± 0.6 | 99.8 ± 0.3 | 100.0 ± 0.0 | 100.00±0.0 | 100.0±0.0 |
| AF027 | 88.5 ± 1.2 | 94.0 ± 1.0 | 99.7 ± 0.3 | 100.0 ± 0.0 | 100.00±0.0 | 100.0±0.0 |
| AF037 | 88.3 ± 1.1 | 95.7 ± 0.6 | 99.7 ± 0.3 | 100.0 ± 0.0 | 100.00±0.0 | 100.0±0.0 |
| ANST | 91.5 ± 1.0 | 100.0±0.0 | 100.0 ±0.0 | 100.0 ± 0.0 | 100.00±0.0 | 100.0±0.0 |
| AN002 | 92.5 ± 0.9 | 99.7 ± 0.6 | 100.0 ±0.0 | 100.0 ± 0.0 | 100.00±0.0 | 100.0±0.0 |
| AFU037 | 99.5 ± 0.0 | 100.0±0.0 | 100.0 ±0.0 | 100.0 ± 0.0 | 100.00±0.0 | 100.0±0.0 |

AFST, *Aspergillus flavus* (ATCC 13697); AF, *Aspergillus flavus*; ANST, *Aspergillus niger* (ATCC 10535); AN, *Aspergillus niger*; AFU, *Aspergillus fumigatus;* Values are mean (n=3) ± standard deviation.

### 3.4.3. Effect of essential oils on the growth of toxigenic *Aspergillus* species (MIC)

The mean score for antifungal activity of *Cymbopogon martinii* oil was revealed by Table 3.10. As it can be seen *Cymbopogon martinii* essential oil has affected the growth. However, in control palates increased mycelial expansion were observed. Statistical result showed that the amount of essential oils have a significant effect at a concentration higher than 0.5µl/ml ($P < 0.05$). Interestingly, for all *Aspergillus* species except *Aspergillus fumigatus* the MIC of *Cymbopogon martinii* oil was recorded at 4µl/ml by agar dilution techniques against toxigenic organisms; while 1µl/ml was recorded for *Aspergillus fumigatus* using sabouraud dextrose agar. At these points; the plant essential oil completely inhibited the growth of all isolated fungal strains *Aspergillus flavus, Aspergillus niger* and *Aspergillus fumigatus*.

**Table 3.10:** MIC of *Cymbopogon martinii* essential oil, against the toxigenic strain of *Aspergillus* spp using agar dilution technique

| *Aspergillus* species | Antifungal activity | | | | | |
|---|---|---|---|---|---|---|
| | Control* | 0.25µl/ml | 0.5µl/ml | 1µl/ml | 2µl/ml | 4µl/ml |
| AFST | 26.0 ± 0.6 | 23.7 ± 0.3 | 22.3 ± 0.8 | 18.7 ± 0.7 | 14.7 ± 0.3 | 0.0 ± 0.0 |
| AF001 | 22.0 ± 0.0 | 22.0 ± 0.0 | 20.3 ± 1.3 | 16.3 ± 0.7 | 16.0 ± 0.6 | 0.0 ± 0.0 |
| AF006' | 24.7 ± 0.7 | 24.7 ± 0.3 | 23.7 ± 0.3 | 20.3 ± 0.3 | 19.0 ± 1.0 | 0.0 ± 0.0 |
| AF009 | 24.7 ± 0.3 | 23.0 ± 0.6 | 22.3 ± 0.8 | 17.7 ± 0.7 | 17.0 ± 0.0 | 0.0 ± 0.0 |
| AF019 | 24.0 ± 0.0 | 23.0 ± 1.2 | 20.3 ± 0.3 | 19.7 ± 0.3 | 17.7 ± 0.8 | 0.0 ± 0.0 |
| AF027 | 23.0 ± 0.6 | 21.0 ± 0.6 | 18.3 ± 1.2 | 18.3 ± 0.3 | 15.3 ± 0.8 | 0.0 ± 0.0 |
| AF037 | 26.3 ± 0.3 | 24.0 ± 1.2 | 21.0 ± 0.6 | 18.7 ± 0.7 | 17.7 ± 0.7 | 0.0 ± 0.0 |
| ANST | 25.0 ± 0.6 | 22.3 ± 1.5 | 19.7 ± 0.3 | 17.7 ± 0.7 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| AN002 | 21.7 ± 0.3 | 19.7 ± 0.3 | 17.7 ± 0.7 | 17.3 ± 0.3 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| AFU037 | 11.0 ± 0.0 | 11.0 ± 0.0 | 9.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |

AFST, *Aspergillus flavus* (ATCC 13697); AF, *Aspergillus flavus*; ANST, *Aspergillus niger* (ATCC 10535); AN, *Aspergillus niger*; AFU, *Aspergillus fumigatus;* *, 5% Tween 20; Values are mean (n=3) ± standard error.

Moderate antifungal activities against the growth of tested organisms were seen by the essential oils of *Foeniculum vulgare* (Table 3.11). In determination of MIC of *Foeniculum vulgare* essential oil by agar dilution revealed that the antifungal effect was not significant ($P$ <0.05) on the test strains of *Aspergillus* species at a concentration of 0.25 and 0.5µl/ml in comparison to the untreated control tests while this essential oils have significant effect ($P$ <0.05) at a concentration of 1, 2, 4, and 8µl/ml. Besides, minimum inhibitory concentration of *Foeniculum vulgare* oil was recorded as 8µl/ml for both toxigenic strains of *Aspergillus flavus* and *Aspergillus niger*; while MIC was recorded 4µl/ml for *Aspergillus fumigatus* with a complete inhibition up to seven days, by agar dilution techniques using sabouraud dextrose agar.

**Table 3.11:** MIC of *Foeniculum vulgare* essential oil, against the toxigenic strain of *Aspergillus* spp using agar dilution technique

| *Aspergillus* species | Antifungal activity | | | | | |
|---|---|---|---|---|---|---|
| | 0.25µl/ml | 0.5µl/ml | 1µl/ml | 2µl/ml | 4µl/ml | 8µl/ml |
| AFST | 24.0 ± 0.6 | 23.3 ± 0.3 | 20.0 ± 0.0 | 18.3 ± 0.3 | 18.3 ± 0.3 | 0.0 ± 0.0 |
| AF001 | 22.0 ± 1.2 | 20.0 ± 0.0 | 17.0 ± 0.0 | 16.0 ± 1.0 | 16.0 ± 1.0 | 0.0 ± 0.0 |
| AF006' | 24.3 ± 0.3 | 23.0 ± 0.6 | 19.7 ± 0.8 | 18.3 ± 0.7 | 18.3 ± 0.7 | 0.0 ± 0.0 |
| AF009 | 22.0 ± 0.0 | 21.0 ± 1.0 | 19.3 ± 0.8 | 14.7 ± 1.8 | 14.7 ± 1.8 | 0.0 ± 0.0 |
| AF019 | 23.0 ± 0.6 | 21.0 ± 1.2 | 19.0 ± 0.0 | 15.3 ± 0.7 | 15.3 ± 0.7 | 0.0 ± 0.0 |
| AF027 | 22.0 ± 0.0 | 21.0 ± 0.6 | 19.0 ± 1.0 | 19.0 ± 0.0 | 19.0 ± 0.0 | 0.0 ± 0.0 |
| AF037 | 24.3 ± 0.7 | 22.0 ± 0.0 | 19.7 ± 0.8 | 18.3 ± 0.7 | 18.3 ± 0.7 | 0.0 ± 0.0 |
| ANST | 23.7 ± 0.7 | 20.3 ± 0.3 | 19.3 ± 0.0 | 12.0 ± 1.5 | 12.0 ± 1.5 | 0.0 ± 0.0 |
| AN002 | 21.0 ± 0.0 | 19.0 ± 0.6 | 16.0 ± 0.0 | 10.7 ± 0.3 | 10.7 ± 0.3 | 0.0 ± 0.0 |
| AFU037 | 10.3 ± 0.3 | 7.7 ± 0.7 | 4.0 ± 0.0 | 3.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |

AFST, *Aspergillus flavus* (ATCC 13697); AF, *Aspergillus flavus*; ANST, *Aspergillus niger* (ATCC 10535); AN, *Aspergillus niger*; AFU, *Aspergillus fumigatus;* Values are mean (n=3) ± standard error.

Strong evidence of antifungal efficacy was found by *Lippia adoensis Hochst. var. koseret* essential oil during our investigation to determine MIC (Table 3.12). It can be clearly seen that the fungitoxic activity of this plant essential oil affected the growth of all tested toxigenic *Aspergillus* spp on sabouraud dextrose agar. Statistical results showed that concentration of essential oils have significant effect ($P<0.05$). Compared to the control, a significant reduction of growth of *Aspergillus* spp were observed after exposure to *Lippia adoensis Hochst. var. koseret* essential oil at concentration of ≥0.5µl/ml ($P<0.05$). These reductions were concentration dependent since the higher concentration resulted in higher suppression of growth of the fungal mycelia extensions. As this table indicated the MIC was recorded at 2µl/ml which showed complete inhibition for up to seven days against all toxigenic *Aspergillus* spp.

**Table 3.12:** MIC of *Lippia adoensis Hochst. var. koseret* essential oil against the toxigenic strain of *Aspergillus* spp using agar dilution technique

| *Aspergillus* species | Antifungal activity | | | | | |
|---|---|---|---|---|---|---|
| | Control* | 0.25µl/ml | 0.5µl/ml | 1µl/ml | 2µl/ml | 4µl/ml |
| AFST | 26.0 ± 0.6 | 23.0 ± 0.0 | 21.3 ± 0.8 | 17.7 ± 0.7 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| AF001 | 22.0 ± 0.0 | 21.3 ± 0.3 | 21.0 ± 0.0 | 18.3 ± 0.3 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| AF006' | 24.7 ± 0.7 | 24.3 ± 0.3 | 22.7 ± 0.3 | 19.0 ± 0.6 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| AF009 | 24.7 ± 0.3 | 23.7 ± 0.3 | 20.3 ± 0.3 | 19.7 ± 0.3 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| AF019 | 24.0 ± 0.0 | 23.0 ± 0.6 | 21.0 ± 0.6 | 19.0 ± 0.6 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| AF027 | 23.0 ± 0.6 | 21.0 ± 0.6 | 19.7 ± 0.3 | 18.7 ± 0.3 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| AF037 | 26.3 ± 0.3 | 22.7 ± 1.5 | 21.3 ± 0.3 | 18.7 ± 0.3 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| ANST | 25.0 ± 0.6 | 22.7 ± 0.7 | 20.3 ± 0.3 | 18.7 ± 0.7 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| AN002 | 21.7 ± 0.3 | 20.7 ± 0.3 | 18.3 ± 0.3 | 17.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| AFU037 | 11.0 ± 0.0 | 11.3 ± 0.3 | 11.0 ± 0.6 | 10.0 ± 0.3 | 0.0 ± 0.0 | 0.0 ± 0.0 |

AFST, *Aspergillus flavus* (ATCC 13697); AF, *Aspergillus flavus;* ANST, *Aspergillus niger* (ATCC 10535); AN, *Aspergillus niger;* AFU, *Aspergillus fumigatus,* *, 5% Tween 20; Values are mean (n=3) ± standard error.

A remarkable antifungal activity against the growth of aflatoxigenic strains of *Aspergillus* spp was recorded after the treatment with different concentration of *Trachyspermum ammi* essential oil (Table 3.13). It is clearly seen that *Aspergillus fumigatus* were the most susceptible strains having no visible mycelial growth expansion at 0.5µl/ml, while similarities in fungitoxic activity were observed between the remaining *Aspergillus* species. Each tested doses of this plants essential oil have showed notable fungitoxic activity against tested organisms. Statistical results (one-way ANOVA) have showed the concentrations of *Trachyspermum ammi* essential oil have significant effect ($P<0.05$), compared to unexposed control plates at dose of ≥0.5µl/ml ($P<0.05$). The most striking result to emerge from the data was that the observation of absolute suppression of growth of the organisms at a concentration of 0.5µl/ml for *Aspergillus fumigatus* followed by 1µl/ml for both *Aspergillus flavus* and *Aspergillus niger* and these concentrations were recorded as MIC.

**Table 3.13:** MIC of *Trachyspermum ammi* essential oil against the toxigenic strain of *Aspergillus* spp using agar dilution technique

| *Aspergillus* species | Antifungal activity | | | | | |
|---|---|---|---|---|---|---|
| | Control* | 0.25µl/ml | 0.5µl/ml | 1µl/ml | 2µl/ml | 4µl/ml |
| AFST | 26.0 ± 0.6 | 23.0 ± 0.0 | 18.3 ± 0.3 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| AF001 | 22.0 ± 0.0 | 21.3 ± 0.3 | 17.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| AF006' | 24.7 ± 0.7 | 24.3 ± 0.3 | 16.3 ± 0.3 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| AF009 | 24.7 ± 0.3 | 23.7 ± 0.3 | 18.3 ± 0.3 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| AF019 | 24.0 ± 0.0 | 23.0 ± 0.6 | 17.7 ± 0.7 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| AF027 | 23.0 ± 0.6 | 21.0 ± 0.6 | 16.0 ± 0.6 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| AF037 | 26.3 ± 0.3 | 22.7 ± 1.5 | 17.3 ± 0.7 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| ANST | 25.0 ± 0.6 | 22.7 ± 0.7 | 18.7 ± 0.3 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| AN002 | 21.7 ± 0.3 | 20.7 ± 0.3 | 16.7 ± 0.8 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| AFU037 | 11.0 ± 0.0 | 11.3 ± 0.3 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |

AFST, *Aspergillus flavus* (ATCC 13697); AF, *Aspergillus flavus*; ANST, *Aspergillus niger* (ATCC 10535); AN, *Aspergillus niger*; AFU, *Aspergillus fumigatus;* *, 5% Tween 20; Values are mean (n=3) ± standard error.

Table 3.14 shows the antifungal efficacy of sodium benzoate a chemical preservative on the growth of toxigenic *Aspergillus* spp using agar dilution assay using sabouraud dextrose agar. It can be seen at glance that growth inhibitory response to the exposure of sodium benzoate synthetic chemical preservative at concentration of 1mg/ml, 2mg/ml and 4mg/ml showed no significant effect on the tested *Aspergillus* spp when compared to the unexposed control ($P$<0.05). Result of statistical analysis (one-way ANOVA) showed sodium benzoate only has displayed a significantly effects at a concentration of 16mg/ml that suppressed the growth of tested *Aspergillus* spp when compared to the control set ($P$<0.05). The minimum inhibitory concentration of this synthetic chemical preservative was recorded at a concentration of 8mg/ml for *Aspergillus fumigatus*, 16mg/ml for *Aspergillus niger* and >16mg/ml for *Aspergillus flavus*. In practical 2% sodium benzoate are used as preservative.

**Table 3.14:** MIC of Sodium benzoate preservative drug against the toxigenic strain of *Aspergillus* spp using agar dilution technique

| *Aspergillus* species | Antifungal activity | | | | | |
|---|---|---|---|---|---|---|
| | Control* | 1 mg/ml | 2 mg/ml | 4 mg/ml | 8 mg/ml | 16mg/ml |
| AFST | 25.3 ± 0.7 | 25.3 ± 0.3 | 22.0 ± 0.0 | 21.0 ± 1.0 | 20.0 ± 0.0 | 17.0 ±0.8 |
| AF001 | 22.0 ± 0.0 | 21.7 ± 0.7 | 21.7 ± 0.3 | 20.7 ± 0.8 | 18.7 ± 0.3 | 14.7 ± 0.3 |
| AF006' | 25.3 ± 0.3 | 24.7 ± 0.3 | 24.3 ±0.3 | 23.3 ± 1.7 | 20.0 ± 0.0 | 15.0 ± 0.0 |
| AF009 | 25.0 ± 0.0 | 23.7 ± 0.3 | 22.3 ± 0.3 | 20.7 ± 0.3 | 19.3 ± 0.3 | 14.7 ± 0.8 |
| AF019 | 24.3 ± 0.3 | 23.0 ± 0.6 | 19.7 ± 0.7 | 19.7 ± 0.3 | 18.3 ± 0.3 | 13.7 ± 0.0 |
| AF027 | 23.7 ± 0.3 | 21.7 ± 0.3 | 20.7 ± 0.3 | 22.7 ± 0.8 | 22.0 ± 0.6 | 15.3 ± 0.3 |
| AF037 | 25.7 ± 0.3 | 24.3 ± 0.3 | 23.7 ± 0.3 | 23.7 ± 0.8 | 23.3 ± 0.3 | 16.3 ± 0.7 |
| ANST | 25.0 ± 0.0 | 24.3 ± 0.3 | 23.3 ± 0.3 | 22.7 ± 0.3 | 8.0 ± 1.0 | 0.0 ± 0.0 |
| AN002 | 22.0 ± 0.0 | 21.7 ± 0.3 | 21.0 ± 0.6 | 20.7 ± 0.3 | 8.3 ± 1.7 | 0.0 ± 0.0 |
| AFU037 | 11.0 ± 0.0 | 10.3 ± 0.3 | 8.7 ± 0.3 | 7.7 ± 0.3 | 0.0 ± 0.0 | 0.0 ± 0.0 |

AFST, *Aspergillus flavus* (ATCC 13697)*;* AF, *Aspergillus flavus;* ANST, *Aspergillus niger* (ATCC 10535*); AN, Aspergillus niger*; AFU, *Aspergillus fumigatus;* *, 5% Tween 20; Values are mean (n=3) ± standard error.

All the four essential oils were documented to have better fungitoxic activity against aflatoxigenic *Aspergillus* spp when compared to synthetic chemical preservative sodium benzoate (Table3.15). Statistical results showed that both the kind and concentration of essential oil have significant effect ($P<0.05$). The most promising MIC was recorded by *Trachyspermum ammi* at 1µl/ml against *Aspergillus flavus* and *Aspergillus niger* while 0.5µl/ml for *Aspergillus fumigatus*; followed by *Lippia adoensis Hochst. var. koseret* essential oil at 2µl/ml against *Aspergillus flavus* and *Aspergillus niger* while 1µl/ml for *Aspergillus fumigatus.* The poor MIC was recorded by *Foeniculum vulgare* essential oils (8µl/ml against *Aspergillus flavus* and *Aspergillus niger* while 2µl/ml for *Aspergillus fumigatus*) when compared with in the four essential oils; while it is far better than the synthetic preservative sodium benzoate used in the study on all the isolated *Aspergillus*

species (16µl/ml against *Aspergillus flavus*, ≥16µl/ml against *Aspergillus niger* and 8µl/ml for *Aspergillus fumigatus*).

**Table 3.15:** Comparative antifungal activity of essential oils with sodium benzoate

| *Aspergillus* species | MIC activity (mg/ml) | | | | |
|---|---|---|---|---|---|
| | Cm | Fv | La | Ta | Sb |
| AFST | 4.00 | 8.00 | 2.00 | 1.00 | >16.00 |
| AF001 | 4.00 | 8.00 | 2.00 | 1.00 | >16.00 |
| AF006' | 4.00 | 8.00 | 2.00 | 1.00 | >16.00 |
| AF009 | 4.00 | 8.00 | 2.00 | 1.00 | >16.00 |
| AF019 | 4.00 | 8.00 | 2.00 | 1.00 | >16.00 |
| AF027 | 4.00 | 8.00 | 2.00 | 1.00 | >16.00 |
| AF037 | 4.00 | 8.00 | 2.00 | 1.00 | >16.00 |
| ANST | 2.00 | 8.00 | 2.00 | 1.00 | 16.00 |
| AN002 | 2.00 | 8.00 | 2.00 | 1.00 | 16.00 |
| AFU037 | 1.00 | 2.00 | 1.00 | 0.50 | 8.00 |

AFST, *Aspergillus flavus* (ATCC 13697)*;* AF, *Aspergillus flavus;* ANST, *Aspergillus niger* (ATCC 10535)*;* AN, *Aspergillus niger*; AFU, *Aspergillus fumigatus*; Cm, *Cymbopogon martinii*; Fv, *Foeniculum vulgare*; La, *Lippia adoensis Hochst. var. koseret*; Sb, Sodium Benzoate; Ta, *Trachyspermum ammi.*

### 3.4.4. Effect of Trachyspermum ammi essential oil on the mycelia dry weight of toxigenic *Aspergillus* species

The effect of *Trachyspermum ammi* essential oil on the dry mycelial weight of *Aspergillus* spp in sabouraud dextrose broth is presented in Table 3.16. Results of statistical analysis showed each tested concentration of essential oils have significantly different mycelial dry weight inhibition ($P < 0.05$). It can be clearly seen that a complete inhibition of mycelial dry weight at a concentration of 1, 2 and 4µl/ml. At least a 12, 43 and 71% of dry mycelia biomass weight suppression were recorded at 0.25, 0.5 and 0.75µl/ml respectively against *Aspergillus* spp. A dose dependent suppression of mycelial growth of *Aspergillus* spp was

observed; as the higher concentration of the *Trachyspermum ammi* essential oil inhibited hundred percent of the mycelial growth.

**Table 3.16:** Percent growth inhibition of *Aspergillus* spp on the basis of dry weight after treatments with different concentration of *Trachyspermum ammi* essential oils

| *Aspergillus* species | Percent growth inhibition (% mycelial dry weight = $\frac{\text{control weight} - \text{sample weight}}{\text{control weight}}$ X 100) | | | | | |
|---|---|---|---|---|---|---|
| | 0.25µl/ml | 0.5µl/ml | 0.75µl/ml | 1µl/ml | 2µl/ml | 4µl/ml |
| AFST | 11.93 | 43.07 | 71.18 | 100.00 | 100.00 | 100.00 |
| AF001 | 24.23 | 51.06 | 73.05 | 100.00 | 100.00 | 100.00 |
| AF006' | 30.65 | 42.69 | 70.15 | 100.00 | 100.00 | 100.00 |
| AF009 | 22.11 | 64.09 | 80.92 | 100.00 | 100.00 | 100.00 |
| AF019 | 30.71 | 65.26 | 81.30 | 100.00 | 100.00 | 100.00 |
| AF027 | 28.87 | 65.89 | 81.83 | 100.00 | 100.00 | 100.00 |
| AF037 | 21.53 | 58.20 | 72.07 | 100.00 | 100.00 | 100.00 |
| ANST | 29.34 | 72.62 | 91.85 | 100.00 | 100.00 | 100.00 |
| AN002 | 26.37 | 75.66 | 92.48 | 100.00 | 100.00 | 100.00 |
| AFU037 | 26.77 | 72.99 | 94.97 | 100.00 | 100.00 | 100.00 |

AFST, *Aspergillus flavus* (ATCC 13697); AF, *Aspergillus flavus;* ANST, *Aspergillus niger* (ATCC 10535); AN, *Aspergillus niger;* AFU, *Aspergillus fumigatus*

### 3.4.5. Aflatoxin arresting potentials of Trachyspermum ammi essential oil

Fig 3.5 shows antiaflatoxigenic potentials of *Trachyspermum ammi* essential oil against aflatoxigenic strains of *Aspergillus* spp (*Aspergillus flavus* and *Aspergillus niger*). It has been documented from this study's chromatogram that the aflatoxin production in SMKY liquid medium was reduced by the essential oils of *Trachyspermum ammi* in dose dependent manner. Aflatoxin production was completely inhibited at a concentration of 0.50µl/ml for strains of *Aspergillus niger* and at the concentration of 0.75µl/ml *for Aspergillus flavus*. These concentrations were less than that are recorded for minimum inhibitory concentration and absolute mycelial dry weight inhibiting concentration (1µl/ml). In all untreated control

(Tween 20 5%), high level of aflatoxin production was observed in all aflatoxigenic *Aspergillus* spp.

Table 3.17: Antiaflatoxigenic activity of *Trachyspermum ammi* essential oils at a concentration of 0.00, 0.25, 0.5, 0.75, 1 and 2µl/ml against toxigenic *Aspergillus* spp.

| *Aspergillus* | Aflatoxin production (fluorescence under UV 366λ) | | | | | |
|---|---|---|---|---|---|---|
| species | Control* | 0.25µl/ml | 0.5µl/ml | 0.75µl/ml | 1µl/ml | 2µl/ml |
| AFST | + | + | + | ND | ND | ND |
| AF001 | + | + | + | ND | ND | ND |
| AF006' | + | + | + | ND | ND | ND |
| AF009 | + | + | + | ND | ND | ND |
| AF019 | + | + | + | ND | ND | ND |
| AF027 | + | + | + | ND | ND | ND |
| AF037 | + | + | + | ND | ND | ND |
| ANST | + | + | ND | ND | ND | ND |
| AN002 | + | + | ND | ND | ND | ND |

AFST*,* *Aspergillus flavus* (ATCC 13697)*;* AF, *Aspergillus flavus;* ANST, *Aspergillus niger* (ATCC 10535)*;* AN, *Aspergillus niger;* AFU, *Aspergillus fumigatus,* +, aflatoxin production detected, ND, aflatoxin production not detected; *Tween 20 (5%)

### 3.5. Safety limit test

During the safety limit tests all the test animals were observed closely for up to 14 days; symptoms of toxicity, recovery and death were noted. No sign of toxicity was recorded for the mice in group of control, 3,000, 3,500 and 4,000µl/kg body weight. The majority of mice from group 4,500, 5000 and 5,500 have showed hypo-activity (decreased motility, debiting effect) and decreased feed intake. Mice from group 6,000 and 7,000µl/kg have showed the sign of prostration, anaesthesia and muscle spasm; within 30 minutes and followed by deaths with in the 24 hour. The mice that have showed hypo-activity and decreased feed intake have recovered within 24 hours.

As it can be observed from Figure 3.6 there was no mortality at the log concentration of 3,000 through 4,000µl/kg while there was one death at 4,500, two mortality at a dose of 5,000, four mortality at 5,500 and seven mortality at 6,000 and 10 mortality from 7,000. The

$LC_{50}$ was determined by drawing a vertical line on the X-axis from the point of the straight line the 50% mortality taken (Figure 3.6) and by calculating the inverse log of the value found on X-axis. The $LC_{50}$ of the essential oils was thus; 5336.297µl/kg body weight.

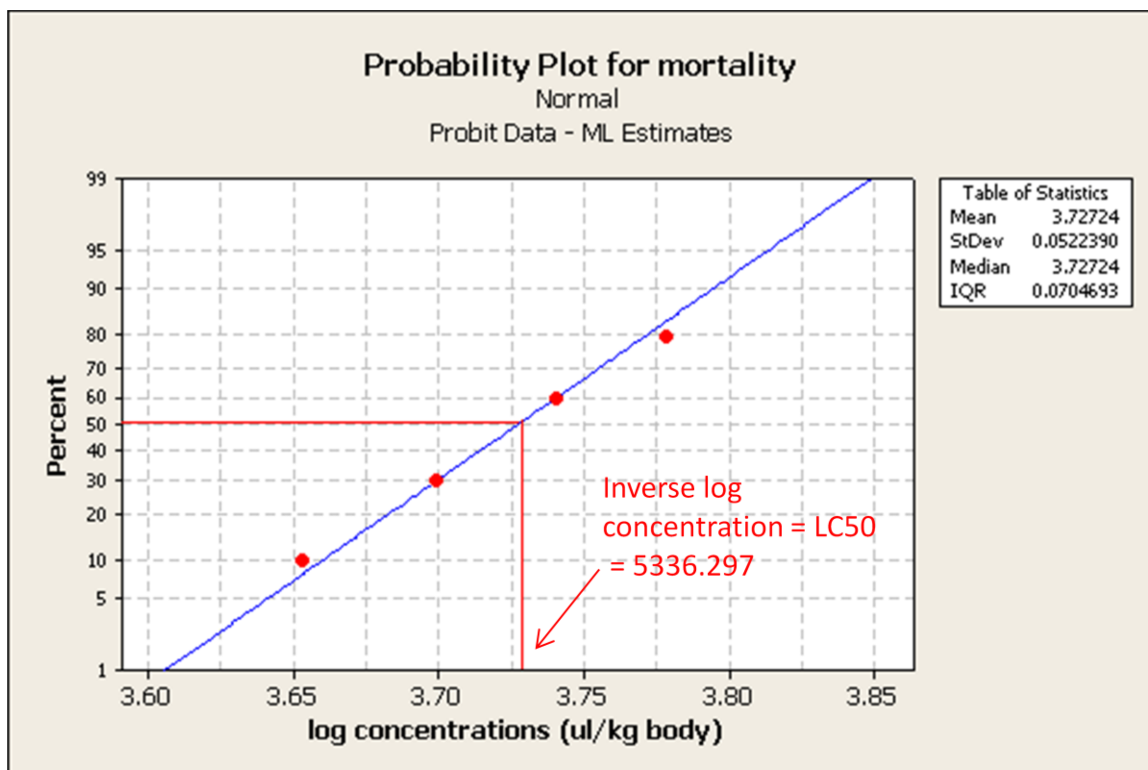


**Figure 3.4**: Probit transformed responses of mice treated with a different concentrations of *T. ammi* essential oils after oral administration

## CHAPTER IV: DISCUSSION, CONCLUSION AND RECOMMENDATIONS

### DISCUSSION

In this millennium infection from *Aspergillus* species become the major public health problem of modern mycology (Pitt and Hocking, 2009). They have a capability to cause directly infection and indirectly mycotoxicosis especially upon the consumption of food contaminated with *Aspergillus* species. Many chemical preservative have been used for the control of *Aspergillus* food contamination (Maier *et al*., 2010). The widespread use of chemical preservative has significant drawbacks including increased cost, handling hazards, concern about pesticide residues on food, and threat to human health and environment (Nollet and Rathore, 2010). Public awareness of these risks has increased interest in finding safer alternatives natural products to replace currently used synthetic chemical preservatives to control *Aspergillus* food contamination. One such alternative is the use of essential oils with antifungal and antiaflatoxigenic activity, since they tend to have low mammalian toxicity, less environmental effects and wide public acceptance (Razzaghi-Abyaneh *et al*., 2009a). *Cymbopogon martinii, Foeniculum vulgare*, *Lippia adoensis Hochst. var. koseret*, *Rosmarinus officinalis, Ruta chalepensis* and *Trachyspermum ammi* are common economic food spices in Ethiopia and thus, it is an advantage to develop safe botanical food preservative against toxigenic *Aspergillus* species that have strong affinity to colonize various food commodities due to its secretion of hydrolytic enzymes (Tadeg, 2004).

In this research we evaluated the essential oil yields of *C. martinii, F. vulgare*, *L. adoensis Hochst. var. koseret*, *R. officinalis, R. chalepensis* and *T. ammi* aromatic plants. All the aromatic plants have different yield of essential oils. Among these plants *T. ammi* gained increased interest as it yielded high amount of essential oil contents. Our finding of evaluations of essential oil yield of aromatic plants of *T. ammi* replicates the result of Javed and his colleagues on nutritional, phytochemical potential and pharmacological evaluation of *Nigella Sativa* and *Trachyspermum ammi* (Javed *et al*., 2012). It is an advantage; having high yield of essential oils in *T. ammi* to develop plant based natural preservative for the large scale control of food contamination by toxicogenic species of *Aspergillus*.

Our TLC analysis confirmed the presence of various components of essential oils which were characterized by the distance they travel in a particular TLC system and their appearance (color) after visualization of the spots. Essential oils are very complex natural mixtures which can contain about 20–60 components at quite different concentrations. They are characterized by two or three major components at fairly high concentrations (20–70%) compared to other components present in trace amounts (Bakkali *et al*., 2008). This chromatogram developed from essential oils with the distinctive spot $R_f$ and color were due to the presence of major component of essential oils i) Alcohols: borneol in *R. officinalis;* geraniol in *C. martini* and linalool in *L. adoensis Hochst. var. koseret*; ii) Phenols: thymol and carvacrol in *T. ammi*; iii) Aldehydes: anisaldchyde in *F. vulgare*; iv) Ketones: fenchone in *F. vulgare;* and undecanone and nonanone in *R. chalepensis*; v) Ethers: 1,8-cineole in *R. officinalis*; vi) Esters: geranyl acetate in *C. martinii*; and vii) Phenylpropanoids: anethole in *F. vulgare* essential oils (Wagner and Bladt, 1996). The presence of various constituent in the essential oils are relevant for clinical application as it is impossible for the organism to develop resistance to all components of the oils. Based on the efficacy of essential oil against *Aspergillus* spp GC analysis of one essential oil (*T. ammi*) was performed. And in our GC analysis we confirmed the presence of thymol (51.5%) and carvacrol (28.5%) as a major component. TLC and GC finger print is the most common chromatographic technique widely available for phytochemical analysis of plant essential oils. They are used in standardizing the constituents of essential oils that are classified as generally recognized as safe by FDA for their use as food additives in controlling food spoilage (Razzaghi-Abyaneh *et al*., 2009a).

Our results confirm that food commodities in Addis Ababa local market are contaminated by *Aspergillus* spp. The microscopy and culture results showed that virtually from all kind of food commodities collected for survey were colonized by *Aspergillus* spp. The samples of peanuts presented a higher number of *Aspergillus* spp, which is a result of not having been processed and stored in appropriate conditions. On the other hand, the “cookies and shiro”, presented a lower number of *Aspergillus* spp, due to the processing of the sample. Our finding of *Aspergillus* spp infection of food commodities replicated the finding of study

conducted in Cameron to see contamination of human food by storage fungi (Njobeh *et al*., 2009). The relative frequencies of *Aspergillus* spp may be higher because of their ability to grow at large range of temperature harvesting and storing conditions of food commodities and also their ability to colonize almost all food commodities. As surveyed in literature review *Aspergillus* spp colonization of food commodities may have occurred at any stage from flowering, harvest, storage, sorting or transport, and processing of the foods. This result actually confirmed that *Aspergillus* spp are really storage fungi. Contamination of commercially purchased food commodities is clearly a cause for concern in our current turbulent time.

Our study also suggests *Aspergillus* spp that infect food commodities in Addis Ababa significantly produce aflatoxin. The aflatoxin-producing *Aspergillus* spp that we were interested in this study were identified on the basis of all lines of evidence examined. However, the color of conidial heads may suffice for their differentiation. Our study confirmed that *Aspergillus flavus* were the most producer of aflatoxin at a concentration (amount) detectable (TLC) in vitro on culture medium as also reported by Legesse (2010). Only some *Aspergillus flavus* strains are genetically capable of producing aflatoxin (external factors play major role in the expression of the gene for aflatoxin production). In this study, out of the *Aspergillus* species isolated from food commodities *Aspergillus flavus* predominated, confirming the reputation of this mould as a ubiquitous spoilage mycotoxin producing organism, even though, *Aspergillus niger* and *Aspergillus fumigatus* were also been isolated. Even though, aflatoxin are the first mycotoxin known to human kind; our finding reminded us that there still a lot to be done in controlling its appearance from food commodities. *Aspergillus* spp that produce them are increasing because of favorable environmental condition, like drought-stress, humidity and insect infestation of the crop at various stages. Moreover, poor harvesting practices, improper storage, and less than optimal conditions during transport and marketing can also contribute to fungal growth and increase the risk of mycotoxin production (DeWaal and Robert, 2005).

Our antifungal screening results confirm that essential oils have fungitoxic potentials against the tested organisms. The essential oils of *C. martinii*, *F. vulgare*, *L. adoensis Hochst. var.*

*koseret*, and *T. ammi* showed higher inhibitory effects on the growth of aflatoxigenic *Aspergillus* spp at a concentration of 2µl/ml. The essential oil composition of plants varies significantly in different genera and species (Tian *et al*., 2011). A variation in antifungal activity of tested plant essential oils against aflatoxigenic strain of *Aspergillus* spp may be due to considerable variation in essential oils constituents. Geraniol have been reported as major components of *C. martinii* essential oil (Rajeswara *et al*., 2005). Trans-anethole has been reported as major components of *F. vulgare (*Aazza *et al.,* 2011*)*. Linalool has been reported as major components of *L. adoensis* Hochst. var. *koseret* (Workalemahu Mikre *et al*., 2007). Piperitone, 1,8-Cineole and a-Pinene have been reported as major components of *R. officinalis* (Moghtader *et al*., 2011, Zaouali *et al*., 2010). Undecanone and decanone have been reported as major components of *R. chalepensis* (Mejri *et al*., 2010). Our finding of antifungal activity of these essential oils support the finding of Bakkiali and his coworker (2008), on the biological effect of essential oils. Screening program using food poisoning technique helps us to reduce the wastage of resources and focus our effort on valuable experiment by providing valuable information. The minimum amount of essential oils to inhibit the fungal population was studied by our MIC to save and/or minimize the wastage of essential oils.

*Cymbopogon martinii* essential oil possesses antifungal activity on *Aspergillus* spp at the concentration tested in our spore germination assay. Our finding suggested all tested doses have significant effect in inhibition of spore germination. Complete fungal spore germination was inhibited at concentration of 1µl/ml for both *Aspergillus fumigatus* and *A. niger;* 2µl/ml *A. flavus*. Our finding that *C. martinii* essential oil have spore germination inhibition effect were in line with the finding of mycosporicidal activity of essential oils from selected herbals against isolates from HIV/AIDS patients by Sivamani & Hameed (2010).They have found the oils of *C.mertinii* effective at a concentration of 1:1000. The impacts of *C. martinii* essential oil on sporulation may reflect effects of the volatiles emitted by oils on surface mycelial development (and thus the 'platform' to support spore production) and/or the perception/ transduction of signals involved in the switch from vegetative to reproductive development (Tzortzakis and Economakis, 2007).

Our result also confirmed the fungitoxic potentials of *F. vulgare* essential oil. Germinations of the spores of all the tested organisms were completely inhibited at 4µl/ml for all strains of *A. flavus* and 2µl/ml for the remaining *Aspergillus* spp. Like other essential oils, sporicidal activity could be due to the interference with the amino acid involved in germination or denaturation of the enzymes responsible for spore germination(Nychas, 1995). Our result that indicated sporicidal potential of *F. vulgare* essential oil was in line with the finding from the research on antifungal effects of essential oils from oregano and fennel on Sclerotinia sclerotiorum (Soylu *et al*., 2007). The inhibition of spore germination by this oil could contribute for the treatment of food commodities to prevent the spread of *Aspergillus* spp.

Our results from spore germination assay of *L. adoensis Hochst. var. koseret* essential oil confirms that it have spore germination inhibition potential against *Aspergillus* spp. Complete inhibitions of spore germination of aflatoxicogenic *Aspergillus* spp were recorded at the concentration of 2µl/ml which could be a suggestive of the fungicidal action of the oil the specified concentration. Our finding that has shown *L. adoensis Hochst. var. koseret* oil have sporicidal activity against *Aspergillus* spp was in agreement with the reputation of this plants in the traditional practices by Oromo and Gurage people in Ethiopia as reported by previous researcher (Tadeg, 2004). The vapor action exerted by volatile constituents of this essential oil on surface mycelial development (and thus the 'platform' to support spore production) and/or the transduction of signals involved in the switch from vegetative to reproductive development could be responsible for the spore germination inhibition activity. Moreover, essential oils sporicidal activity could be due to the interference with the amino acid involved in germination or denaturation of the enzymes responsible for spore germination(Nychas, 1995).

Another important finding of our research was that essential oils from *T. ammi* have a remarkable sporicidal activity against toxigenic strains of organisms tested. Hundred percent inhibitions of spore germination of *A. fumigatus* at 0.5µl/ml, *A. niger* at 1µl/ml and *A. flavus*

at 2µl/ml was recorded. Our finding that indicatedd spore germination inhibition potentials of *T. ammi* essential oil replicates the findings of antimicrobial activity of *T.ammi* essential oils in influencing membrane permeability and surface characteristics in inhibiting food-borne pathogen (Paul *et al*., 2011). The result could be in the form denaturation of the enzymes responsible for spore germination or interference with the aminoacid involved in germination (Nychas, 1995). The vapor action exerted by volatile constituents of this essential oil on surface mycelial development and/or the transduction of signals involved in the switch from vegetative to reproductive development could also be responsible for the spore germination inhibition activity.

*C. martinii* essential oils had a clear dose-dependent antifungal activity on *Aspergillus* spp at the concentration tested in our agar dilution assay to determine MIC. Our finding suggests the increment of dose to 1, 2 and 4µl/ml have a significant effect on the inhibition of fungal in reduction of fungal growth while all tested doses have significant effect in inhibition of spore germination. Complete inhibition of fungal growth was seen at a dose of 1, 2 and 4µl/ml for *A. fumigatus, A. niger* and *A. flavus* respectively. Certainly, differences in major and minor constituents of the oils that are responsible for their biological activity by geographical location and seasons of collection of the plants could contribute to the difference in MIC of *C. martinii* essential oils between the present study and that of Bansod and Rai (2008). Finding the minimum amount (MIC) of oils that inhibit fungal population is clinically important for saving and or minimizing the wastage of essential oils.

Our result also confirmed that *F. vulgare* essential oils have fungitoxic potentials against test aflatoxigenic strains of *Aspergillus* spp. It has shown absolute inhibition at MIC value of 8µl/ml for the strains of both *A. niger* and *A. flavus* and at 4µl/ml for *A. fumigatus*. The activity could be as a result of damages on the enzymes responsible for the energy production and synthesis of structural compounds. Our finding that *F. vulgare* essential oils have fungitoxic potential replicate the finding of other researches on the phenolic major component (anethole) which are responsible for the antifungal activity of the oils (Marei *et al*., 2012, Singh *et al*., 2006). Considering activity from this plant is promising result for the applicability of the finding as the plants are easily cultivable.

From other plants our result confirmed the fungitoxic potentials on the growth *Aspergillus* spp was shown by essential oils of *L. adoensis Hochst. var. koseret*. A complete inhibition of growth were recorded at a concentration of 2µl/ml against all aflatoxigenic organism tested. This finding of efficacy of this plant against storage organism were supported the report of traditional use of the plants by Oromo and Gurage people in Ethiopia by Tadeg (2004). Even though, there was no previous studies conducted reporting the antifungal activity of this essential oil, there exists the antifungal activity of the major constituents' linalool from other plants. It may be responsible for the fantastic fungitoxic activity of the plant. Moreover, the vapor action exerted by volatile constituents of this essential oil may support the fungitoxic activity. As the traditional use of this plant is most common in Ethiopia, having growth inhibitory effect at small amount was promising finding that will avoid the risk of residual effect of the oil on health and environment upon use as botanical preservative.

Another notable finding of our research was that essential oils from *T. ammi* have outstanding antifungal activity against toxigenic strains of organisms tested. Complete inhibition of *Aspergillus* species growth was recorded at 0.5µl/ml for *A. fumigatus*; at 1µl/ml for *A. niger* and 2µl/ml for *A. flavus*. Complex volatile components seen in our phytochemical study could be responsible for the activity of this essential oil. Certainly, differences in major and minor constituents of the oils that are responsible for their biological activity and the difference in test organisms used in the study could contributes to the difference in MIC of *T. ammi* essential oils between the present study and that of Javed and his colleagues (2012). The *A. flavus* and *A. niger* in our effect of essential oils on *Aspergillus* spore germination, growth and mycotoxin production was similar with that of Javed and his colleagues but different technique for the determination of MIC was used. Moreover, antifungal activity of its major component (thymol and charvacrol) was reported by Kordali and his coworker (2008), which could also be responsible for the variation as there may exists variation in the percentage yield variation of these major components. Volatile constituent of the essential oils create vapor action that could be the responsible for

the activity. The finding of our MIC at lowest value has public importance at it reduce the loss of essential oil for the practical application.

Interesting results of our study was recorded for antifungal activity of sodium benzoate chemical preservative. The growth of the organisms tested was completely inhibited at a concentration of 8mg/ml for *A. fumigatus*, 16mg/ml for *A. niger* and above 16mg/ml for *A. flavus*. The fungitoxic activity of the chemical preservative was dose dependent as the significant inhibition was observed at higher concentration. The other may be due to hurdle conditions that sodium benzoate may have created, which the organisms could not overcome. This may causes physiological, homeostatic and metabolic distortion in the organisms. Moreover, further attempts to overcome these hurdle micro environments had led to increased stress which in turn brought about metabolic exhaustion, death and subsequent decrease in population observed. MIC of sodium benzoate was higher than previously reported studies on spoilage fungi (Lopez-Malo *et al*., 2005). Different explanations may be applied for the reductions of the fungitoxic potentials of sodium benzoate. First, sodium benzoate was highly active at acidic (pH3.5) environment but we conducted at neutral pH. Secondly, the organisms may develop resistance to these chemical preservatives (Brul and Coote, 1999, Stanojevic *et al*., 2009).

In our study we tried to compare the preservative potentials of plant essential oils with the prevalent synthetic preservative by using their MIC value. For easy of comparison we took the weight of each essential oils For easy of comparison we took the weight of 1µl of each essential oil and found to be 1.10mg, 1.04mg, 1.01mg and 1.09mg for *C. martinii, F. vulgare*, *L. adoensis Hochst. var. koseret*, *s* and *T. ammi*, respectively. The four essential oils were more active than sodium benzoate. These results that indicated essential oils have better fungitoxic activity than synthetic preservative are in accordance with the finding of previous study (Bakkali *et al*., 2008). The authors of have showed highest activity of sodium benzoate at pH 3.5. This may be due to mode of action of the chemicals, i.e. salt of sodium and benzoic acid. Weak acid compounds are more lipophilic in their non dissociated form which enables them to cross the cell membrane that led to pH lowering of cytoplamic cell

with rupture of certain metabolic reactions of the microorganism, permeabilization of the cytoplasmic membrane and cell death. Other authors' demonstrated benzoic acid has membrane-perturbing potentials. In addition, these acids induce loss of mitochondrial function, and one possibility that we entertained was that this could be the result of mitochondrial autophagy (Hazan *et al*., 2004).

Moreover, essential oils are complex mixtures of numerous molecules, and one might wonder if their biological effects are the result of a synergism of all molecules or reflect only those of the main molecules present at the highest levels. In the literature in most cases, only the main constituents of certain essential oils like thymol, carvacrol, carvone, geraniol, linalool, trans-anethole, were analyzed (Bakkali *et al*., 2008). Thus, synergistic functions of the various molecules contained in an essential oil, in comparison to the action of one or two main components of the oil, seems questionable. However, it is possible that the activity of the main components is modulated by other minor molecules (Bakkali *et al*., 2008). Moreover, it is likely that several components of the essential oils play a role in cell penetration, lipophilic or hydrophilic attraction and fixation on cell walls and membranes, and cellular distribution (Bakkali *et al*., 2008).

Moreover, our study also suggested that *T. ammi* essential oil have pronounced activity in reducing mycelial biomass. It can be clearly seen that the effect are dose dependent, as up on the increment of the concentration of the oils the mycelia dry weights of the tested *Aspergillus* spp were recorded. As shown in our result a hundred percent mycelia growth was inhibited at concentration similar to the 1µl/ml. The reduction in fungal mycelia biomass may be due to the presence of phenolic compounds in the essential oils. Our study that showed this essential oils have the ability to reduce mycelial dry weight is in line study of Ahmed and his collogues (2010). It was documented these author that at low dose, phenols affected enzyme activity, especially of those enzymes associated with energy production, while at greater concentrations, caused protein denaturation as reported by Ahmed and his collogues (2010). In addition, lypophilic nature of the essential oils helps to cross cell membrane of the fungal cell interacting with the enzymes and proteins of the

membrane, so producing a flux of protons towards the cell exterior which induces changes in the cells and, ultimately leading to death. Determining the activity of this essential oil against the mycelial dry weight will have public importance by minimizing the wastage of food commodities by the presence of visible mycelial growth.

Another crucial aim of this study was confirmed by our result that have shown us the aflatoxin arresting potentials of essential oils extracted from *T. ammi* against aflatoxicogenic *Aspergillus* spp tested in our study. Aflatoxin can be produced by *Aspergillus* spp but the fungus may no longer be present in the food, hence preservative used for the control of aflatoxin should act on both fungus and the mycotoxin they produce. Interestingly, the productions of aflatoxin were inhibited by *T. ammi* essential oil at concentrations lower than recorded for MIC, spore germination inhibition and mycelial dry weight. Thus, the inhibition of aflatoxin production cannot be completely attributed to reduced fungal growth, but may be because of inhibition of carbohydrate catabolism in *Aspergillus* spp by acting on some key enzymes, reducing its ability to produce aflatoxins. Our finding that *T. ammi* has aflatoxin arresting potential replicate the finding of Hajare and his collaborators on the aqueous extract of *T. ammi* seed have aflatoxin inactivation potential (2005). Once again as mycelial growth and spore germination inhibition; aflatoxin production inhibition could be due to the presence of thymol and carvacrol (phenolic OH group) that form hydrogen bonds with target enzyme active site (Tian *et al*., 2011). As aflatoxin is the most potent carcinogenic and teratogenic chemical, the finding of our result indicated that its production was terminated by oil can have a major public importance. Currently the contaminations of food commodities by toxigenic species of storage fungi are common. Our public should be aware of the risk of consuming aflatoxin with food and the value of using this essential oil as remedial for the risk.

Our result of safety limit on mice shows that *T. ammi* is essential unlikely to present acute toxicity supporting the consumption of this plant seed as spice in Ethiopia. All preservatives used against food spoilage moulds should not be harmful for human beings upon consumption. The safety limit of the *T. ammi* essential oil was also determined through its

oral administration (acute oral toxicity) on mice and its $LC_{50}$ value was found to be 5336.297µl/kg body weight and classified using WHO recommended classification in the U group (WHO, 2009). The high value of $LC_{50}$ is a symbol of the non-mammalian toxicity of the *T. ammi* essential oil. And hence it may be recommended as a safe preservative of foods; as 5,000.00µl of the test substance/kg body weight is the practical upper limit for the amount of test material that can be administered in one oral gavages dose to a rodent. Now a day's using essential oils as food additives is common throughout the world (Razzaghi-Abyaneh *et al*., 2009b).

## CONCLUSION

Our study conducted to evaluate the presence of toxigenic strains of *Aspergillus* spp in food commodities in Addis Ababa, has indicated the existence of these strains. Moreover, our study was also conducted to control these toxigenic organisms by medicinal plants that have folkloric reputation in our community. In this part we found essential oils of *C. martini*, *F. vulgare*, *L. adoensis Hochst. var. koseret* and *T. ammi* have fungitoxic potential. Moreover, essential oils of *T. ammi* have antiaflatoxigenic potentials and no mammalian toxicity on mice. Therefore, essential oils of *T. ammi* could be the best safe botanical alternative of food preservative.

There are many opportunities of the use of *T. ammi* essential oil as food preservative; one is the antifungal as well as antiaflatoxigenic activity, broad spectrum fungitoxicity, superiority over synthetic fungicides and non-mammalian toxicity. The other important opportunity is the practical applicability of this essential oil as fumigant of food commodities due to their aromatic volatility nature. Moreover, we can also minimize the residual effect of this plant in food commodities by drying food staffs using sun light before consumption. As a result of these finding and opportunities we suggest *T. ammi* essential oil as a potential source of safe botanical food preservative that inhibit *Aspergillus* spore germination, growth and mycotoxin production inhibition.

## RECOMMENDATIONS

1. *Aspergillus* and their mycotoxin are the growing threat to the health around the world, Ethiopia is not exceptional. Hence tests to detect and diagnose them need to be adopted on wide scale in the country.
2. Because of volatile nature, easy applicability, pronounced antifungal and antiaflatoxigenic effect with no mammalian toxicity we recommend *Trachyspermum ammi* essential oil as food preservative of food commodities.
3. Further study should be conducted to explore large scale utilization of *T ammi* essential oil as food preservative.
   - 3.1. Exploring the efficacy of *T. ammi* essential oils using other toxigenic organism that contaminate food commodities.
   - 3.2. Needs for tests using quantitative method to evaluate the in vitro aflatoxin arresting potential and in the field condition to see aflatoxin arresting potential of the essential oils.
   - 3.3. Study on other toxin producer strains of storage microorganism (other than aflatoxin).
   - 3.4. Mechanism of action and chronic toxicity studies to determine its safety on term usage

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## Annex I. Macroscopic and Microscopic Identification Key of Aspergillus spp.

### 1. Isolation using Macroscopic Features

**Principle**

Variations in growth rate, color of the colony, and thermotolerance can be used in identification of Aspergillus spp. *Aspergillus* colonies are downy to powdery in texture. The surface color may vary depending on the species. The reverse is uncolored to pale yellow in most of the isolates. *Aspergillus fumigatus* is a thermotolerant fungus and grows well at temperatures over 40°C. This property is unique to *Aspergillus fumigatus* among the *Aspergillus* species. *Aspergillus fumigatus* can grow at a temperature range of 20 to 50°C. *Aspergillus flavus* can be readily distinguished from other *Aspergillus* species, by lack of growth at 5 °C, by rapid growth at both 25 and 37°C, and by the production of a bright yellow-green conidial color.  Some species of specific colors are given the table below.

**Equipment & materials**

PDA

Petri dish

Pipette filler

Micropipette

Vortex mixer

Tissue paper

Processed and unprocessed sample

**Procedure**

| Step | Action |
|---|---|
| 1 | Inoculate the sample in to the PDA medium. |
| 2 | wrap the Petri dish by paraffin film |
| 3 | Incubate the plate's at5, 25, 37 and 45$^{o}$C for 10 days. |
| 4 | Isolate the *Aspergillus* spp based on macroscopic features. |
| 5 | Perform microscopic feature to support the identification |

**Interpretation**

| SPECIES | SURFACE | REVERSE |
|---|---|---|
| *A. clavatus* | Blue-green | White, brownish with age |
| *A. flavus* | Yellow-green | Goldish to red brown |
| *A. fumigatus* | Blue-green to gray | White to tan |
| *A. glaucus* group | Green with yellow areas | Yellowish to brown |
| *A. nidulans* | Green, buff to yellow | Purplish red to olive |
| *A. niger* | Black | White to yellow |
| *A. terreus* | Cinnamon to brown | White to brown |
| *A. versicolor* | White at the beginning, turns to yellow, tan, pale green or pink | White to yellow or purplish red |

**2. Isolation using Microscopic feauture**

**Principle**

The basic microscopic morphology is same for all species. However, some other microscopic structures are unique to certain species and constitute the key features for species identification together with the color of the colony

**Equipment & materials**

Transparent Scotch tape

Slides

Lactophenol cotton blue

Forceps

Tissue paper

*Aspergillus* colony on agar plate

Microscope

**Procedure**

| Step | Action |
|---|---|
| 1. | Place a drop of lactophenol cotton blue on a microscope slide. |
| 2. | Cut a piece of Scotch tape and hold it between the thumb and forefinger of each hand with the sticky side down. Forceps can be used for this operation. |
| 3. | Press the centre of the sticky side firmly onto the surface of the mould colony and pull it gently away from the colony.<br>The aerial hyphae of the colony will remain glued onto the tape surface. |
| 4. | Place the tape sticky side down on the drop of lactophenol cotton blue previously placed at the centre of the slide. |
| 5. | Ends of the Scotch tape will extend beyond the end of the slide. These are folded over the ends of the slide. |
| 6. | Remove the excess of lactophenol cotton blue with tissue paper if needed. |
| 7. | Examine microscopically the whole preparation under X10, X40, and X100 if required. |

**Interpretation**

| SPECIES | CONIDIOPHORE | PHIALIDES | VESICLE |
|---|---|---|---|
| *A. clavatus* | Long, smooth | Uniseriate | Huge, clavate-shaped |
| *A. flavus* | Colorless, rough | Uni-/biseriate | Round, radiate head |
| *A. fumigatus* | Short (<300 µm), smooth, colorless or greenish | Uniseriate | Round, columnar head |
| *A. glaucus* group | Variable length, smooth, colorless | Uniseriate | Round, radiate to very loosely columnar head |
| *A. nidulans* | Short (<250 µm), smooth, brown | Biseriate, short | Round, columnar head |
| *A. niger* | Long, smooth, colorless or brown | Biseriate | Round, radiate head |
| *A. terreus* | Short (<250 µm), smooth, colorless | Biseriate | Round, compactly columnar head |
| *A. versicolor* | Long, smooth, colorless | Biseriate | Round, loosely radiate head |

**Annex II. Laboratory Data sheet**

Results from microscopy and culture

Sample number:_____

1. Microscopic features of *Aspergillus species* stained by lacto-phenol cotton blue:
   :____________________________________________________________

   :____________________________________________________________

   :____________________________________________________________

2. The color of the colony in *Aspergillus* species on Sabouraud Dextrose Agar:

   :____________________________________________________________

   :____________________________________________________________

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Comments______________________________________________________

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Name of principal investigator ______________________________________________

Signature _________________ Date _______________

## Annex III. Media, Chemicals and Solvents

### Medias

Peptone Dextrose Agar (BNo. ZG236, HiMedia Laboratories Pvt. Ltd., India), Sabouraud Dextrose Agar (BNo. 697074, Oxoid Ltd., England), Sabouraud Dextrose Broth (BNo. 0382-01, Defco., Ltd., England), Sucrose (Lot No. 0209/10240/03, Labort fine Chem Pvt. Ltd, India), Yeast Extract (Lot No. 0209/10246/03, Labort fine Chem Pvt. Ltd, India)

### Chemical

Aflatoxin Mix Kit-M (No. 46304-U, Supelco), anhydrous Sodium sulphate (Lot No. 7462785, Merck-Schuchardt, Germany), MgSO4.7$H_2O$ (Merck-Schuchardt, Germany), Potassium hydroxide (Lot No. 3239, Merck-Schuchardt, Germany), Potasium nitrate (Lot No. 66160, Rhone Poulenc, US), Sodium benzoate (Lot No. 366757, Codex® Farmacopia, Italy), Thymol (Lot No. 36H360, Sigma-Aldrich Chemie, Germany), Vanillin (Lot No. 3309160, Merck-Schuchardt, Germany), Silicagel60 Thin Layer Chromatography plate 0.2mm (Merck, Germany)

### Solvent

Acetone (Lot No. 2208/10219/031L, Labort Fine Chem PVT. Ltd, India), Chloroform (BNo. 024071, Sigma-Aldrich Chemie, Germany), Ethanol absolute (BNo. A5692, Finkem, India), Ethyl acetate (Sigma-Aldrich Chemie, Germany), Methanol (BNo. A7156, Finkem, India), Sulphuric acid (Lot No. 30890100, Sigma-Aldrich Chemie GMbH, Germany), Tolune (Lot No. 363K19097750, AnalaR®, England), Tween20 (Lot No. 289160, Sigma-Aldrich Chemie GMbH, Germany), Tween80 (Lot No. TS038309TS, Sigma-Aldrich Chemie, Germany)

**Declaration**

I, under signed, declare that this M.Sc. thesis is my original work, has not been presented for a degree in other university and that all sources of materials used for the thesis have been duly acknowledged.

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