ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
FOOD SCIENCE AND NUTRITION PROGRAM

GROWTH INHIBITION OF GRAIN SPOILAGE FUNGI BY SOME HERB AND SPICE ESSENTIAL OILS GROWN IN ETHIOPIA

By

Diriba Chewaka Tura

A Thesis Submitted to Graduate Studies Program, Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Food science and Nutrition

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LIST OF ABBREVIATIONS

AFB	Aflatoxin B
ANOVA	Analysis Of Variance
ATCC	American Type Culture Collection
EHNRI	Ethiopian Health and Nutrition Research Institute
EIAR	Ethiopian Institute of Agricultural Research
FDA	Food and Drug Administration
GRAS	Generally Regarded as Safe
HACCP	Hazard Analysis and Critical Control Points
MIC	Minimum Inhibitory Concentration
NCCLS	National Committee for Clinical Laboratory Standard
OD	Optical Density
PDA	Potato Dextrose Agar
RPM	Revolutions Per Minute
SAD	Sabroud Dextrose Agar
SD	Standard Deviation
SPSS	Statistical Products and Service Solutions

ABSTRACT

Microbial food contamination is an on-going limiting factor in crop production that can determine the shelf life of processed and unprocessed foods. Spice plants and herbs are commonly used as food flavoring and seasoning agents. Their antimicrobial properties as food preservatives are also well documented. In this study, essential oils of seven spice plants were tested for their antimicrobial properties against *Aspergillus flavus* and *A. niger*, two of the most important food and feed spoilage organisms. Agar disk diffusion assay was used for screening of the most effective essential oils, agar dilution assay was used to determine Minimum Inhibitory Concentration (MIC) of the essential oils and broth dilution assay was employed to the spore germination inhibition assay. Tests were also conducted to examine the effects of the essential oils for sorghum kernel protection against the tested fungi, and the optimal protective dosages on the sorghum grains were also determined. From the preliminary tests, essential oils of *Cinnamomum zeylanicum* (Cinnamon) and *Thymus schimperi* (thymus) were found to be the most effective. However *piper nigrum* (black pepper) had no effect on the test organisms. In MIC, spore germination inhibition and grain protection assay, cinnamon essential oil was found to be superior where its MIC on the isolates was found to be 0.0156% and its optimum protective dosage on the grain was 5%. It inhibited spore germination at a concentration of 3 µL/ml. The effect of thymus oil was also very much comparable to these results (no significant difference at $P < 0.05$).

Finally, it could be concluded that some plant essential oils can be a useful source of antifungal agents for protection of grain spoilage by fungi.

1 INTRODUCTION

1.1 Background

1.1.1 Microbial food contamination

Microbial food contamination is an on-going limiting factor in crop production that can determine the shelf life of processed and unprocessed foods (Yulia, 2005). Substantial yield losses occur due to fungi, bacteria and virus contamination and are of increasing importance as to food production needed for the fast expansion of world population (Kordali *et al*., 2008).

Fresh produce may become contaminated at any point along the farm-to-table continuum. The activity of the microbes can cause undesirable effects in grains including discoloration, contribute to heating and losses in dry matter through the utilization of carbohydrates as energy sources, degrade lipids and proteins or alter their digestibility, produce volatile metabolites giving off-odours, cause loss of germination, baking and malting quality and affect use as animal feed and food or as seed (Velluti *et al*., 2004; Magan and Aldred, 2007).

Contamination of various foodstuffs and agricultural commodities is a major problem in the tropics and sub-tropics, where climatic conditions and agricultural and storage practices are conducive to fungal growth and toxin production (Kumar *et al*., 2008). Thus fungal contamination of grains can occur in the field or in store with the extent of contamination largely determining the rate of deterioration of stored grains (Samapundo *et al*., 2007; Amiri *et al*., 2008; Rasoolia *et al*., 2008). Those grains stored for a long time before consumption or

distribution are more prone to contamination with mycotoxins, though formation of toxins could also start before produce is harvested and put in storage (Samapundo *et al*., 2007).

The most important species of field fungi are from the genera *Alternaria*, *Cladosporium*, *Fusarium* and *Drechsler*a (Amare, 2002). *Fusaria* and *Aspergilli* are the most commonly isolated contaminants of corn worldwide, with the most important species being *F. verticillioides*, *F. proliferatum*, *A. flavus* and *A. paraciticus* (Velluti *et al.,* 2004; Samapundo *et al*., 2007; Amiri *et al*., 2008).

Estimated losses of grain, especially staple food grains in store, caused from insects and pathogens vary widely. They may amount to 10% worldwide but can reach 50% in tropical regions. For example an estimated loss of high moisture rice in southern India was reported to be about 15–25% in only 9 days (Velluti *et al*., 2004; Zhang *et al*., 2009). Agricultural commodities such as peanuts, corn, maize and others are the highly affected crops by fungi and ultimately end up unfit for consumption on long time storage (Rasoolia *et al*., 2008).

Contamination of cereal commodities by moulds and mycotoxins results in dry matter, quality, and nutritional losses and represents a significant hazard to the food chain (Paster *et al*., 1994; Nguefack *et al*., 2004). Their effect fall more heavily on some countries than others (Calvin, 2007). They pose major problem especially in developing countries (Nguefack *et al*., 2004; Kaefer *et al*., 2008). These are commodities associated with such countries where food supplies are frequently limited and malnutrition, especially protein malnutrition, is prevalent (Towers, 1979). They influence nutrition in two ways; quantitatively by their action as food spoilage

organisms and qualitatively by ways of their toxins. For example in developing countries post harvest economic losses reach 25–40% or even more by fungal contamination and the mycotoxins produced by them (Kumar *et al*., 2008; Ngoko *et al*., 2008; Rasoolia *et al*., 2008).

Fungi can be extremely destructive for several reasons :1) they sporulate prolifically; 2) the infection cycle (i.e., the time between infection and the production of further infectious propagules – usually spores) may be only few days; 3) the fungi can rapidly mutate to develop resistance to fungicides ; 4) the spores themselves may be carried long distance by the wind ; 5) the fungus may produce compounds that are highly phytotoxic, drawing nutrients away from the economically valuable agricultural commodities such as grains, and thus depress yields (Yulia, 2005).

Recently, scientists have focused on the increase of food needed for the fast expansion of world population. Unfortunately, substantial yield losses occur due to insects and crop diseases caused by fungi, bacteria and viruses (Kordali *et al*., 2008). Once fresh produce has been contaminated, removing or killing the microbial pathogens is very difficult. Hence numerous food products require protection against microbial contamination during their shelf life (Goni *et al*., 2009).

1.1.2 Mycotoxin producing fungi

Besides the possible food decaying caused by moulds and ultimate changes in its nutritional and organoleptical characters, the moldiness in foodstuffs is toxicologically significant since the

mould species growing on such products is known as potentially mycotoxicogenic (Carmo *et al.*, 2008).

The major mycotoxin-producing fungi are species of *Aspergillus*, *Fusarium* and *Penicillium* and the important mycotoxins are aflatoxins, fumonisins, ochratoxins, cyclopiazonic acid, deoxynivalenol/nivalenol, patulin and zearalenone (Omidbeygi *et al.*, 2007; Bluma and Etcheverry, 2008). Studies on the ecology of these species has resulted in modeling of germination, growth and mycotoxin minima and prediction of fungal contamination levels which may lead to mycotoxin contamination above the tolerable legislative limits (Magan and Aldred, 2007).

Mycotoxins are secondary metabolites produced by specific filamentous fungi that contaminate agricultural commodities during storage (Nielsen and Rios**,** 2000; Nguefack *et al.,* 2004; Atanda *et al*., 2007; Carmo *et al*., 2008; Kumar *et al*., 2008; Rasoolia *et al*., 2008). The primary metabolites of fungi as well as for other organisms are those compounds that are essential for growth. Secondary metabolites are formed during the end of the exponential growth phase and have no apparent significance to the producing organism relative to growth or metabolism. In general, it appears that they are formed when large pools of primary metabolic precursors such as amino acids, acetate, pyruvate, and so on, accumulate. The synthesis of mycotoxins represents one way the fungus has of reducing the pool of metabolic precursors that it no longer requires for metabolism (Jay, 2000).

Particular mycotoxins are produced by one or a limited number of fungi species, and not all isolates of a given toxigenic species can produce its specific toxin. Hence only *Aspergillus*

flavus, A.paraciticus and A.nomius produce afltoxin and the ability to produce afltoxins is most consistent in the latter two species (Amare, 2002).

These metabolites have been detected in several food commodities. Levels of mycotoxins and mycotoxicogenic moulds, which can cause risk to population, are refused by consumers and many countries have set regulations in various agricultural foods (Carmo *et al*., 2008).

Toxin-producing fungi may invade at pre-harvesting period, harvest-time, during post-harvest handling and in storage. According to the site where fungi infest grains, toxinogenic fungi can be divided into three groups: (a) field fungi; (b) storage fungi; and (c) advanced deterioration fungi. Given the proper humidity and temperature afltoxigenic fungi are capable of growing and producing afltoxins on almost any organic substances (Amare, 2002).

1.1.3 Health impact of mycotoxin contaminated food

Contamination of food and feed is an important but unrecognized risk to public health and can have long-term health implications (Ngoko *et al*., 2008). It has been estimated that as many as 30% of people in industrialized countries suffer from a food-borne disease each year, and at least two million people died from diarrheal diseases worldwide in 2000 (Gutierreza *et al*., 2008).

Crop production worldwide has a close relationship with the demand. Therefore mycotixin contaminated crops are eventually always consumed either by human beings or by animals. Countries without specific legislation and low enforcement are most likely to end up using them

(Juglal *et al*., 2002). Several of these mycotoxins in agricultural products cause health hazards to people and animals (Kumar *et al*., 2008). It is reported that consumption of mycotoxin-contaminated foods has been associated with several cases of human poisoning, or mycotoxicosis, sometimes resulting in death (Rasoolia *et al*., 2008). The most important groups of mycotoxins that occur quite often in food are: aflatoxins, ochratoxins, trichothecenes (deoxynivalenol, nivalenol), zearalenone and fumonisins. The food-borne mycotoxins likely to be of greatest significance for human health in tropical developing countries are the aflatoxins and fumonisins (Omidbeygi *et al*., 2007; Kumar *et al.,* 2008).

Among the mycotoxins, aflatoxins chiefly produced by strains of *Aspergillus flavus* are the most dangerous and about 4.5 billion people in underdeveloped countries are exposed to aflatoxicoses (Amare, 2002; Kumar *et al*., 2008). They are clearly the most widely studied of all mycotoxins. Knowledge of their existence dates from 1960, when more than 100,000 turkey poults died in England after eating peanut meal imported from Africa and South America. From the poisonous feed were isolated *Aspergillus flavus,* and a toxin produced by this organism that was designated aflatoxin *(Aspergillus flavus* toxin A-fla-toxin (Jay, 2002). It carries the most potent carcinogenic activity as a natural product. It also carries acute toxicity to various human cells such as hepatocytes, renal cells, lung epithelioid cells, cause liver damage to humans and to most experimental animals (Chalfoun *et al*., 2003; Omidbeygi *et al*., 2007; Rasooli *et al.,* 2008; Nguefacka *et al*., 2009; Tatsadjieu *et al*., 2009) etc., as well as various immunosuppressive activities (Kamei and Watanabe, 2005).

The most important aflatoxin are aflatoxins B1, B2, G1, and G2 (M1) out of which aflatoxin B_1 (AFB_1) is a highly toxic and carcinogenic metabolite produced by *Aspergillus* species on food and agricultural commodities such as: cereals, dried fruits, milk, coffee and fruit juice (Chalfoun *et al*., 2003; Bluma and Etcheverry, 2008; Carmo *et al*., 2008; Rasooli *et al*., 2008).

Generally, the presence of toxigenic fungi and mycotoxins in foods and grains stored for long periods of time presents a potential hazard to human and animal health (Omidbeygi *et al*., 2007). Therefore, the contamination of foods and feeds by mycotoxins should be minimized by designing a series of measures of prevention and control.

Spice and herbs such as *Cinnamomum zeylanicum* (Cinnamon), *Thymus schimperi* (Thymus), *Zingiber officinalis (*Ginger), *Allium sativum* (Garlic), *Laggera tomentosa* (Keskese), *Piper nigrum* (Black pepper) and *Citrus limon* (Lemon) are essential oil plants commonly grown in various parts of Ethiopia. At the same time they are extensively used by local people as food preservatives, cure for various ailments and food flavoring and seasonings (Fullas, 2003).

However, the antifungal activity of this plant extracts has not been widely investigated and a little quantitative data on the antimicrobial activities of most plant extracts is reported. Therefore, in order to fill this gap of information, essential oils of some traditional spices and herbs are to be investigated for their potential in the control of mold growth in culture medium and also for their ability to cause fungal inhibition in sorghum grains.

1.2 REASEARCH OBJECTIVE

1.2.1 General objective

- To evaluate the antifungal activity of some essential oils from common spices and herbs of Ethiopia.

1.2.2 Specific objectives

- To evaluate the effectiveness of the selected essential oil extracts in inhibiting the growth of *Aspergillas flavus* and *A. niger*.
- To determine the MIC (minimum inhibitory concentration) of the essential oil extracts.
- To confirm the effectiveness of the essential oil extracts causing fungal inhibition on sorghum grains (In-vivo test).

2 LITERATURE REVIEW

2.1 Introduction

Various plants have an important historical tradition in healing or are particularly valued for their medicinal, savory and aromatic quality. They are considered as medicinal plants if they are collected only for their medicinal or aromatic properties (Yulia, 2005). Essential oils are aromatic, volatile extracts from components of such plants (leaves, flowers, fruits, bark, roots, rhizomes, and wood) that are usually obtained by technologically simple processes of maceration and water solution, steam or hydro distillation (Nguefack *et al*., 2004).

The extracts are used as flovours, fragnaces and for medicinal purposes or health care purposes. Nevertheless, the term volatile is preferred because it refers to the fact that most component of the oils, which are stored in extracellular space in the epidermis or mesophyll, have low boiling points and can be recovered from the plant tissues by steam or hydro-distillation (Yulia, 2005).

Modern research has shown that medicinal plants act through a relatively small number of constituents called active principles. In a number of cases, tannins have a more extensive action than the isolated active principles.

The general categories of plant products are as follows: 1) the lipids including the simple and functionalized hydrocarbons, as well as terpenes; 2) aromatic compounds, including phenols;

3) carbohydrates; 4) amines, amino acids and proteins; 5) alkaloids; and 6) nucleosides, nucleotides and nucleic acids (Yulia, 2005).

There is no reason to doubt that evidence for the antifungal and antimicrobial activity of some volatile oils components in vitro exhibits the same antibiotic activity in nature. The extent of recent interest in antimicrobial activity is shown by the wide range of organisms which have been tested against volatile oils, including filaments fungi, yeasts and plant viruses (Lopes-Lutz *et al*., 2008). The major constituents of many of these oils are phenolic compounds (terpenoids and phenylpropanoids) like thymol, carvacrol or eugenol, of which antimicrobial and antioxidant activities are well documented (Nielsen and Rios, 2000).

In general, terpenoid molecules predominate but many oils contain components originating from other biosynthetic pathways. The terpene components of oils are particularly significant. Volatile oils used for culinary, pharmaceutical and perfumery purposes are composed almost entirely of two classes of compounds, terpenes and phenylpropenes. Of these, the terpenes are by far the more abundant but, where they occur, phenylpropenes are usually the major flavor or odor factors (e.g., anise, fennel, clove cinnamon and basil (Yulia, 2005).

2.2 Plant extracts and essential oils

Essential oils are aromatic and volatile oily liquids obtained from plant material. They are normally formed in special cells or groups of cells, found in leaves and stems, and commonly concentrated in one particular region such as leaves, bark, fruit flowers, seeds, twigs, herbs wood fruits or roots (Gutierrez *et al*., 2008). They accumulate in specialized structures such as oil cells, glandular trichomes, and oil or resin ducts (Simon, 1990) and can be obtained by expression, fermentation, or extraction (hydro-distillation, water and steam distillation, direct steam distillation, and solvent extraction) but the method of steam distillation is most commonly used for commercial production of essential oils (Simon, 1990).

The term 'essential oil' is thought to be derived from the name coined in the 16th century by the Swiss reformer of medicine, Paracelsus von Hohenheim; he named the effective component of a drug *Quinta essential* (Tzortzakis and Economakis, 2007).

They are a heterogeneous group of complex mixtures of organic substances whose quality and quantity vary with the growth stages, ecological conditions and other factors of the plant from which the essential oil is extracted (Ozcan and Erkmen, 2001; Tzortzakis and Economakis, 2007). The complexity in essential oils is due to terpene hydrocarbons as well as their oxygenated derivatives, such as alcohols, aldehydes, ketones, acids and esters (Tzortzakis and Economakis, 2007).

Chemically, the essential oils are primarily composed of mono- and sesquiterpenes and aromatic polypropanoids synthesized via the mevalonic acid pathway for terpenes and the shikimic acid pathway for aromatic polypropanoids (Simon, 1990).

An estimated 3000 essential oils are known, of which about 300 commercially important are destined chiefly for the flavors and fragrances market (Yulia, 2005).

2.3 Uses of plant extracts and oils

Plant oils and extracts have been used for a wide variety of purposes for many thousands of years. These purposes vary from the use of rosewood and cedar wood in perfumery or flavoring drinks with lime, fennel or juniper berry oil, and the application of lemon grass oil for the preservation of stored food crops (Hammer *et al*., 1999; Singh *et al*., 2008). Because of their antibacterial, antifungal, antioxidant, and anticarcinogenic properties, they can be used as natural additives in different foods (Bluma and Etcheverry, 2008).

The plants can be collected from the wild, or cultivated in crop fields, home gardens or in modern large scale plantations (Fullas, 2003; Gutierreza *et al*., 2008). Spices and their derivatives such as essential oils and oleoresins are used with the primary purpose of flavoring foods and beverages although it has long been known that some spices have an antimicrobial activity.

Many of the spices and herbs used today were known to the people of ancient cultures throughout the world and they were valued for their preservative and medicinal powers as well

as their flavor and odor qualities (Ozcan and Erkmen, 2001; Atanda *et al*., 2007; Shan *et al*., 2007; Kaefer and Milner, 2008). Archeologists discovered evidence that as early as 50,000 B.C., humans used the leaves of plants for flavoring meats and around 2300 B.C. for wine making (Kaefer and Milner, 2008).

A growing body of epidemiological and preclinical evidence points to culinary herbs and spices as minor dietary constituents with multiple anticancer characteristics (Kaefer and Milner, 2008). At present, it is estimated that about 80% of the world population relies on botanical preparations as medicines to meet their health needs (Singh *et al*., 2008).

Herbs and spices are generally considered as safe and proved to be effective against certain ailments and have therapeutic potential, mainly in diseases involving mucosal, cutaneous and respiratory tract infections (Lopes-Lutz *et al*., 2008). Fortunately, even long-term consumption of these substances is not known to produce any side effects. They have been extensively used in many Asian and African countries since ancient times. In recent years, in view of their beneficial effects, use of spices/herbs has been gradually increasing in developed countries also. In traditional Indian medicine or Ayurveda, ginger and many other spices have been used as medicine (Singh *et al*., 2008).

Traditionally, natural compounds from plants have been used to preserve food in some countries like India, Japan and Russia (Montes-Belmont and Carvajal, 1998; Atanda *et al*., 2007). They are proven to be inhibitory against a wide range of food spoiling microorganism, dependent upon their concentration, testing method, and active constituents present (Burt, 2004;

Hayounia *et al*., 2008). Laboratory studies of extracts and oils of plant species have revealed powerfull fungitoxicities in relation to many fungal phatogens. For example, oils obtained from seeds of several plants such as sunflower, olive and soybean have given excellent control against fungal pathogens. They were also effective against other pathogens such as Ascomycota and Bascidomycota leaf pathogens. Some were also active against strains of bacteria (Yulia, 2005).

2.4 Antimicrobial activity of plant essential oils

Most plants produce antimicrobial secondary metabolites, either as part of their normal program of growth and development or in response to pathogen attack or stress (Bluma and Etcheverry, 2008). These compounds when present in foods can extend shelf-life of unprocessed or processed foods by reducing microbial growth rate or viability (Holley and patel, 2005). Originally they are added to change or improve taste. Some of these substances are also known to contribute to the self-defense of plants against infectious organisms (Holley and Patel, 2005). In their wild state, plants possess defense mechanisms against bacterial and fungal invasions. Domestication of wild plants resulted in decreased amounts of unpleasant-tasting chemicals, which are part of their natural defense mechanisms, but still contain some inducible chemical defense activity (Lopez-Malo *et al*., 2007).

The composition, structure as well as functional groups of the oils play an important role in determining their antimicrobial activity (Holley and Patel, 2005). It seems that the anti-fungal and anti-microbial effects are the result of many compounds acting synergistically. These

means that the individual components by themselves are not as effective (Jobling, 2004; Holley and Patel, 2005; Tzortzakis, 2009). Among these natural antimicrobials are eugenol from cloves, thymol from thyme, carvacrol from oregano, vanillin from vanilla, allicin from garlic, cinnamic aldehyde from cinnamon, and allyl isothiocyanate from mustard (Lopez-Malo *et al*., 2007; Tzortzakis, 2009).

Burt (2004) suggested that the minor components present in the essential oils extracts are more critical to the activity than essential oils main components mixed, and may have a synergistic effect or potentiating influence. In many cases the result was an "additive" effect (Rodriguez *et al*., 2007; Gutierreza *et al*., 2008).

Little information is available on interaction among constituents in essential oils and the effects they have on antimicrobial activity (Nguefack *et al*., 2009). Essential oils presenting high amount of phenolic compounds have the greatest anti-mould activities (Carmo *et al*., 2008; Kaefer and Milner, 2008; Lopes-Lutz *et al*., 2008; Goni *et al*., 2009; Nguefack *et al*., 2009). Among these, the oils of clove, oregano, rosemary, sage and vanillin have been found to contain phenolic compounds and are consistently effective against microorganisms (Cosentino *et al*., 199).

These compounds have in the past shown great promise as potential antifungal agents to protect stored grain from fungal invasion and the associated production of mycotoxins (Samapundo *et al*., 2007). The presence of these compounds in cinnamon and cloves, when added to bakery items, function as mold inhibitors in addition to adding flavor and aroma to baked products.

There are also some non-phenolic constituents of oils which are more effective (allyl isothiocyanate) is quite effective against Gram-negative bacteria (Yin and Cheng, 2003; Lopez-Malo *et al*., 2007). Moreover, essential oils of oregano and thyme (which contain carvacrol and thymol) are effective as fumigants against fungi on stored grain (Snyder, 1997)

Generally, it is the phenolic components in the essential oils which exhibit the antimicrobial activity (Deans *et al*., 1995). Exceptions include: mustard where allyl and related isothiocyanates are responsible; and allicin in garlic and onion. These are both non-phenolic, aliphatic compounds. Phenolic compounds in olive oil (oleuropein) and tea-tree oil (terpenes), which are not classified as either spices or herbs, also show antimicrobial activity (Holley and Patel, 2005).

2.5 Mechanism of antimicrobial action of plant essential oils

Expression of antimicrobial activity of essential oils is often very clear, but the mechanism of antimicrobial action is incompletely understood (Holley and Patel, 2005; Lopez-Malo *et al*., 2007; Tzortzakis and Economakis, 2007; Hayouni *et al*., 2008; Kaefer and Milner, 2008 Tatsadjieu *et al*., 2009; Tzortzakis, 2009).

Some studies suggest that compounds penetrate inside the cell, where they interfere with cellular metabolism. Other studies suggest that they disturb the cellular membrane and react with active sites of enzymes or act as an H^+ carrier, depleting adenosine triphosphate pool (Tatsadjieu *et al*., 2009).

In addition, the antimicrobial activity of these compounds has been suggested to be attributed to the presence of an aromatic nucleus and an OH group known to be reactive and to form hydrogen bonds with active sites of target enzymes (Carmo *et al*., 2008). This is generally considered to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force, electron flow, active transport and coagulation of cell contents (Brul and Coote, 1999; Hayouni *et al*., 2008; Nguefack *et al*., 2009).

Other events which could lead to membrane disruption include dissipation of the two components of the proton motive force in cells (the pH gradient and the electrical potential) either by changes in ion transport or depolarization through structural changes in the membrane; interference with the energy (ATP) generation system in the cell; or enzyme inhibition preventing substrate utilization for energy production. Certainly, the ability of phenolics to interfere with cellular metabolism through a number of mechanisms (substrate complexing, membrane disruption, enzyme inactivation and metal chelation) is well known (Holley and Patel, 2005).

If a pair of antimicrobials with different inactivation mechanisms is applied together, better microbial control could be achieved, if additive or synergistic effects take place. However, if antagonistic effects occur, the effectiveness of the antimicrobials would be greatly reduced (Lopez-Malo *et al*., 2007).

According to Carmo *et al*. (2008), essential oils with anti-mould effectiveness are able to consistently cause morphological changes in *Aspergillus* species including lack of sporulation,

loss of pigmentation, aberrant development of conidiophores (flattened and squashed) and distortion of hyphaes (budding, lack of cytoplasm, swelling anomalous apex bifurcation). They suggested that the mode of antifungal activity of essential oils could include an attack on the cell wall and retraction of the cytoplasm in the hyphae ultimately resulting in death of the mycelium. In addition, it was also related to the interference of the essential oil components in enzymatic reactions of wall cell synthesis, which affects the fungal growth and morphogenesis.

In general, inhibitory action of natural products on mould involves cytoplasm granulation, cytoplasmic membrane rupture and inactivation and/or inhibition of intercellular and extracellular enzymes. These biological events could take place separately or concomitantly culminating with mycelium germination inhibition (Souza *et al*., 2005).

2.6 Synthetic chemicals versus natural product fungicides

In recent years consumers have become more concerned about the safety of the foods they buy and eat (Lopez-Malo *et al*., 2007; Tzortzakis, 2009). Several biological, chemical and physical pre- and post-harvest strategies have been proposed to minimize the loss of food and agricultural commodities as a result of fungal spoilage or poisoning by their mycotoxins (Samapundo *et al*., 2007; Kumar *et al*., 2008; Kotan *et al*., 2009).

At present, quick and effective management of microbial contamination in several agricultural commodities is generally achieved by the use of synthetic fungicides (Yulia, 2005). A wide range of food grade chemicals has been added during food manufacturing to extend shelf-life by stabilizing chemical change or by preventing or inhibiting microbial growth (Holley and

Patel, 2005). Antimicrobial chemicals belonging to the groups of benzimidazoles, aromatic hydrocarbons and sterol biosynthesis inhibitors are often used, as post harvest treatments (Kotan *et al*., 2009; Tatsadjieu *et al*., 2009). They are being used for the protection of crops, food products and pest control in many countries (Sharma and Tripathi, 2008).

The chemical control measures remain the main strategy to reduce the incidence of post harvest diseases in various foods (Atanda *et al*., 2007; Carmo *et al*., 2008; Tatsadjieu *et al*., 2009). Indeed, the tremendous increase in crop yields associated with the 'green' revolution would not have been achieved without the contribution of these synthetic compounds (Dayan *et al*., 2009).

However, the indiscriminate application of chemicals has caused significant drawbacks including increased cost, handling hazards, concern about pesticide residues on food, and threat to human and animal health and environment (Daferera *et al*., 2003; Tzortzakis and Economakis, 2007; Bluma and Etcheverry, 2008; Tzortzakis, 2009). Because of this indiscriminate use, some microorganisms have developed resistance to most widely used synthetic fungi toxicants rendering them out of date. This seriously hinders the management of microbial contamination of crops and agricultural products by synthetic chemicals (Daferera *et al*., 2003; Amiri *et al*., 2008; Kumar *et al*., 2008).

In recent years, a large number of synthetic pesticides has been banned in the Western world because of their undesirable attributes such as high and acute toxicity, long degradation periods, accumulation in the food chain, and an extension of their power to destroy both useful and harm

full pests. In developing countries, they are still being used despite their harmful effects (Yulia, 2005).

Because of greater consumer awareness and concern regarding synthetic chemical additives, there is a need to develop new fungicides/preservatives with improved performance, safe, as well as ecofriendly in nature (Omidbeygi *et al*., 2007; Kumer *et al*., 2008; Tatsadjieu *et al*., 2009). This provokes many researchers and food processors to investigate new sources of ingredients and/or food additives (Viuda-Martos *et al*., 2008) with a broad spectrum of antimicrobial activity (Holley and Patel, 2005). One such source may be agro-food co-products (Viuda-Martos *et al*., 2008) and foods preserved with natural additives have become popular (Mishra and Dubey, 1994; Holley and Patel, 2005; Irkin and Korukluoglu, 2007; Omidbeygi *et al*., 2007; Carmo *et al*., 2008; Rasooli *et al*., 2008; Tzortzakis, 2009).

Traditional and natural antimicrobial agents with potential or current value for use in foods as "secondary preservatives" were reviewed (Holley and Patel, 2005). Natural plant extracts are of interest as a source of safer or more effective substitutes for synthetically produced antimicrobial agents and may provide an alternative way to prevent food or feed from fungal contamination (Tzortzakis, 2009).

Phyto-compounds are expected to be far more advantageous than synthetic pesticides for sheer magnitude of complexity, diversity and novelty of chemicals, reactions and phenomenon as they are bio-degradable in nature, non-pollutant and posses no residual or phytotoxic properties (Sharma and Tripathi, 2008). An important benefit of natural product-based pesticides is their

relatively short environmental half-lives, which is due to the fact that they do not possess 'unnatural' ring structures and contain relatively few halogen substituents (Dayan *et al*., 2009).

Powders and extracts of various herbs, spices and their essential oils have been reported to have antimicrobial activity and some also to inhibit aflatoxin formation (Hall and Fernandez, 2004; Thanaboripat *et al*., 2007). Numerous investigations have confirmed the antimicrobial action of essential oils in model food systems and in real food (Rasooli *et al*., 2008).

More than 280 plant species have been investigated for their effect on toxigenic *Asperillus spp* and nearly 100 of them had some activity on growth or toxin production of these fungi (Montes-Belmont and Carvajal, 1998).

There is evidence that essential oils are more strongly antimicrobial than is accounted for by the additive effect of their major antimicrobial components; minor components appear, therefore, to play a significant role. Since essential oils are considered as generally regarded as safe (GRAS) by the FDA, at least at concentrations commonly found in foods (Nguefack *et al*., 2004; Shan *et al*., 2007; Tatsadjieu *et al*., 2009), the possibility of reinforcing their natural antimicrobial effects by the addition of small amounts of other natural preservatives may be a way to attain a balance between sensory acceptability and antimicrobial efficacy (Rasooli *et al*., 2008; Goni *et al*., 2009).

Being natural foodstuffs, spices and herbs appeal to many consumers who question the safety of synthetic food additives (Shan *et al*., 2007). Their antimicrobial activities form the basis for many applications, including raw and processed food preservation, pharmaceuticals, alternative

medicines and natural therapies (Shan *et al*., 2007). The applications of these natural plants are especially attractive to home gardeners and growers of organic produce (Yulia, 2005).

3 GENERAL CHARACTERISTICS OF THE MEDICINAL PLANTS

3.1 Ginger (*Zingiber officinale*)

"*Zingiber*" is derived from the Sanskrit singabera, which means "shaped like a horn", because of the resemblance of the roots of the plant to a deer's antler. "Officinale" is obtained from the Latin Officina, which means "Work shop" or "in the pharmacy" to indicate medicinal importance of the plant (Fullas, 2003).

Botanically, *Zingiber* gives its name to the whole ginger family, Zingiberaceae. Several Zingiberaceae plants have been reported to be useful in traditional medicines (Madya *et al*., 2006). The *Zingiberaceae* is a plant family made up of wild and cultivated species. It comprises about 1200 species of which about 1000 occur in tropical Asia. It is one of the oldest herbs known by the people and is one of the earliest spices to be known in the east (Madya *et al*., 2006).

Ginger is a creeping plant on a thick tuberous rhizome, which spread under ground. In the first year a green, erect, reed like stem about 60 cm high grows from the rhizome. The flower terminates in a long, curved spike, from which white, yellow, or purple flowers grow (Fullas, 2003). The plant is indigenous to warm tropical climates, particularly southeastern Asia. It is now extensively cultivated in India, China, Africa, Jamaica, Mexico and Hawaii.

In Ethiopia, ginger has been known perhaps since the 13th century, when the Arabs brought it from India to East Africa (Fullas, 2003). Mostly gingers in cultivation are sterile cultivars

grown for the edible rhizomes and flowers are rarely seen. The rhizomes (spice of commerce) are aromatic, thick lobed, branched and scaly structures with a spicy lemon-like scent. It is well known that ginger rhizomes contain both aromatic and pungent components. The essential oil and oleoresins extracted from ginger rhizomes are very valuable products responsible for the characteristic ginger flavor and pungency. Both oil and oleoresins are used in many food items, soft drinks, beverages and many types of medicinal substances (Madya *et al*., 2006; Singh *et al*., 2008).

It has good demand in the international and even local market for preparing ginger oil, oleoresin, ginger juice and essence. Ginger rhizome contains a mixture of an essential oil, a fixed oil, pungent compounds, starch, and others, such as saccharides, proteins, cellulose, waxes, colouring matter, trace minerals and etc (Madya *et al*., 2006).

3.1.1 Chemical constituents of ginger

Ginger contains an aromatic volatile oil in a concentration of 1% to 3%. The pungent vanilloids are in general called gingerols. They occur at levels of less than 1% of the root by weight. It also contains two other phenolic compounds called shagaols and zingerone in addition to gingerol. The other pharmacologically active constituents include (6)-shogaol [dehydroxylated analog of (6)-gingerol], (6)-and (10)-dehydrogingeridone and zingerone. High amount of iron (54-62 mg /100g) and calcium (1% - 1.5%) are found in ginger rhizomes (Fullas, 2003). 6-Gingerol and 6-Shogaol are the most important pungent compounds of ginger products (Madya *et al*., 2006).

3.1.2 Uses of ginger

Ginger has a wide spectrum of uses as a culinary spice. It is available in ground, cracked (broken into pieces) forms or as a whole. It is used in gingerbread, pies, cookies, pickles, pickling vinegar, flavored syrups, and in the preparation of ornamental meat-based recipes. Ginger is also used as ingredients of curry powder, to flavor ginger beer and ginger wine and various confections. In the USA, fresh and dried roots of ginger have a GRAS status in foods (Simon, 1990).

In Ethiopia ginger is a popular spice. The fresh rhizome is washed, scrapped, chopped into bits, pound and the resulting product is used before or after drying. The spice obtained in this manner is used along with other spices in “wot”. It is also used in alcoholic drinks and to flavor tea (Fullas, 2003).

3.2 Garlic (*Allium sativum)*

Garlic is a perennial bulbous plant, with a tall stem that grows to 2 to 3 ft. It produces pink to purple flowers. The garlic bulb is a compound bulb, which is covered with silky white or green skin. It has a stronger odor and is considered to have originated in central Asia and later introduced to the Mediterranean region (Fullas, 2003).

3.2.1 Chemical constituents of garlic

Garlic contains vitamins, minerals and trace elements (germanium and selenium) of all *Allium* species; *A.sativum* (garlic) contains the highest sulfur content. Up on crushing or cutting garlic bulb, the odorless and colorless, sulfur-containing amino acid constituents allicin is transformed by enzymatic action to the pungent compound allicin (Fullas, 2003).

3.2.2 Uses of garlic

Fresh garlic, garlic powder and garlic oil are used in foods and beverages as flavoring components worldwide. In Ethiopia, garlic is used widely as food additives. It is used in the preparation of barley flour ("ye gepsi duqet"), beans flour ("ye baqela duqet"), herbed butter ("nitiri qibe"), green pepper paste ("tiqure qaria awaze"), red pepper paste ("awaze"), spiced oil ("ye tenetere zeit") and many other foods (Fullas, 2003).

In folk medicine, garlic is used for the treatment of high blood pressure, atherosclerosis, colds coughs, whooping cough and bronchitis. As indicated by the survey conducted in Ethiopia, garlic is used for the treatment of common cold, malaria, cough, lung TB, hypertension wounds, sexually transmitted diseases, mental illness, kidney and liver disease, abdominal colic, gastritis, eye disease, toothache diabetes, skin disease, headache, typhus, swelling, back pain and hemorrhoids (Dilbato and Tito, 1999). Various investigators have reported on the antimicrobial, antifungal and anticancer activity of garlic.

Generally garlic is one of the most important herbs used worldwide both to flavor food and as a medicinal agent. It is perhaps one of the most studied herbs. There is ample justification for the many uses that garlic finds in Ethiopian traditional medicine.

3.3 Cinnamon (*Cinnamomum Zeylanicum*)

Cinnamon is a bushy, evergreen tree of the laurel family reaching a height of up to 30 to 40 ft (Fullas, 2003), with numerous branches, long, leathery, bright green leaves, small yellowish flowers and ovoid blackish fruits. There is normally a single centered stem to 20 m. The leaves measures up to 5 to 7 ft long and are dark glossy green at the top surface and lighter underneath. They are opposite, 5-18 x 3-10 cm, with round bases and accumulate tips. They are green to bright green above, dull grey- green below, but young leaves may initially have a reddish tint (Fullas, 2003;Yulia, 2005).

The flowers are up to 3 mm diameter, pale yellow or cream and have unpleasant fetid smell. The fruit is a black or bluish, fleshy, ovoid drupe, 1.5-2.0 cm when ripe and the enlarged calyx persists at its base. The dried bark is the “true” cinnamon that is commercially used as a spice (Fullas, 2003). The bark on young shoots is smooth and pale brown, on mature branches and stems is rough, dark brown or brownish grey. *C.zeylanicum*, also known as “true cinnamon” is a tree of the wet tropics with somewhat restricted natural range in Sri Lanka, India and South East Asia (Fullas, 2003;Yulia, 2005).

3.3.1 Chemical constituents of cinnamon

The bark contains 0.5-2 % oil, of which the main constituent is cinnamaldehyde (60-70%). Green leaves yields about 1% essential oil, whose main constituents is eugenol (70-95%). Fruits mature in 3-5 months, and the globular brown seeds contain approximately 33% fixed oil (Fullas, 2003).

3.3.2 Uses of Cinnamon

Cinnamon is used in foods and beverages. It has been used as a spice and taste enhancer for centuries. In Ethiopia, cinnamon sticks have been used to flavor tea. Its powder is used as an ingredient for “berbere” (red pepper spice mix) and “awaze” (red pepper paste) and to spice foods such as “mitin shiro”(spiced, hot, powdered peas) “yeminshet abish dabo” (bread made with spiced minced beef sauce), and “ginfilfil” (“injera” in sauce mixed over low heat) (Fullas, 2003).

Medicinally, cinnamon bark is used for gastrointestinal upsets and dysmenorrheal. Topically, it is used as an ingredient of a multi-component preparation to treat premature ejaculation.
In Ethiopia, although “qarafa” has been used in teas by people to treat cold symptoms, it appears that there are no medicinal uses reported in the literature (Fullas, 2003).

Generally cinnamon has been used as spice, fragrance and folk medicine for centuries. It is possibly effective as an antiflatulent, antispasmodic, and appetite stimulant

3.4 Lemon (*Citrus limon*)

The Lemon tree (*C. limon*) is an evergreen that grows to over 6 m tall. The leaves are toothed and light green in color. The fruit is small, green to yellow in color and oval in shape. *C.limon* originated in southeast, probably in India, Asia and Southern China. The plant is cultivated in the Mediterranean region and in subtropical climates (Fullas, 2003).

3.4.1 Chemical constituents of Lemon

Generally, citrus fruits contain sugars, polysaccharides, organic acids, lipids, carotenoids (responsible for the color), vitamins, minerals flavonoids limonoids (import bitterness) and volatile oil constituents. *C.limon* contains potassium calcium, and vitamins A, B1, B2, B_3 and C. Other constituent include volatile oils (2.5% of the peel), limonene, Alpha-terpinene, alpha-pinene, citral, coumarins, Mucilage, pectin and flavoinds (mostly in the pith and peel). Lemon oil (the essential oil component) is extracted from the skin (Fullas, 2003).

3.4.2 Uses of lemon

Lemon is used as food and a flavoring agent. It has been used in food preparation and the agricultural industry to gel and stabilize foods. It is also an important source of vitamin C.

Known by the vernacular name “lomi” both *C. Limon* and *C.aurantifolia* are edible in Ethiopia. The juice is directly sucked from the fresh fruits. Lemon juice is used in the preparation of various Ethiopian foods, such as dried fish (“yasa quanta”), boiled beets (“yeqeysir qiqil”), fresh tomatoes and green pepper (“teematim beqaria”), spiced chicken pea bread (“yeshimbira

kitfo"), and fresh chopped tomatoes blended in 'injera' and spices ('yetimatim fitfit') (Fullas, 2003).

Medicinally, lemon is used as a source of vitamin C in the treatment of scurvy, low resistance, and colds. It is also used as anti inflammatory, diuretic and to improve vascular permeability. Lemon juice has long been used as a diuretic diaphoretic, astringent, tonic, lotion and gargle.

Vitamin C, a constituent of lemon, is necessary to boost the body's resistance to infection and to improve the healing of wounds. Despite its acidity, lemon has an alkaline effect in the body, thus helping alleviate rheumatism, arthritis and gout. Lemon antimicrobial activity is reported. The pectin content of lemon is hydrophilic and hence useful to treat vomiting and diarrhea, by thickening gastric contents and regulating transit. The bioflavonoid constituents strengthen the inner lining of blood vessels, thus helping in the management of varicose veins (Fullas, 2003).

In Ethiopia, lemon is used to spice a number of traditional dishes and to give them palatability. In traditional medicine, it is used in many preparations as a vehicle or medicinal agents (Fullas, 2003).

3.5 Thymus (*Thymus schimperi*)

Thymus schimperi is a perennial herb, woody at the base and 5 to 40 cm high. They have a crowded inflorescence with pink corollas and have ovate to elliptic leaves with entire margins.

Its name is derived from Greek, which refers to courage, sacrifice and fumigation. In Ethiopia, it is commonly called *'Abyssinian thyme'*. It was an essence used to purify temples. In ancient Greek, it was regarded as a symbol of courage and bravery. It is believed that thymus was found in the hay and straw bed in which Virgin Mary and Chiled Christ rested (Fullas, 2003).

Well over a hundred species of thyme and their hybrids are known to occur throughout the world. However they are uncommon to African tropics. Outside of Ethiopia, known by the common names thyme, rubbed thyme, Spanish thyme, and thyme herba, the species *Thymus vulgaris* and *Thymus zygis* are the commercial sources of thyme and thymole oil.

T. schimperi and *T. serrulatus* are indigenous to Ethiopia .They are endemic to the highlands, and are found on edges of roads, in open grass lands and on slops. They occur at altitude range of 2,200 and 4000 m above sea level. *T.schimperi* wide spread in central eastern and northern Ethiopia, while *T. serrulatus* is restricted to the northern parts of the country (Fullas, 2003).

3.5.1 Chemical constituents of Thymus

Chemically analysis of the volatile oil of *T.schimperi* collected in Bale, Gonder, Shewa and Wello indicated the presence of P-cymene (9.23%), Sigma-terpinene (8% to 17%), thymol (6% to 38%) and carvacol (5% to 63%). The oil from *T.serrulatus* was found to contain p-cymene (13%), thymol (49%) as major constituents (Fullas, 2003).

T. vulgaris contains a volatile oil (1.0% to 2.5%) whose constituents are p-cymene(14% to 45%) thymol (20% to 55%), carvacol (1% to 10%), borneol (up to 8%), and linalool (up to 8%).

Other non volatile constituents include caffeic acid derivatives (rosmarinic acid, etc.), flavonoids (luteolin, apigenin, naringenin, circilineol, circimatrin and thymonin) and triterpenes (ursolic acid and oleanolic acid) (Fullas, 2003).

3.5.2 Uses of Thymus

Thyme and thymol oil are GRAS-listed in the USA. When used in specified amount of food, the maximum allowable level of thyme in foods is 0.172% and that of thyme oil is 0.003%. In manufacturing, red thyme is used in soaps, cosmetics, tooth pasts, and as a flavor component in foods. In Ethiopia the fresh and dried leaves of both *T.schimperi* and *T.serrulatus* are used as flavoring ingredients of chilli powder ("berbere") stew, bread and tea (Fullas, 2003).

Thyme (from *T.vulgaris* and *T.zygis*) is primary used for cough and bronchitis. It is also used for pertussis (whooping cough), soar throat, colic dyspepsia chronic gastritis, diarrhea and enuresis in children, dyspenea, rheumatism skin disorders, as an appetite, stimulant, ant flatulent, diuretic, urinary disinfectant and anthelminthic. It is also used optically for upper respiratory tract mucous membrane inflammation, laryngitis, tonsillitis stomatitis, and halitosis. In Ethiopia, both *T. schimperi* and *T. serrulatus* find many traditional uses (Fullas, 2003).

The solution from the boiled leaves is drunk for cough, headache, stomachache, and gonorrhea. *T.serrulatus* is topically used for chloasma. Internally used for ascariasis, taeniasis, tinea captis, and toothache, rheumatic pain mental illness as an emenagogue, vermifuge, antispasmodic and emetic (Fullas, 2003).

There is some evidence to indicate that thymol in thymus species has anti-worm activities. The volatile oil and flavonoid constituents are responsible for the antispasmodic, antitussive and expectorant effects. Thymol and carvacol have antibacterial and antifungal activities.

Generally, thyme is an important culinary herb. Among many of its medicinal uses are included its use for whooping cough, gastrointestinal problems and as an anthelmintic.

In Ethiopia, *Abyssinian thyme* (*T.schimperi* and *T.serrrulatus*) is used as food flavor and in teas. It is also used in folk medicine. The use of thyme for cough, stomachache and worm infection in Ethiopia corresponds to the comparable use of the related *T.zygis* (botanically and chemotaxonomically) in other societies (Fullas, 2003).

3.6 Black pepper (*Piper nigrum*)

Piper nigrum is a vine that produces berries in clusters. The berries are picked either green (not fully ripe) or after they are ripened. The unripe pepper corns give black pepper black after processing, while that ripened berries give white pepper. A woody cliber *Piper nigrum* grows to 30 feet high or more, with grayish stem of up to 0.5 cm in diameter. The leaves are dark green above and pale yellow underneath (Fullas, 2003).

Piper nigrum is native to the damp jungle of south-western India. It is cultivated in tropical areas and grows in moist hot climate at altitude of up to 1,500ft above sea level.

In Ethiopia, trials to grow *P. nigrum* at Jimma Agricultural research center had shown promising results and it was from there that the sample for this study was taken.

3.6.1 Chemical constituents of black pepper

Chemically *P.nigrum* contains piperne.

3.6.2 Uses of black pepper

P.nigrum is the world most important spice, due to its availability and versatility. It is often added to food just before preparation or as seasoning when food is served. Whole pepper corn spices are added to meats, soups, fish and pickles. The spice has a hot biting and pungent taste. It is also added to beverages as flavoring.

In Ethiopia, it is used in the preparation of spiced powdered pepper, collared green mixed in spiced cottage cheese (‘ayib begomen’), dried meat (‘quanta’), spiced barley meal (‘chiko’) bread made with special minced fish sauce (“yasa dabbo”) and grilled ribs and beef stew (‘insirsir’) (Fullas, 2003).

Medicinally, black pepper and white pepper are used orally to treat cancer traditionally. Other uses include for treatment of stomach disorders, digestive problems and bronchitis. Topically it is used for treating neuralgia and scabies (Fullas, 2003).

In Ethiopia, *P.nigrum* is used as ingredients in a multi-spice preparation which is used for the treatment of mental illness. It is also used by itself and in combination with other two plants as an aid in fortune telling and to acquire wealth/have good married life, respectively.

Generally, *P.nigrum* is a versatile spice. It is used in foods prior to or during preparation. It can also added as a seasoning after food is served. There is no reliable evidence on the effectiveness of *P.nigrum* when used medicinally.

In Ethiopia piper nigrum is used as spice and in Ethiopian traditional medicine for superstitious benefits (Fullas, 2003).

4 MATERIALS AND METHODS

4.1 Plant sample collection

Seven various types of herbs and spice plants were collected from highlands and low lands of the country from November, 2009 – January, 2009 (Table 1). Different parts of the test plants of *piper nigrum* (seed), *Zingiber officinale* (root) and *Cinnamomum zylianicum* (bark) were collected from low land areas of Jimma/Tepi, southwestern Ethiopia. *Thymus schimperi* (leaf with areal parts) and *Laggera tomentosa* (leaf) were collected from north Showa around Chancho town. *Allium sativum* (bulb) was collected from east Showa, Debre Zeit and *Citrus limon* (peel) was collected from Addis Ababa, Atikilt tera.

The selection was based on the traditional practices that local people use these plants as food preservatives, food flavoring and seasoning agents. Furthermore, incorporation of the plants in food system with promising antifungal activity was assumed.

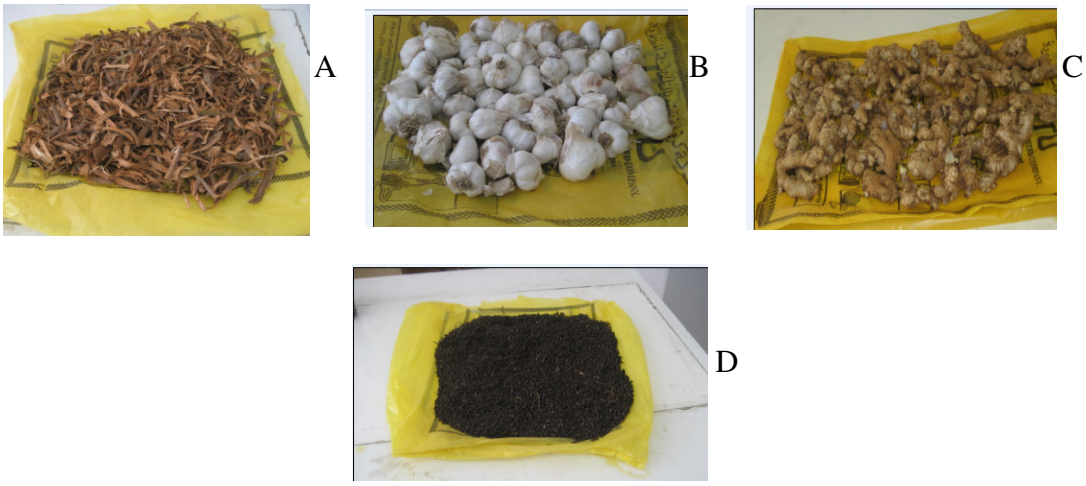


Fig.1. Some of the spice plants collected (A= cinnamon, B= garlic, C=ginger, D =black pepper)

4.2 Sample preparation

The collected plant materials were dried at room temperature and ground using a grinder.

Table1. Plants used as a source of essential oils.

Species	Part of plant used	Code	source	Known components of the essential oils
Cinnamomum zeylanicum (cinnamon)	Bark	Cn	Jima/Tepi	Cinnamaldehyde, 2-hydroxycinnamaldehyde and eugenol.
Allium sativum (garlic)	Bulb	Gc	Debre Zeit	Allicin, diallyl disulfide, allyl isothiocyanate
Zingiber officinale (ginger)	root	Gn	Jima/tepi	Sesquiterpens,bisabolene,farnesene and monoterpenoids
(*Thymus schimperi* (thymus)	Leaf and areal part	Ty	Chancho	P-cymene,Sigma-terpinene ,thymol and carvacol
Laggera tomentosa (Keskese)	Leaf	Lt	Chancho	-
piper nigrum (black pepper)	seed	Pn	Jima/tepi	Monoterpene-hydrocarbon, beta-pinene, limonene,
Citrus limon (lemon)	peel	Lm	Addis Ababa	limonene, Alpha-terpinene, alpha-pinene, citral, coumarins, Mucilage, pectin and flavoinds

Sources for known components of essential oils: (Fullas, 2003; Kaefer and Milner, 2008)

4.3 Extraction of essential oils

The extraction of the spices and herbs were conducted through the process of hydro-distillation at Ethiopian Health and Nutrition Research Institute (EHNRI).

The essential oils from different parts of the plants described in section 4.1 were extracted by hydro-distillation (using Clevenger 77-550 type apparatus) following the procedure outlined by Hettiarachichi (2008) with little modification. About 500 g of these materials were packed in a distillation flask with approximately four times water (w/w) of the test materials. The distillation chamber was heated to 40^{o}c to 50^{o}c and allowed to boil until completed (4-5 hrs).

The distillate was collected in the separating funnel in which the aqueous portion was separated from the volatile oil. The water (lower) layer was slowly drowned off until the oil layer remained. Colored oils, with pleasant odors, were obtained. Finally the oils were collected in sterile container, dried over minimum amount of anhydrous sodium sulfate to remove traces of moisture and preserved in refrigerator until it was used for further experiments.

The yields of the oils as percent of plant material weight by weight were as follows: 0.85%, 1.1%, 0.65%, 0.78%, 1%, 0.14% and 1.2% for the essential oils from *Piper nigrum, Zingiber officinale, Cinnamomum zylianicum, Thymus schimperi, Laggera tomentosa, Allium sativum* and *Citrus limon* peel, respectively.

Fig.2. Hydro-distillation apparatus set up

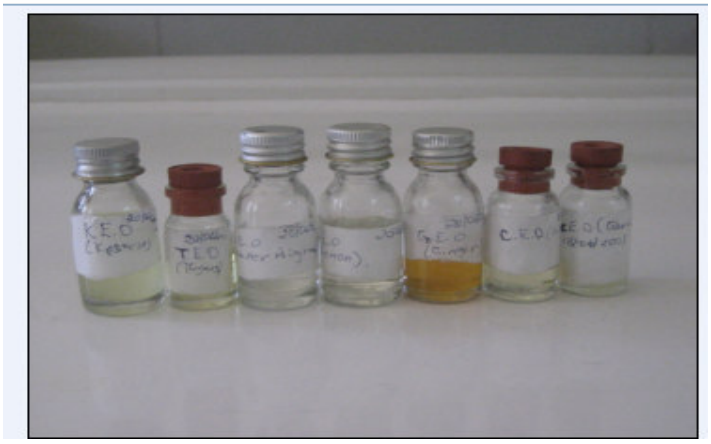


Fig.3. The extracted essential oils

4.4 Grain sample collection

Visibly healthy sorghum grains were purchased from the local market to carry out the grain protection assay. The grains that are not damaged by insects, which do not have any broken parts and other physical injury, were visually inspected seriously for the decision to buy them.

4.5 Fungi sample collection and maintenance

For antimicrobial testing, the test fungi *Aspergillus flavus* (ATCC 13697) and *Aspergillus niger* (ATCC 10535) standards were kindly supplied by Ethiopian Health and Nutrition Institute (EHNRI), while local isolates of *Aspergillus flavus* and *A. niger* were obtained from Addis Ababa University Department of Biology, Mycology Lab.

The fungi were maintained on potato dextrose agar (PDA; Himedia Ltd., India) slant at refrigerator temperature and periodic transfers were done to keep the microorganism viable.

4.6 Inoculums preparation and standardization

The tested fungi were grown on PDA (Himedia Ltd., India) medium for sporulation on petri dishes for 5–7 days. Fresh stock suspension of the organisms were prepared in 10 ml of sterile normal saline solution and were adjusted to 0.8-1 OD range with 6051-type colourmeter at 625 nm wave length to obtain a concentration of 10^4 spores/ml and was utilized the same day.

4.7 Antifungal analysis

The extent of antifungal activity of the various plant oils is evaluated on two of the more important molds of food borne disease and /or food spoilage. Antifungal activities of the oils with respect to the isolates were studied with special reference to the inhibition of the *standard organisms.*

The National Committee for Clinical Laboratory Standard (NCCLS) method for antibacterial susceptibility testing, which is principally aimed at the testing of antibiotics has been modified for testing essential oils (Hammer *et al.,* 1999).

The antifungal assays conducted were:

1.Evaluation of antifungal activity of essential oils by disk diffusion method.
2.Determination of the minimal inhibitory concentrations (MIC) by agar dilution method
3.Spore germination inhibition assay
4.Grain protection assay
5.Optimal protective dosage of the essential oils.

4.7.1 Disk diffusion assay

Screening of essential oils for antifungal activity was carried out by disc diffusion method, which is normally used as a preliminary screening of efficient essential oils (Burt, 2004) following the procedure approved by NCCLS with little modification: the tested fungi (*Aspergillus flavus* and *A. niger* isolate) were grown on PDA (Himedia Ltd., India) medium and the spore suspension was prepared as described in section 4.6.

The standard fungi, *Aspergillus flavus* (ATCC 13697) and *Aspergillus niger* (ATCC 10535) of both isolates were used for special reference or test validation. Filter paper discs (Whatman no.1, 6 mm in diameter) were prepared and sterilized. 5 µL of each essential oil extracts were

impregnated on to the disk by sterile micropipette tips. Using an ethanol dipped, flamed and cooled forceps; these disks were aseptically placed soon individually over the middle of freshly prepared PDA plates, already seeded with the respective test microorganisms and gently pressed down on to the agar. Sterile distilled water was added on the discs to provide negative control. The plates were left for 30 minutes at room temperature to allow the oil diffusion, turned upside down and were incubated at 27^{o}C for 3 days. At the end of the incubation period, antifungal activity was evaluated by measuring zone of complete inhibition (including diameter of the disk) against the test fungi using a ruler. All treatments consisted of three replicates, and the averages values were determined.

4.7.2 Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of all the essential oils was conducted by agar dilution method (Hammer *et al*., 1999, Cao *et al*., 2009; Rusenova and Parvanov, 2009). The agar dilution method followed that approved by the National Committee for Clinical Laboratory Standards (NCCLS) with little modification: first stock solutions of the oils were prepared in 2% Tween-80 and different concentration of the oils from the stock ranging from 2% (v/v) to 0.0078% (v/v) was prepared in sabroud-dextrose agar (SDA; Oxoid, England) and vortexed for 30 seconds.

The oils to be tested were added to the culture medium at a temperature of 30–40^{o}C. The content was poured into Petri-dishes and allowed to solidify at room temperature for 30 minutes. After the plate was solidified, one loop full of the fungi suspension containing

approximately 10^4 cfu was spot inoculated using sterile loop. 2% Tween-80 but no oil was used as a positive growth control. Inoculated plates were incubated for 72 hours at 28^{o}C. At the end of the incubation period, the plates were evaluated for the presence or absence of microbial growth. The MIC was determined as the lowest concentration of oil inhibiting the visible growth of each organism on the plates (Tullio *et al*., 2006; Cao *et al*., 2009; Goni *et al.,* 2009).

4.7.3 Spore germination inhibition assay

Different concentrations of the most effective essential oils were tested for spore germination inhibition of the assayed fungi. The fungi were grown on PDA medium for sporulation on Petri dishes for 5–7 days and the suspension was prepared in 10 ml sterile distilled water containing 0.1% (v/v) Tween 80 (for better spore separation; Tzortzakis and Economakis, 2007) by aseptically dislodging the spores with a sterile inoculating loop. The spore suspensions were aseptically filtered off the mycelia in a funnel containing sterile cotton wool and adjusted with sterile water to give a final spore concentration of approximately 10^4 spore/mL.

Various concentrations of the oils (20 µL, 15 µL, 10 µL and 5 µL) were added to 5 mL of nutrient broth in a small flat bottom flask and 1ml of the spore suspension was added to each flask. The flasks were then incubated for 24 h at 25°C on a rotary shaker (121 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 400 x magnification.

Each assay was performed three times and the extent of spore germination was assessed by looking for the presence of germ tubes. The nutrient broth without the essential oils was served as positive control. Results were expressed in terms of the percentage of spores germinated as compared to the control from the average of the triplicats.

4.7.4 Grain protection assay

In order to confirm the effectiveness of the oils in causing fungal inhibition and to suggest its possible practical application, sorghum grains were immersed in the essential oils, one of the most widely grown and consumed cereal crops in Ethiopia (Aduga, 2007) and tested using the methods of Montes-Belmont and Carvajal, 1998; Juglal *et al*., 2002; Atanda *et al*., 2007.

The grain protection assay was carried out using visibly healthy sorghum grains following the procedure outlined by Montes-Belmont and Carvajal, 1998; Juglal *et al*., 2002; Atanda *et al*., 2007. The grains were selected by sorting 120 seeds of the grain per treatment and were immersed in the essential oils for 30 min, dried for another 30 min at room temperature and distributed in three Petri-dishes with sterile wet cotton wool. They were then inoculated (sprayed) with fungal spore suspension of *A.flavus* and *A.niger* that was prepared by scraping spore material from the surfaces of the colonies by sterile loop (approximately 10^4 spores per mL) and incubated at a temperature of 27^OC for seven days. Binocular microscope (40 x magnifications) was used to see growth of the tested fungi on the surface of the grain.

To examine proliferation of the fungi into the kernel, the grain was surface sterilized using 1% commercial sodium hypochlorite solution for 2 min to surface sterilize the grains (Amare, 2002;

Dikbas *et al*., 2008) and subsequently rinsed three times in sterile distilled water, dried for an hour over sterile filter paper and placed on freshly prepared PDA plates (30 grains each) using ethanol dipped and flamed forceps. The plates were then incubated for 3 to 5 days and the effects of each oil were observed for fungal growth from the grains. The percentage of contaminated grains was obtained from three replications.

4.7.5 Optimum protective dosage of the essential oils

The same procedure as described in section 4.7.4 was followed but at different concentrations of the most effective essential oils (cinnamon and thymus) at a concentration of 2%, 3%, 4%, 5%, 6%, 7%, and 8% was prepared using hexane as a solvent. The range of concentration of the oils used in the present study (2% - 8%) was based on concentration ranges adopted from a previous study of sweet basil (Atanda *et al*., 2007) oil at concentration that is known to be safe and acceptable sensory levels.

4.8 Data analysis

All the measurements were replicated three times for each assay and the results are presented as mean ± SD. The statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Post Hoc Multiple Comparison Tests using statistical software (SPSS) package version 15.0 for windows and P values < 0.05 were considered as significant.

5 RESULTS

5.1 Screening for antimicrobial activity using disk diffusion assay

All essential oils extracted from the common herb and spice plants have shown antifungal activity on the test organisms, except *Piper nigrum* that did not inhibit the growth of the tested fungi (Table 2). The essential oils from the remaining spice and herbs showed from weak to strong antifungal activity. Essential oil extracts from *Thymus schimperi* (thymus) and *Cinnamomum zeylanicum* (cinnamon) were found to be the most effective as compared to the others revealed by the broader zone of inhibition followed by *Allium sativum* (garlic). *Thymus schimperi* has shown the greatest inhibition zone diameter of 62.3 mm against *A.niger* standard which is the largest zone diameter recorded in this study.

The three essential oils, *Zingiber officinale (*ginger), *Citrus limon* (lemon) and *Laggera tomentosa* (keskese) had comparable results and showed a lesser inhibitory effect relative to the two most effective essential oils. Result of ANOVA showed that there is no significant difference between the three essential oils against *Aspergillus flavus* standard and *A. niger* standard (Table 2). All the tests were conducted in parallel to the control which didn't show any fungal activity. All of the assayed fungi were similarly (almost equally) sensitive to each of the respective essential oil extracts as proofed from the results of one-way ANOVA.

Table 2. Antifungal activity of seven essential oils against the tested fungi using disk diffusion assay. (Mean inhibition zone diameter including disk diameter)

Tested fungi	Inhibition zone diameter in mm (Mean ± SD)						
	Cinnamon EO	Thymus EO	Ginger EO	Keskese EO	Lemon EO	Garlic EO	Piper nigrum
A. flavus local isolates	35.57±0.60 (+++)a	58.33±0.42 (+++)	11.0±1.00 (+)c	11.5±1.32 (+)c	12.5±0.50 (+)d	20.33±0.58 (++)e	0.00± 0.00 (-)f
A. flavus standard	42.33± 0.58 (+++)	61.00±1.00 (+++)b	12.5±0.50 (+)d	13.50±0.5 (+)d	13.33±0.58 (+)d	23.0±1.00 (+)	0.00± 0.00 (-)f
A.niger local *isolates*	35.47±0.70 (+++)a	61.00±1.00 (+++)b	11.0±1.00 (+)c	7.8±0.280 (+)	13.17±0.29 (+)d	20.0±1.00 (++)e	0.00±0.00 (-)f
A. niger standard	38.33± 0.58 (+++)	62.33±1.52 (+++)	14.5±0.50 (+)d	12.5±0.50 (+)d	15.33±0.58 (+)d	26.0±1.00 (++)	0.00± 0.00 (-)f

(-) essential oils having no antifungal activity

(+) essential oils having weaker antifungal activity

(++) essential oils having intermediate activity

(+++) essential oils having stronger antifungal activity

* Means with the same superscripts are not significantly different ($P < 0.05$)

(EO = essential oil)

Fig.4. Growth inhibition zone diameter on *Aspergillus niger* and *Aspergillus flavus* by cinnamon essential oil

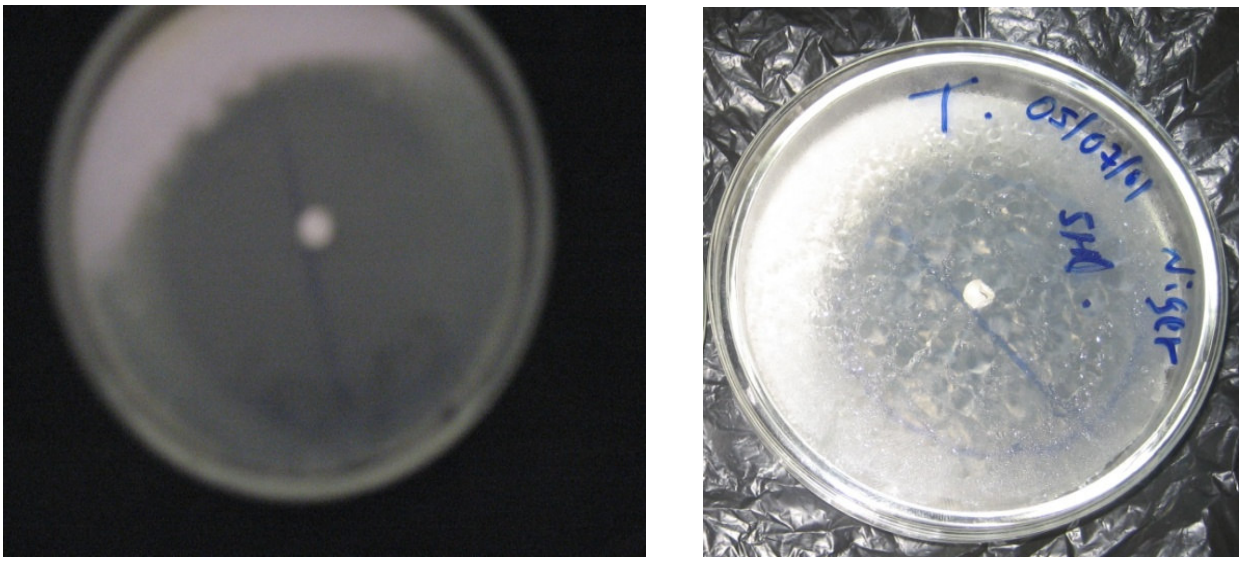


Fig.5. Growth inhibition zone diameter on *Aspergillus niger* and *Aspergillus flavus* by thymus essential oil

5.2 Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of all the essential oils was determined by agar dilution method (Table 3). All the essential oils inhibited the mycelia growth at a concentration of less than 2% except *piper nigrum* that did not inhibit the growth of *Aspergillus flavus* and *A. niger* local isolates at concentration less than 2%, while it inhibited the standard organisms at a concentration of 1%. As can be noted from the table, cinnamon essential oil inhibited growth of the organisms at a very low concentration compared to the others followed by thymus essential oil.

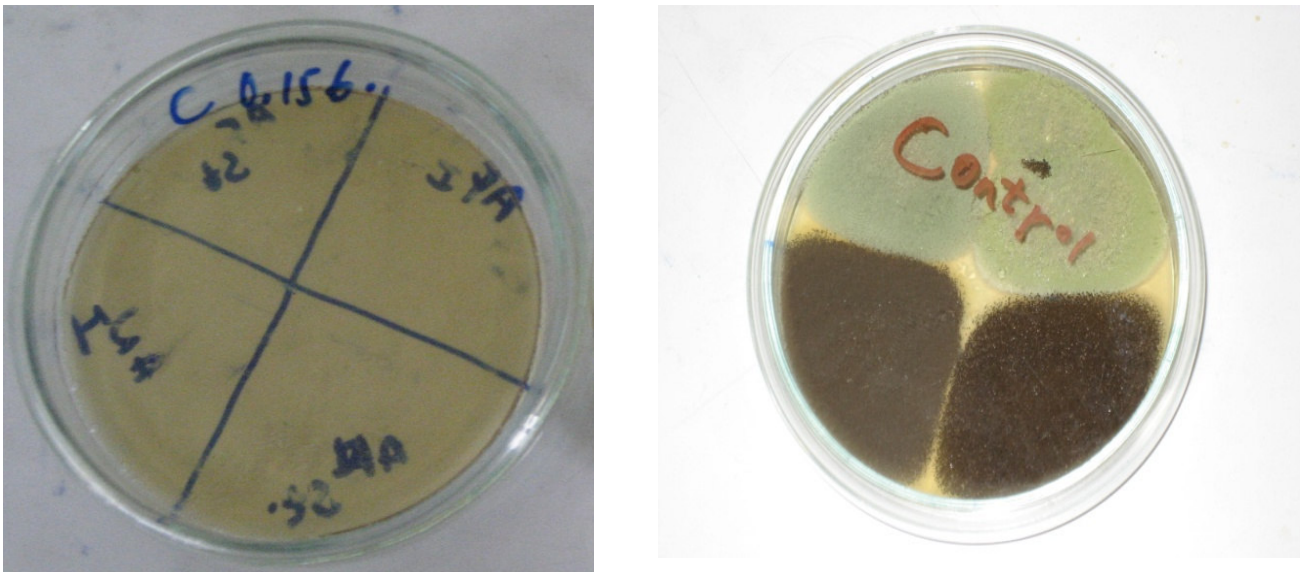
The lowest concentration of the cinnamon essential oil at which *Aspergillus flavus* standard was unable to grow was found to be 0.0078 % even after further incubation for another three days. Its MIC for the rest of the tested organisms (*Aspergillus flavus* isolates*, A. niger* isolates and *A. niger* standard) was found to be 0.0156%.

Thymus essential oil is the second most mycelia growth inhibitor with regard to this assay. It has the lowest MIC value of 0.0315% (v/v) against *Aspergillus flavus* standard. Generally from the table, the test organisms are sensitive to all the essential oils and the concentration at which they are unable to grow under the test condition varies (Table 3).

Table 3. Minimum inhibitory concentrations (MIC) of the selected essential oils against the tested microorganisms.

Details of plant oils			Test organisms and corresponding MIC (%)			
Plant species	Common name	source	AFI	AFS	ANI	ANS
Cinnamomum Zeylanicum	Cinnamon	Jima/Tepi	0.0156	0.0078	0.0156	0.0156
Thymus schimperi	Thymus	Chancho	0.0625	0.0315	0.0625	0.0625
Zingiber officinale	Ginger	Jima/Tepi	0.5	0.125	0.25	0.25
Laggera tomentosa	Keskese	Chancho	1	0.25	0.5	0.5
Citrus limon	Lemon	Addis Ababa	1	0.5	1	1
Allium sativum	Garlic	Debrezeit	0.25	0.0625	0.125	0.125
Piper nigrum	Black pepper	Jima/Tepi	>2	1	>2	1

(AFI=*Aspergillus flavus* isolate; AFS= *Aspergillus flavus* standard; ANI= *Aspergillus niger* isolate; ANS= *Aspergillus niger* standard)



A B

Fig.6. MIC value of cinnamon (no visible growth observed on the plate, plate A) and the control assay (plate B)

5.3 Spore germination inhibition assay

In the present study, properties of the two most effective essential oils, thymus and cinnamon oils were studied for their potential to inhibit germination of spores of the fungi by broth dilution assay.

The results of the effects of both essential oils at concentrations of 1 to 4 µL/mL on the spore germination of *A. flavus* and *A. niger* isolates are shown in table 4. As can be noted from the table, the essential oils exhibited a strong inhibition of spore germination of the tested fungi by the assayed concentrations.

Table 4. Percentage of spore germination of the tested fungi (results expressed in percent of spore germinated in comparison with the control assay, Mean±SD) at different concentration of the most active essential oils.

Tested fungi	Thymus essential oils				Cinnamon oils			
	1 µL/mL	2 µL/mL	3 µL/mL	4 µL/ml	1 µL/mL	2µL/mL	3 µL/mL	4 µL/mL
A.flavus isolates	51.98±*0.*46	20.76±0.88	5.71±0.27[a]	0.00[b]	34.04±0.66	9.62± 0.40	0.00[b]	0.00[b]
A.niger isolates	39.76±0.46	19.05±1.01	0.00[b]	0.00[b]	28.07±0.37	5.06 ±0.11[a]	0.00[b]	0.00[b]

Means with the same superscript are not significantly different using Post Hoc multiple Comparison test ($P < 0.05$).

5.4 Grain protection assay

To confirm the effectiveness of the oils in causing fungal inhibition and to suggest its possible practical application, the inhibitory potential of oils from all plant materials were examined on sorghum grains.

The effects of the essential oils on sorghum grains could be categorized into four groups (Table 5). In the first group, the tested fungi were completely inhibited by cinnamon essential oil and almost completely by thymus essential oils. In the second group, garlic and ginger essential oils recorded scanty growth while in the third group lemon peel essential oil had minimal effect on the contaminated grains and in the fourth group black pepper and Keskese essential oils were almost similar to the control.

The result from the analysis of ANOVA shows that there is no significant difference between cinnamon and thymus essential oils, ginger and garlic essential oils and between black pepper, kesekse and the control (Table 5) on percentage contamination of the grain.

Table 5. Effect of the essential oils on sorghum grain protection against fungal invasion (Mean±SD)

Treatment (used at 5% concentration)	*A. niger*		*A. flavus*	
	Contamination (%)	Reduction of contamination (%)	Contamination (%)	Reduction of contamination (%)
Cinnamon	0 ± 0.00[a]	100	0 ± 0.00[a]	100
Thymus	0 ± 0.00 [a]	100	1.66±0.835 [a]	98.34
Garlic	29.72± 4.59 [b]	40.83	31.39± 2.09[b]	48.33
Ginger	25.00±1.67 [b]	45.83	25.56± 3.47 [b]	54.16
Black pepper	68.33± 2.21 [c]	2.50	80± 3.33[c]	0
Keskese	62.56±3.41	8.27	74.72± 6.02 [c]	5.28
Lemon	44.77± 9.61 [e]	25.83	48.61± 7.74[e]	31.66
Control	70.55± 2.68 [c]	-	80 ± 3.33[c]	-

Mean values followed by same superscripts within a column are not significantly different using Post Hoc multiple Comparison test ($P < 0.05$)

5.5 Optimum protective dosage

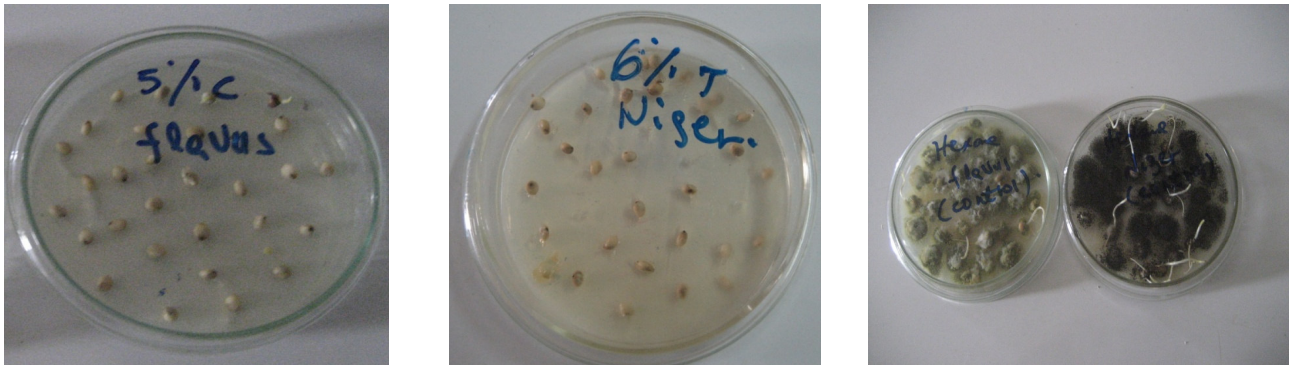
The optimal protection dosage of the two most effective essential oils studied was between 4% (v/v) and 6% (v/v) (Table 6). Both of the tested fungi were sensitive to 5% cinnamon essential oil; likewise both are sensitive to 6% thymus essential oil.

Table 6. Optimum sorghum grain protective dosage by the most effective essential oils (result expressed in Mean±SD).

Treatment	Contaminated kernels(%) at: Oil concentration (%)						
	2	3	4	5	6	7	8
CAF	45 ± 5.00^{a}	21.67 ± 3.81^{b}	2.5 ± 0.86^{c}	0^{c}	0^{c}	0^{c}	0^{c}
CAN	33.67 ± 3.21^{d}	18.33 ± 4.41^{b}	0^{c}	0^{c}	0^{c}	0^{c}	0^{c}
TAF	53.61 ± 5.55^{e}	40 ± 1.67^{f}	23.89 ± 2.55^{b}	3.33 ± 0.83^{c}	0^{c}	0^{c}	0^{c}
TAN	46.94 ± 3.93^{a}	33.61 ± 1.27^{d}	14.45 ± 2.09^{g}	0^{c}	0^{c}	0^{c}	0^{c}

(CAF=Cinnamon EO against *A.flavus*, CAN = Cinnamon EO against *A.niger*, TAF=Thymus EO against *A.flavus*, TAN=Thymus EO against *A.niger*)

(Means with the same superscript are not significantly different at $P < 0.05$)



A B C

Fig.7.Optimum protective dosage of the two most effective EOs (plate A = cinnamon essential oil, plate B = thymus essential oil and Plate C = the control plate)

6 DISCUSSION

The disk diffusion assay, which is generally used as a preliminary screening for antimicrobial activity prior to more detailed studies (Hammer et al., 1999), was employed to identify the most effective essential oils. The usefulness of this method is limited to the generation of preliminary quantitative data only (Lopes-Lutz et al., 2008; Burt, 2004) as the hydrophobic nature of most essential oils and plant extract components prevent their uniform diffusion through the agar medium (Burt et al., 2005).

The differences in the susceptibility of the test organisms to essential oils could be attributed to variation in the rate of essential oil constituent's penetration through the cell wall and cell membrane structures, the site where their antimicrobial action is suggested to be expressed (Tatsadjieu *et al*., 2009). The ability of essential oil to disrupt the permeability barrier of cell membrane structures and the accompanying loss of chemiosmotic control are the mostly likely reasons for its lethal action (Matasyoha *et al*., 2009).

Furthermore, the composition, structure as well as functional groups of the essential oils which play an important role in determining the antimicrobial activity of essential oils (Holley and Patel, 2005) is one of the major factor for difference in action of the essential oils. Usually compounds containing phenolic functional groups are the most effective (Carmo *et al*., 2008). The presence of these groups such thymol, carvacrol, cineole, α-pinene and eugenoin in thymus (Rasooli *et al*., 2008) and Cinnamaldehyde, 2-hydroxycinnamaldehyde and eugenol in cinnamon are responsible for their effectiveness.

In agreement with the present work, previous studies have revealed that several essential oils have shown important antimicrobial activity against bacteria, yeasts, dermatophyte and *Aspergillus* strains. For example, Lopes-Lutz *et al.* (2008) studied the antimicrobial efficacy of *Artemisia absinthium* L., *Artemisia biennis* Willd., *Artemisia cana* Pursh, *Artemisia dracunculus* L., *Artemisia frigida* Willd., *Artemisia longifolia* Nutt. and *Artemisia ludoviciana* Nutt. and reported that *Artemisia* oils have inhibitory effects on the growth of bacteria (*Escherichia coli*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*), yeasts (*Candida albicans*, *Cryptococcus neoformans*), dermatophytes (*Trichophyton rubrum*, *Microsporum canis*, and *Microsporum gypseum*), *Fonsecaea pedrosoi* and *Aspergillus niger*.

Ashenafi Goshu (2007) tested the efficacy of Ethiopian spice essential oils against food-borne bacteria and found that *Cinnamomum zeylanicum* and *Carum copticum* oils had the strongest antibacterial activity.

Dikbas *et al*. (2008) conducted an antifungal activity of *Satureja hortensis* oil on *Aspergillus flavus* and reported an inhibition zone of the oil to be 61mm by disk diffusion assay. Juglal *et al*. (2002) reported that clove oil (eugenol) was the most inhibitory oil against growth of *F.moniliforme* and *A.paraciticus* followed by cinnamon, oregano, mace, nutmeg turmeric and aniseed oils.

On the other hand, some of the present findings are in the contrary to past results from screening of some plant essential oils. Singh *et al*. (2008) tested the efficacy of ginger essential oils against *A. flavus* and *A. niger* and reported a higher inhibition zone diameter of 44.4 mm on *A. flavus* and 27.9 mm on *A. niger*.

The contrary result may be attributed to the differences in the essential oil contents of the test plants that can be influenced by different agro-ecological conditions. Such differences with regard to local climate and environmental conditions have been well documented. Burt *et al*. 2005; Clausen and Yang, 2008, out lined that the geographical area of production and weather conditions during the growing season and particularly at harvest can have a significant effect on the content of active ingredients found in plant.

The amount of rainfall and daylight to which plants are exposed, and the soil conditions, humidity, elevation, even the time of day at which the plants are harvested have an effect on chemical constituents of the plants (Burt *et al*., 2005; Nguefack *et al*., 2009).

Generally, essential oils produced from herbs harvested during or immediately after flowering possess the strongest antimicrobial activity (Burt, 2004). Furthermore, methods of extraction, the bioassay used and differences in the types of test strains can be attributed to different results (Holley and Patel, 2005; Kaefer and Milner, 2008; Kumar *et al*., 2008**).**

Although *Piper nigrum* (black pepper), is traditionally used as a remedy for various ailments such as carminatives and antimicrobial activities (Magness *et al*., 1971), the present study did not show any antimicrobial activities against the tested organisms. Dorman and Deans, (2000) suggested that absence of phenolic compounds from volatile oil is associated with poor or no antimicrobial properties against food-borne pathogens.

In this study, the minimum inhibitory concentration (MIC) of all the essential oils under considerations was determined. Though different researchers defined MIC differently, here it is defined as the lowest concentration of oil inhibiting the visible growth of test organism on the plates (Hammer *et al*., 1999; Tullio *et al*., 2006; Cao *et al*., 2009).

Consequently, visible growth of the test organisms on the plates was assessed and cinnamon essential oil was found to be the strongest mycelia growth inhibitor by the assay technique followed by thymus essential oil. The MIC value of cinnamon is 0.0078% against *Aspergillus flavus* standard and that of thymus oil is 0.0315%. This indicates that essential oil extracts from *Cinnamomum zeylanicum* is more potent than that of *Thymus schimperi.*

This result is in line with the work of Hammer *et al*. (1999) who had reported that *Thymus vulgaris* showed the lowest MIC value of 0.03% (v/v) against *C.albicans.* Rusenova and Parvanov (2009), studied the antimicrobial activity of twelve essential oils against twelve Gram-positive bacteria and yeasts (*Candida albicans* and *Malassezia pachydermatis*) and reported that cinnamon, oregano, lemongrass, and thyme exhibit the strongest activity against the selected strains tested and yeasts were more sensitive to cinnamon, oregano and lemongrass with MICs of 0.03-0.06% (v/v).

However, agar disk diffusion method for screening test in this study revealed that *Thymus schimperi* oil showed larger inhibition zone than *Cinnamomum zeylanicum* on all the tested fungi. This may due to difference in the viscosity of the two essential oils that impede the mobility of the oils on the agar surface. The viscosity of *Cinnamomum zeylanicum* oil is

medium to watery, whereas *Thymus schimperi* oil resembeles purely water-like, indicating that *Thymus schimperi* oil is highly mobile than *Cinnamomum zeylanicum* oil (Order, 2007).

The result of spore germination inhibition assay shows that fungal spore germination was highly suppressed by both the effective essential oils. The inhibitory effect of the oils increased in proportion to their concentrations. A 100% inhibition (zero spore germination) was found at 3 µL/mL on *A. niger*, by both the essential oils. Thymus essential oil completely inhibited spore germination of *A. flavus* at 4 µL/mL while cinnamon essential oil completely inhibited germination of *A. flavus* at 3 µL/mL which shows that *A. flavus* was more sensitive to cinnamon essential oil than thymus. In addition, it was noted that no hyphae growth was observed by further incubating the treatment for another 36 hours at concentrations where complete inhibition occurred.

The cinnamon essential oil inhibited the spore germination of the tested fungi at a concentration less than that of thymus which shows that cinnamon oil is more potent than thymus. This can be attributed to the dispersion and persistence of the oil in the nutrient broth (Ashenafi, 2007).

In agreement with this work, previous researches reported the inhibition of spore germination caused by some essential oils was in a dosage response manner (Rasooli *et al*., 2008; Carmo *et al*., 2008). Chalfoun *et al*., (2003) analyzed the inhibitory effect of ten powdered spices on mycelial growth, sporulation and production of aflatoxins by toxigenic fungi at the concentrations of 1, 2, 3 and 4%, and reported that clove and cinnamon powders promoted

total inhibition of *A. niger* mycelia development in all doses tested. They also showed that level of inhibition was always proportional to the concentration used.

Tzortzakis and Economakis (2007) tested the impact of lemongrass (*Cympopogon citratus L.*) essential oil against key post harvest pathogens (*Colletotrichum coccodes, Botrytis cinerea, Cladosporium herbarum, Rhizopus stolonifer and Aspergillus niger*) and reported that lemongrass oil-enrichment on fungal sporulation in PDA revealed spore production to be significantly inhibited when compared with equivalent plates stored in ambient air, with spore production depressed by 70% for *B. cinerea*, 58% for *C. coccodes,* 41% for *A. niger*, 40% for *C. herbarum*, and 35% for *R. stolonifer* at 25 ppm. Moreover, spore production was completely inhibited at the highest oil concentration (500 ppm) examined for all of the pathogens.

Spore germination is one of the most important life cycle stages of fungi as the spore is the principal agent of dispersal. Nutrients in the growth medium, especially carbon and nitrogen have a major effect on the growth and sporulation of fungi. Consequently, any consideration of the ecology or the spread of fungi must take fungus nutrition and spore germination into account. Hence the control of crop disease by protectant fungicides is, in essence, a matter of inhibiting the germination of spores (Yulia, 2005).

For many compounds, spore germination is the growth stage that is most sensitive to inhibition (Richard,et al., 2002). With this general background, the tested essential oils in this study were good spore germination inhibitors.

In a similar way to the screening of the essential oils by disk diffusion assay, in the grain protection assay cinnamon and thymus essential oils were found to be the most active in reducing contamination of the grain. Ginger and garlic essential oils have comparable effect unlike the result obtained by disk diffusion but black pepper has shown similar effect to the disk diffusion assay result. The findings were categorized in to four groups as described in section 5.4.

The variation in the result of the grain protection could probably be related to the volatility of the respective active compounds of the oils. For example, onion and garlic sulfides may not be retained for more than 24 h and their effect is only partial and if they don't kill all the spores, the remaining spores germinate and mycelia develop (Montes-Belmont and Carvajal, 1998).

Similar problem was also reported on black pepper (*P. nigrum*) essential oil that even if all the spores might be killed, the surviving one germinate and mycelia grow (Montes-Belmont and Carvajal, 1998).

The present finding is in agreement with the work of Montes-Belmont and Carvajal (1998) who used *Cinnamomum zeylanicum, Thymus volgaris* and *piper nigrum* for contaminated maize grains. They also used essential oil constituents of cineole, cinnamic aldehyde and linalool to obtain a reduction of 11.4%, 17.4% and 0%, respectively, for the contaminated maize grains. Juglal *et al*. (2002), studied the effectiveness of nine essential oils to control the growth of mycotoxin producing moulds and noted that clove, cinnamon and oregano were able to prevent the growth of *Aspergillus parasiticus* and *Fusarium moniliforme*, while clove (ground and essential oil) markedly reduced the aflatoxin synthesis in infected grains.

Bluma and Etcheverry (2008), studied impact of five essential oils on growth of *Aspergillus section Flavi* isolates in sterile maize grain at different water activity conditions and showed that there is a significant inhibitory effect on lag phase, growth rate, and aflatoxin accumulation by the oils. They also demonstrated that the effectiveness of these essential oils was influenced by changes in their concentrations, substrate water availability, and time of incubation.

From the study of the optimum protective dosage it is revealed that cinnamon essential oil prevented contamination of the grain by *A.niger* isolate at 4% (v/v) while it inhibited contamination of the grain by *A. flavus* isolate at concentration of 5% (v/v) dosage. On the other hand, thymus essential oil had a protective dosage of 6% (v/v) against *A.flavus* isolate and 5% (v/v) against *A.niger* isolates. This result again shows the potency of cinnamon essential oil over thymus.

The optimal protective dosage of $\leq$ 6% (v/v) was obtained for both *C.zeylanicum* and *T.schimperi* oils for treatment of sorghum grains.

As compared to the MIC levels (section 5.2) recorded for both the essential oils, the optimum dosage required to protect the grain from fungal contamination is higher. It was outlined that the levels of essential oils and their compounds necessary to inhibit microbial growth are higher in foods than in culture media and this is due to interactions between phenolic compounds and the complex food matrix (Omidbeygi *et al*., 2007; Tzortzakis and Economakis, 2007).

Furthermore, other factors such as pH environment, lipids that decrease activity of hydrophobic compounds and protein that may cause binding of some compounds influence the activity in food system and reduce the effectiveness of the active compounds (Thanaboripat *et al*., 2004).

It was suggested that fat in food could form a protective coat around microorganism, thereby protecting them from antimicrobial agents. Additionally the lipid fraction of the food absorbs the antimicrobial agent, thus decreasing the concentration in the aqueous phase and hence its antimicrobial action. Furthermore, the reduced water content in food compared to laboratory media could hamper the transfer of antimicrobial agents to the active site in the microbial cell (Omidbeygi *et al*., 2007).

In agreement to the present findings, studies in the past have demonstrated the efficacy of some essential oils in protecting grains. Atanda *et al*. (2007) studied the potency of sweet basil (*Ocimum basilicum*), cassia (*Cinnamomum cassia*), coriander (*Coriandrum sativum*) and bay leaf (*Laurusnobilis*) essential oils to protect sorghum grains and reported that sweet basil oil was fungistatic on *A. parasiticus* CFR 223 at 5% (v/v) which was found to be an optimum protective dosage. They also showed that sweet basil and cassia oil combinations gave complete inhibition of the fungus at dosages below their optimal levels when they were combined and suggested that the properties of the active principles in the combination containing the oils were maintained and indeed increased by a synergistic effect.

Furthermore they studied the feasibility of incorporating whole and ground basil in sorghum grains and found that the addition of whole and ground basil leaves markedly reduced aflatoxin contamination at 10% (w/w).

Thanaboripat *et al*. (2004) demonstrated that the *A. flavus* IMI 242684 growth and AFB production were inhibited for only 3 days at concentration of 1% of citronella essential oil whereas fungal growth and AFB1 production were completely inhibited for 28 days at concentration of 5% in maize grain with various concentrations of citronella essential oil (1–5%).

7 CONCLUSION AND RECOMMENDATION

7.1 Conclusion

In addition to boosting flavor, herbs and spices are known for their preservative, antioxidative, antimicrobial and various other medicinal values (Singh *et al*., 2008). With this background, it can be concluded from the present study that some of the herbs and spices grown in different regions of Ethiopia have strong potential to control microbial growth. *Cinnamomum zeylanicum* and *Thymus schimperi* essential oils possess better antifungal activity against two of the more important food spoilage microorganisms, but there are also some spices like *Piper nigrum* essential oils which do not have or possess poor antifungal activity.

Though, the antifungal activity of the remaining essential oils are less effective than the two most effective oils, their antimicrobial activity are not regarded as useless. It is documented in many literatures that they have comparable antimicrobial potential on the same or other microorganisms like gram positive and gram negative bacteria and yeasts.

The variability of antifungal action of the spice plants is attributed to the type and constituents of the essential oils and the difference from some previous reports could be attributed to the variation in ecological and weather conditions in which the spices are grown.

Generally, the levels of essential oils necessary to inhibit microbial growth are higher in foods than in culture media and this is due to interactions between phenolic compounds and the complex food matrix as well as other factors.

In the grain protection assay, thymus oil is one the strongest reducer of contamination of the grain by the sprayed fungi. The broad antifungal spectrum exhibited by this oil in this study provides its recommendation as ideal antimicrobial for food preservation. In addition, some literatures documented that thyme essential oil showed pronounced efficacy in arresting aflatoxin B1 production by the toxigenic strain of *A. flavus* and its safety limit is even much higher than the safety scale of some chemicals in control of storage pests. Based on this information and the abundant availability of the plant in the country, it can be pointed that people can use locally available resources as an alternative to other fungicides for inhibiting growth of spoilage molds on some agricultural commodities such as sorghum grains and prolong their shelf life during storage. This would provide economic value of the study.

7.2 Recommendations

The evaluation of the plants used in this study on other pathogenic microorganisms has to be made to point their potential efficacy. There are also various types of other spices and herbs grown in the country. So the evaluation of these plants against fungi and others microbes is recommended.

Synergistic effects of the combinations of the essential oils to maximize the antimicrobial activity and to minimize the concentrations required to achieve a particular antimicrobial effect need to be studied.

There is need for further in depth studies to formulate essential oils of common herb and spice plants grown in Ethiopia into an efficient, cost effective and ecologically friendly biopreservative to improve the storage life of some of our staple foods especially grains.

Since higher concentrations of plant essential oils are generally required when added to foods, the application of essential oils in food may be limited due to changes in organoleptic and textural quality of food or interactions of essential oils with food components. Therefore, an optimized low dose should be applied to maintain product safety and shelf-life, thereby minimizing the undesirable flavor and sensory changes that might be associated with the addition of high concentrations of essential oils.

In addition to using natural preservatives to protect foods, the prevention strategies which are predominantly based on using the HACCP approach are recommended to control the pre- and post-harvest food losses.

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