VAPOUR-PHASE SYNERGISTIC ANTIFUNGAL ACTIVITY OF SELECTED ESSENTIAL OILS AGAINST *ASPERGILLUS* SP., *CURVULARIA* SP. AND *FUSARIUM* SP.

By
MR. Alex AHEBWA

A Thesis Submitted in Partial Fulfillment of the Requirements
for Master of Science (BIOSCIENCE FOR SUSTAINABLE AGRICULTURE)
Graduate School, Silpakorn University
Academic Year 2019

[illegible] [illegible] *Aspergillus* sp.,
Curvularia sp. [illegible] *Fusarium* sp.

โดย
MR.Alex Ahebwa

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรวิทยาศาสตรมหาบัณฑิต
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Advisor	Associate Professor Mana Kanjanamaneesathian

Graduate School Silpakorn University in Partial Fulfillment of the Requirements for the Master of Science

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MR. ALEX AHEBWA : VAPOUR-PHASE SYNERGISTIC ANTIFUNGAL ACTIVITY OF SELECTED ESSENTIAL OILS AGAINST *ASPERGILLUS* SP., *CURVULARIA* SP. AND *FUSARIUM* SP. THESIS ADVISOR : ASSOCIATE PROFESSOR MANA KANJANAMANEESATHIAN

Grain storage plays a crucial role in ensuring food security to many households around the world. However, food insecurity continues harm the most vulnerable groups of our communities who are farmers themselves due to grain losses to fungal infection before and during storage. Essential oils (EOs) have demonstrated a broad spectrum fumigant antifungal activity against most storage fungi and generally acceptable as safe. This study investigated the *in vitro* efficacy of individual and mixed EOs vapour against mycelial growth of *Aspergillus* sp., *A. niger*, *Fusarium* sp. and *Curvularia lunata* and inhibition of aflatoxin production by *Aspergillus* sp. The fungi were isolated from dried rice and maize grains by blotting paper and direct agar plating methods. EOs were extracted by hydrodistillation method from dried buds of clove (*Syzygium aromaticum*), fruit peels and leaves of makrut lime (*Citrus hystrix*), fresh leaves of eucalyptus (*Eucalyptus* sp.) and fresh stems of lemongrass (*Cymbopogon citratus*) grown in Thailand. Antifungal and antiaflatoxin assays for individual and combined EOs were carried out in plastic cups by disc volatilisation technique. Minimum inhibitory concentration (MIC) and fractional inhibitory concentration index (FICI) values were determined. Results showed that individual 10 µL EO vapour of lemongrass (LG) and makrut lime leaf (ML) showed a complete mycelial growth inhibition for all tested fungi and aflatoxin production inhibition. LG and ML EOs had the lowest MIC values of 0.09 µL/mL against *C. lunata* and 0.19 µL/mL against *Aspergillus* sp., *A. niger* and *Fusarium* sp. Eucalyptus produced the lowest growth inhibition (MIC: 0.56 to >0.74 µL/mL) and was ineffective against *A. niger*. In combination, the EO vapours of LG and ML acted synergistically (FICI =0.5) against all the tested but only additively against aflatoxin production. *C. lunata* was most sensitive pathogen to all the EOs individually and in combination. The combination of LG and ML EOs showed synergism against all the tested fungi with a fractional inhibitory concentration index (FICI) of 0.5. Lemongrass and makrut lime leaf EOs have potential, individually and in combination, against storage fungal growth and aflatoxin production by *Aspergillus* sp. Further studies are needed to assess the suitability of individual and combined ML and LG for fumigation against fungi in a real grain storage setting used by small scale farmers.

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Alex AHEBWA

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviation	Description
µL	Microlitre
mL	Millilitre
L	Litre
g	Gram
kg	Kilogram
mm	Milimetre
nm	Nanometre
et al.	And others
β	Beta
α	Alpha
&	And
sp.	Species
^0C	Degrees centigrade
h	Hours
psi	Pound-force per square inch
Na_2SO_4	Sodium sulphate
NaOH	Sodium hydroxide
HCl	Hydrochloric acid
CO_2	Carbondioxide
v/v	Volume to volume ratio
w/w	Weight to weight ratio
w/v	Weight to volume ratio
%	Percent
vs	Versus
EO	Essential oil
MIC	Minimum inhibitory concentration
SID	Smallest inhibitory dose
FIC	Fractional inhibitory concentration
FICI	Fractional inhibitory concentration index

No. 1	Number one
PDA	Potato dextrose agar
CMA	Coconut milk agar
LAV	Liquid ammonia vapour
TLC	Thin layer chromatography
AFPA	*Aspergillus flavus* and *parasiticus* agar
UV	Ultraviolet
GC-MS	Gas chromatography coupled with mass spectrometry
ML	Makrut lime leaves
LG	Lemongrass stems
MP	Makrut lime fruit peels
C	Clove buds
EU	Eucalyptus leaves
CRD	Completely randomized design
ANOVA	Analysis of variance
DMRT	Duncan multiple range test
ASAT	Faculty of Animal Sciences and Agricultural Technology
OECD	Organisation for Economic Cooperation and Development
FAO	Food and Agriculture Organisation
FDA	Food and drug administration
WHO	World Health Organisation
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
USD	United States dollar
IARC	International Agency for Research on Cancer
CDC	Centres for Disease Control
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic acid

CHAPTER ONE

INTRODUCTION

1.1 Rationale of the study

Cereal grains are important foods globally. Rice (*Oryza sativa*), maize (*Zea mays*) and wheat (*Triticum aestivum*) provide at least 30% of the daily food calories to more than 4.5 billion people in developing countries (OECD/FAO, 2019). Rice is the staple food for Asian people, and together with corn (baby corn and milled corn), are major raw materials used in food and feed industries (Prasertsri, 2019). However, a number of abiotic and biotic stresses have caused significant losses especially under storage (Kumar & Kalita, 2017). Although most storage methods have been designed to protect grains from deterioration, the stored grains have been damaged by various fungi during storage (Mendoza et al., 2017). The fungi such as *Aspergillus sp., Curvularia sp., Fusarium sp., Penicillium sp.* and *Trichonosis caudate* were reported to cause economic losses worldwide (Douglas & Tullis, 1950; Manandhar et al., 2018; Williams & McDonald, 1983). It was reported that seedling mortality and poor seed germination of rice was associated with fungal infection (Arshad et al., 2009). Moreover, some *Aspergillus* sp. are concomitant with some of the most dangerous mycotoxins called aflatoxins

Aflatoxins are difuranocoumarin derivatives produced in a polyketide pathway by many strains of *A. flavus* and *A. parasiticus*, in particular, *A. flavus* which is a common contaminant in agriculture. Aflatoxins B_1, & G_1 and their dihydroxy derivatives B_2 & G_2 naturally contaminate food and feeds (Oluwafemi et al., 2012). The presence of Aflatoxin M_1 and M_2, the 4-hydroxy metabolites of aflatoxins B_1 and B_2 respectively in biological fluids including milk and tissues was reported to be due feeding of animals on *A. flavus* contaminated feed stuffs (Bellio et al., 2016). When ingested, inhaled, or absorbed through the skin, aflatoxins have carcinogenic, hepatotoxic, teratogenic, and mutagenic effects on human health, even at very small concentrations**.** The United States Food and Drug Administration (FDA) established 20 parts per billion (ppb) as the maximum acceptable level of aflatoxin in all foods other than milk. World bank estimated the economic losses encountered by Africans exporting foods to Europe, due to aflatoxin regulations, to be about USD 670 million

annually (Otsuki et al., 2001) while Davis and Lubulwa (1994) estimated an annual crop and livestock loss of over USD 700 million.

Synthetic chemical fungicides have been effectively used in controlling fungi and their metabolites (Kumar & Kalita, 2017). Synthetic fungicides such as mancozeb are major grain protectants used in some countries (Tawatsin, 2015) despite the various reported health risks they pose to the farmers and agricultural workers (Corsini et al., 2005; Dhananjayan & Ravichandran, 2018; Panuwet et al., 2008; Thundiyil et al., 2008) and the government legislations to reduce use of these chemicals in agriculture production (Panuwet et al., 2012). There has been a reported increasing demand for safer foods with particular interest in organic products (Jiumpanyarach, 2018; Kongsom & Panyakul, 2016) and increased investment in biopesticides use, registration and research (Kanjanamaneesathian & Nimanong, 2019; Thobunluepop et al., 2008).

Essential oils (EOs) also known as “essence oils” and “volatile oils” are steadily emerging as crop, food protectants and preservatives because of the increased public demand for safer and natural food. EOs are biosynthetic oily products of secondary metabolism in living organisms especially plants’ various parts such as roots, rhizomes, stems, leaves, flowers, flower buds, fruits and seeds. They can be extracted from the living tissue matrix by hydro-, and steam-distillation, use of organic solvents, and supercritical CO_2 extraction (Danh et al., 2013; Memarzadeh et al., 2015). They are composed of various chemical compounds (differing in structure and function) that give them their beneficial characteristics such as fungicidal, bactericidal, virucidal, insecticidal, medicinal and application in the food and cosmetics industries (Bakkali et al., 2008; Elshafie & Camele, 2017; Irshad et al., 2019). EOs are generally acceptable as safe for use in food, however concerns for their potential health risks have attracted various food and drug regulatory institutions to limit acceptable daily intakes (ADIs) when added to food (Baser & Buchbauer, 2015; Hashemi et al., 2017). This has been because their major dispensation methods by direct contact through spraying and seed treatment (Karaca et al., 2017; Nguefack et al., 2008; Okonkwo & Okoye, 1996; Riccioni et al., 2019; Somda et al., 2007) require large quantities to achieve control purpose as well may leave residues on food surfaces that affect their sensory properties (Serra et al., 2016) and can be phytotoxic

(Boukaew et al., 2017; Somda et al., 2007). Therefore, strategies to reduce the quantity of EOs in direct contact with grains, while maintaining or improving their efficacy as antimicrobial agents need further research.

EOs are complex mixtures of volatile secondary metabolites which can vaporise at ordinary temperatures (Burt, 2004). Their application by vapour phase has potential to be an appropriate delivery system because control efficacy can be achieved with a reduced application quantity (Avila-Sosa et al., 2012; Tyagi & Malik, 2011; Velázquez-Nuñez et al., 2013). Moreover, the volatile compounds in vapour are reported to act additively/synergistically to increase the efficacy against microorganisms (Ji et al., 2019). The application of two EOs simultaneously, with each EO originating from separate sources, showed an increased efficacy at the reduced concentration compared with the oils applied individually (Edris & Farrag, 2003; Hossain et al., 2016; Stević et al., 2014). Sukatta et al. (2008) reported a higher antifungal activity for a combination than individual of clove and cinnamon EO vapours in inhibiting the growth of postharvest fungi isolated from berries of white Malaga grapes from Thailand. In Iran, duo and triple combinations of thyme, cinnamon, rosemary and marjoram EO vapours were synergistic against *Botrytis cinera* and *Penicillium expansum* in vitro and *in vivo* (Nikkhah et al., 2017).

The aim of this study was to assess the efficacy of selected EO vapours, applied both individually and in combinations, 1) to inhibit growth of *Aspergillus* sp., *Curvularia* sp. and *Fusarium sp*., 2) to inhibit aflatoxin production by *Aspergillus* sp.

1.2 Objectives of the study

1. To determine the efficacy of EOs vapours in suppressing fungal growth with focus on *Aspergillus* sp., *Curvularia* sp. *and Fusarium* sp.
2. To assess synergistic potential of EOs vapours against growth of the fungi.
3. To assess potential of selected EOs to inhibit aflatoxin production by *Aspergillus* sp

1.3 Hypotheses of the study

1. Essential oil vapours have effective as antimicrobial agents
2. Essential oils with various chemical constituents in their vapour phase can combine to produce synergistic efficacy against fungal growth.
3. Effective EO vapours against fungal growth can inhibit aflatoxin production by *Aspergillus* sp.

CHAPTER TWO

LITERATURE REVIEW

2.1 Global importance of grains and peanuts

Maize, wheat and rice are the most produced grains for food and feed all over the world. In parts of Africa and Mesoamerica, maize alone contributes over 20% of food calories (Shiferaw et al., 2013) while rice is a staple food to more than half of the global population. In Sub-Saharan Africa (SSA), Central America and other developing world, demand for maize is majorly for human consumption as compared to developed world where majority of maize. The changing trends in the world's major economies coupled with urbanisation have increased diversification in feeding patterns – demanding more of livestock products (Pingali, 2015). And consequently, most of the maize produced in the United States, China and Brazil goes to livestock production and industry. This makes maize a very important cereal crop to the world as people depend on it directly as food (mostly in less developed countries) and indirectly through livestock and industrial products. Peanuts also known as groundnuts (*Arachis hypogaea* L.) are among the most protein rich crops produced in all parts of the world mainly for food in different forms and for oil production (Arya et al., 2016). China and India are the world's leading producers of peanuts but peanuts are major exports of most small scale producing countries (Valentine, 2016).

Chegere (2017) reported 11.7 percent of maize from small holder farmers in SSA to be lost through postharvest handling practices which fall with the range of 10-20 percent reported by the World Bank. The World Bank in 2011 equated the loss to USD 4 billion and a caloric value deprivation of 48 million people (Zorya et al., 2011). And storage remains a key stage of postharvest losses in developed and developing countries with moulds and insects ranking highest in quantity and quality losses.

2.1.1 Conditions for storage that lead to fungal spoilage in grains and nuts

The conditions for storage ecosystem are influenced by several factors. These can be categorised as abiotic and biotic stresses, and socioeconomic factors. Abiotic stresses include temperature, relative humidity, moisture content, water activity, air composition (O_2 or CO_2) (Dubale et al., 2014; Eeckhout et al., 2013). Biotic stressors

include moulds, insects, rodents, mites and birds. These factors are interdependent and work hand-in-hand to influence the development of moulds in stored maize (Suleiman et al., 2013).

2.2 Genus *Aspergillus* in agriculture

Aspergillus is a group of ubiquitous, generally asexually reproducing with characteristic conidiophore bearing a vesicle that produces spores (conidia). *Aspergillus* sp. have been largely isolated from the soil, crop debris and food substances and consequently can infect susceptible crops such maize (*Z. mays* L.) in the field (Figure 1), through harvest and postharvest handling and during storage (Abbas et al., 2009). Morphological (macroscopic and microscopic), biochemical (analysing production of exudates and metabolites) and molecular techniques have been used in the identification of fungi to species level (Klich & Pitt, 1988; Pitt & Hocking, 1997; Zulkifli & Zakaria, 2017). Morphological and biochemical analyses are the commonest used and culturing plus microscopy are essentially the readily available means for identification of fungi in the laboratory (Camiletti et al., 2017; Perrone et al., 2007).

The significance of *Aspergillus* in agriculture is mainly due to their potential to produce poisonous substances called mycotoxins that are harmful to humans and animals. Closely related species – *A. flavus, A. parasiticus* and *A. nomius* (Samson et al., 2014) are known to produce most noxious toxins of all mycotoxins – aflatoxins and have been labelled as group one agents for cancer by the International Agency for Research on Cancer (IARC) (IARC, 2012).

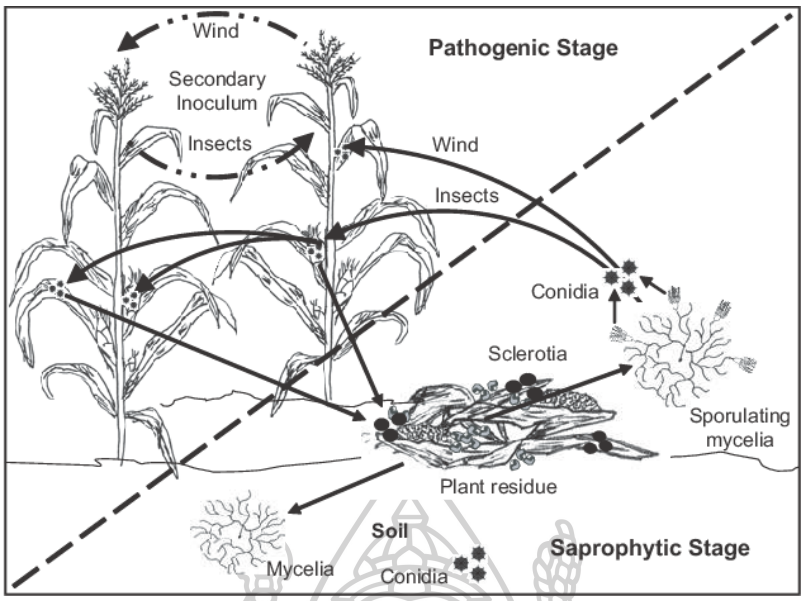


Figure 1 Lifecycle of *A. flavus* in a maize cropping system showing the saprophytic and pathogenic stages of the fungal ecology

Source: Abbas et al. (2009)

2.3 Aflatoxins contamination in grains and associated health, economic losses

Aflatoxins are di-furanocoumarin derivatives produced in a polyketide pathway by many strains of *A. flavus* and *A. parasiticus*, in particular, *A. flavus* which is a common contaminant in agriculture. Aflatoxin B_1, G_1 and their dihydroxy derivatives B_2 and G_2 (Figure 2) naturally contaminate food and feeds (Oluwafemi et al., 2012). Aflatoxin B_1 is the most toxic mycotoxin known, labelled group one carcinogen by IARC. The incidence of aflatoxin B_1 metabolite (aflatoxin M_1) in milk (Bellio et al., 2016) has also furthered the interest of research in aflatoxins and their control.

Aflatoxins contaminate a range of food and feed stuffs and their metabolites have been identified from products of animals (especially milk) consuming these toxins and have been regarded by joint food and agriculture organisation/world health organisation expert committee on food additives (JECFA) as the most serious toxicants of maize food and feed (JECFA, 2016). Direct exposure to the aspergillus propagules by both humans and animals is implicated in respiratory failure of the victims. Several fatalities have been reported in relation to *Aspergillus* and their

metabolism since the first incidence was reported in 1960 (CDC, 2004; Krishnamachari et al., 1975; Ngindu et al., 1982; Okoye et al., 1989; Richard, 2008).

Toxicity of aflatoxins has been largely reported in areas of cytotoxicity and hepatotoxicity (Liu et al., 2015; Sun et al., 2015) for major mycotoxins with less regard to phytotoxicity. Phytotoxicity however has also been sparsely reported to cause significant losses in maize production (Ismaiel & Papenbrock, 2015; McLean, 1995). McLean et al. (1992) reported that aflatoxins significantly disrupted maize seed germination and seedling growth by distorting the embryo and inhibition of radicle and shoot elongation. Aflatoxin B_1 significantly reduced the DNA and RNA, and protein content of germinating maize seed in addition to reduction in the chlorophyll content (Sinha & Prasad, 1996). In a recent study, RNA *in situ* hybridization revealed a possible down regulation of sucrose synthase-encoding gene in maize seeds inoculated with *A. flavus* and *F. verticillioides* (Shu et al., 2015). In terms of hepatotoxicity and cytotoxicity, mycotoxins have been reported to have four basic kinds of toxicity viz. acute, chronic, mutagenic and teratogenic (Benkerroum, 2020; Pitt, 2000). Acute aflatoxin poisoning leads to deterioration of liver and kidney and sometimes leading to death (CDC, 2004). Some toxins affect the genome morphology and gene expression and can be regarded as mutagenic or teratogenic (Bressac et al., 1991; Mary et al., 2017). In general, aflatoxins have been regarded as serious health hazards to human health (CDC, 2004; Chain et al., 2020; IARC, 2002; JECFA, 2016). The commonest detection and isolation methods for aflatoxins are chromatography, immunochemical, selective media and molecular characterisation (Norlia et al., 2019; Rodrigues et al., 2009). A combination of one or all the techniques have been employed to increase efficiency in the detection and isolation of the mycotoxins (Kamle et al., 2019).

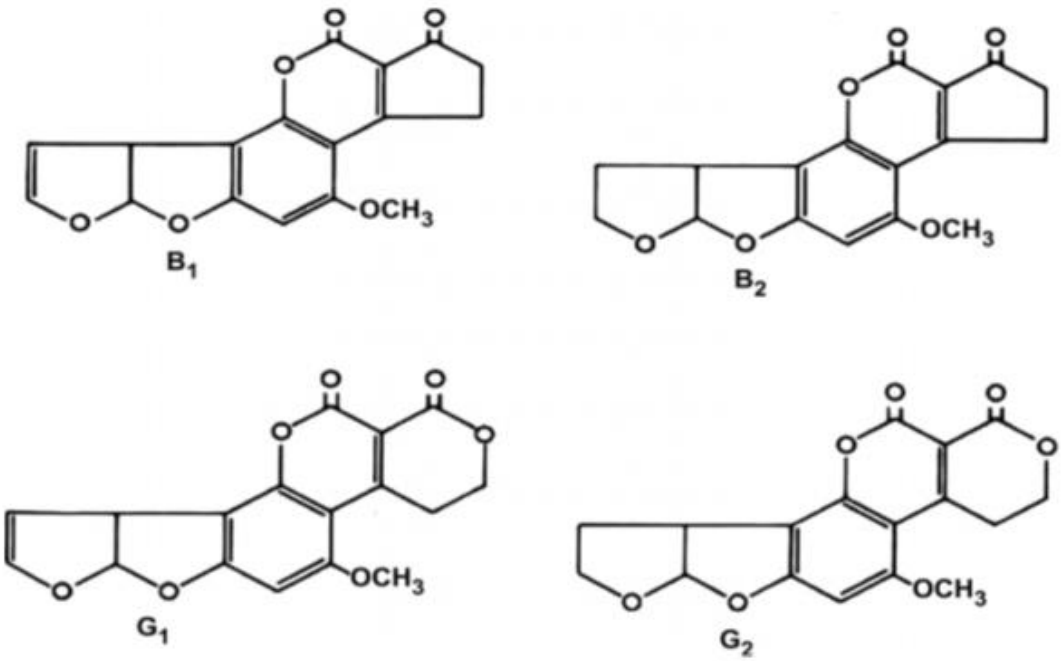


Figure 2 Chemical structures of the four major naturally produced aflatoxins B_1, B_2, G_1 & G_2.

Source: Coppock et al. (2018); Wogan (1966)

2.3.1 Aflatoxin control strategies

As diverse as their chemical structures are, their varied toxicity and occurrence, so is how hard it is to manage mycotoxins. Effective mycotoxin management strategies begin with detection and isolation.

Prevention through conventional crop production practices, breeding for resistance, and legislation; chemical control, decontamination, botanical and biological control; surveillance and awareness creation are some of the aflatoxin control strategies (Ellis et al., 1991; Hell & Mutegi, 2011; Hell et al., 2010; Soares et al., 2016; Tola & Kebede, 2016). Potential bio-control agents include atoxigenic strains of *A. flavus* and *A. parasiticus* which upon introduction to soil of developing crops resulted in 74.3 to 99.9% reduction in aflatoxin contamination in peanuts in the United States of America (Dorner et al., 1998). Co-inoculation of atoxigenic alongside toxigenic *A. flavus* isolates on maize grown in the field in Nigeria were reported to significantly reduce aflatoxin accumulation in harvested maize (Atehnkeng et al., 2008). Two azole fungicides tebuconazole and prochloraz individually and in combination were reported to effectively control *A. flavus* growth

and aflatoxin production (Mateo et al., 2017). However, residues of these chemicals in foods have been implicated in disruption of the food chain and impacting on human/animal health while the use of biological control begs so much of technical knowledge.

As a matter of fact, the prevalence of aflatoxins in foods and feeds is highest in developing countries with limited resources to counter such challenges (Allen et al., 1992; Peers et al., 1987; Sirma et al., 2019). Investing in plant-based control agents such as EOs is considered a safe and sustainable alternative to controlling aflatoxins in stored products (R. Bluma et al., 2008; Chulze, 2010). Fumigation of oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) EOs was used to control *Aspergillus* sp. on wheat during storage (Nachman Paster et al., 1995). The treatment of maize and soybean seeds with EOs of *O. vulgare* and *Ageratum conyzoides* significantly reduced aflatoxin accumulation in the seeds (Esper et al., 2014).

2.5 Minimum Inhibitory Concentration (MIC)

Antimicrobial susceptibility testing (AST) is an important phenomenon in drug efficacy testing. To establish the standards for using antimicrobial agents, the European commission on antimicrobial sensitivity testing (EUCAST) (for Europe) and clinical and laboratory standards institute (CLSI) (for United States of America) have been at the forefront of establishing breakpoints for their MICs. AST for bacteria has received wider attention compared to fungi. The first antifungal susceptibility testing (AFST) standard report was published by CLSI in 1997 (Clinical Laboratory Standards Institute, 2008) and EUCAST in 2008 (Rodriquez-Tudela et al., 2008).

According to EUCAST (E.DEF 9.3), MIC is the lowest concentration of an antifungal agent that limits the growth of moulds within a defined period of time (Arendrup et al., 2015). Also, MIC is defined as the lowest concentration of an antimicrobial agent that prevents the visible growth of a test microorganism in a specified period of time. Broth dilution, broth micro-dilution and disc diffusion are the methods recommended by EUCAST and CLSI in laboratory screening for the MICs for both bacteria and moulds, and azole agar screening for *Aspergillus* sp. resistance testing (Berkow et al., 2020). Irrespective of the method employed, MIC determination is dependent on several dilutions of the antimicrobial agent of which the highest dilution (least concentration) that prevents visible growth of test microbe

is used as the MIC (Aguilar-González et al., 2015; Gunes et al., 2016). The standards for AFST are mainly clinical based, however their applications are widespread to include agriculture in general. Various *in vitro* studies have employed broth dilution (Abbaszadeh et al., 2014; Babiah et al., 2015; Jamdagni et al., 2018; Johnson et al., 1998) and disc diffusion (Das et al., 2016; Dobre et al., 2011; Patel & Jasrai, 2013) methods to determine MICs for antifungal agents used against economically important pathogens in agriculture production. The disc diffusion method has been modified by various researchers into disc volatilisation technique (Avila-Sosa et al., 2012; Dobre et al., 2011; López et al., 2005).

With the recent innovations of determining antifungal efficacy of volatile compounds (Feyaerts et al., 2018; Ji et al., 2019; Manssouri et al., 2016; Soylu et al., 2010), MICs are determined by the inverted-petri-dish method (Aguilar-González et al., 2015; Velázquez-Nuñez et al., 2013), inhibition measurements are done as described by CLSI and EUCAST. Ji et al. (2019) determined the MICs of several EOs vapours against *Penicillium corylophilum* by diluting preliminary inhibitory concentrations into five concentrations. Each EO dilution was deposited on a paper disc that was impregnated in the upper well of the used vial, fungal spore suspension was inoculated on PDA in the lower well of the vial. The vials were incubated for 120 h and the MIC (µL/mL) was determined by considering the vial with the smallest concentration of the oil that did not permit fungal mycelial growth.

2.6 Essential Oils

Essential oils (EOs) are complex mixtures of volatile secondary metabolites biosynthesized by plants. They are also known as “volatile oils” for their ability to vaporise. The rich history for various EO chemistry and applications in aromatherapy dates way back to before the 16th century (Guenther & Althausen, 1948). The major properties of EOs are that they are clear liquids, generally hydrophobic and less dense than water. The EOs have been known for various applications since long ago including food preservation, aromatherapy, antiseptic agents i.e. antibacterial, antifungal and antiviral. With over 300 EOs now available commercially, EOs are important ingredients in pharmaceutical, agronomic, food, sanitary, perfumes and cosmetics industries. The biosynthesis of EOs can be traced to almost all parts of plants such as roots (vetiver grass), rhizomes (galangal), barks (cinnamon), seeds

(fennel), fruits (citrus), buds (clove), leaves (makrut lime), bulbs (garlic) twigs (tea tree) and flowers (acacia). Oils or pure chemical compounds of clove (*Syzygium aromaticum*), thyme (*Thymus vulgaris*), eucalyptus (*Eucalyptus* sp.), lemongrass (*Cymbopogon* sp.), peppermint (*Mentha* sp.), rosemary (*Rosmarinus officinalis*), lemon (*Citrus* sp.), lime (Citrus sp.), sage (*Salivia officinalis*), oregano (*Origanum vulgare*), ginger (*Zingiber officinalis*), vetiver and cinnamon (*Cinnamommum* sp.) etc. are now commercially available (Hua et al., 2014; Powers et al., 2018; Sharma et al., 2017).

2.5.1 Extraction of EOs

Isolation of EOs from plant specimens is by various techniques such as solvent extraction, accelerated solvent extraction, supercritical fluid extraction (SFE), solid-phase microextraction (SPME), and hydrodistillation (Richter & Schellenberg, 2007). Steam distillation, microwave-assisted steam hydro-diffusion and microwave-assisted hydrodiffusion were used in extraction of EO from savory (*Satureja bachtiarica* Bunge.) (Memarzadeh et al., 2015). However, hydro distillation the most commonly used laboratory scale EO isolation technique (Tongnuanchan & Benjakul, 2014). Nekoei and Mohammadhosseini (2016) employed hydrodistillation, solvent-free microwave extraction, headspace solid-phase micro-extraction and microwave-assisted hydrodistillation to extract EO from aerial parts of the medicinal herb yarrow (*Achillea wilhelmsii* C. Koch). Although new advances in extraction of EOs such as SFE utilize lesser/no solvents and are less time consuming (Herrero et al., 2010), hydrodistillation of *Valeriana officinalis* L. roots yielded oils significantly richer in chemical composition than supercritical CO_2 extraction (Safaralie et al., 2010). The Clevenger-type apparatus, although with various modifications, is the major apparatus used in the laboratory to extract EO by hydrodistillation technique (Pingret et al., 2014; Wesołowska et al., 2014).

2.5.2 Chemical composition of EOs

A wide range of chemical compounds (Table 1) can be found in diverse groups of plants but exist majorly as terpenes (hydrocarbons) and terpenoides (oxygenated hydrocarbons) (Guenther & Althausen, 1948; Oliveira et al., 2018). The chemical composition of EOs varies among species, age and parts of plants used,

season, location and the extraction method used (Bakkali et al., 2008). Also, genetic regulation of some EO compounds biosynthesis was reported (Mahmoud & Croteau, 2003).

EO biosynthesis follows definitive pathways that may differ within and/or among species (Ganjewala & Luthra, 2010). Isopentenyl pyrophosphate (IPP) is the precursor of the terpenoidal constituents of EOs that are formed through condensation of isoprene usits e.g two isoprene uints for monoterpenes (-oides) and three for sesiquiterpenes (-oides) (Başer & Demirci, 2007). Examples of terpenes are limonene, pinene, sabinene, terpinene, caryophyllene and myrcene. Terpenoides are further grouped into alcohols (such as geraniol, linalool, menthol, terpineol, citronellol and nerol), aldehydes (such as citral, citronellal, cinnamaldehyde, geranial and neral), ketones (such as jasmine, menthone, carvone, and camphor), phenols (such as eugenol, carvacrol and thymol) and phenolic esters (myristin and anethole), oxides (such as 1,8 – Cineole), acids, esters, and coumarines (Clarke, 2008; Koutsaviti et al., 2018; Stefanello et al., 2011). Different phytochemical compounds are represented by related chemical structures that are differentiated by their functional groups as discussed above (Figure 3).

Although EOs are composed of various phytochemicals, one or two compounds are mostly dominant as major composition in excess of atleast 20% in concentration (Table 1) with the rest being represented as minor components (Koutsaviti et al., 2018). Correspondingly, antimicrobial activity of EOs is influenced by the constituent chemical compounds acting individually, in synergism, additively or in antagonism (Songsamoe et al., 2017).

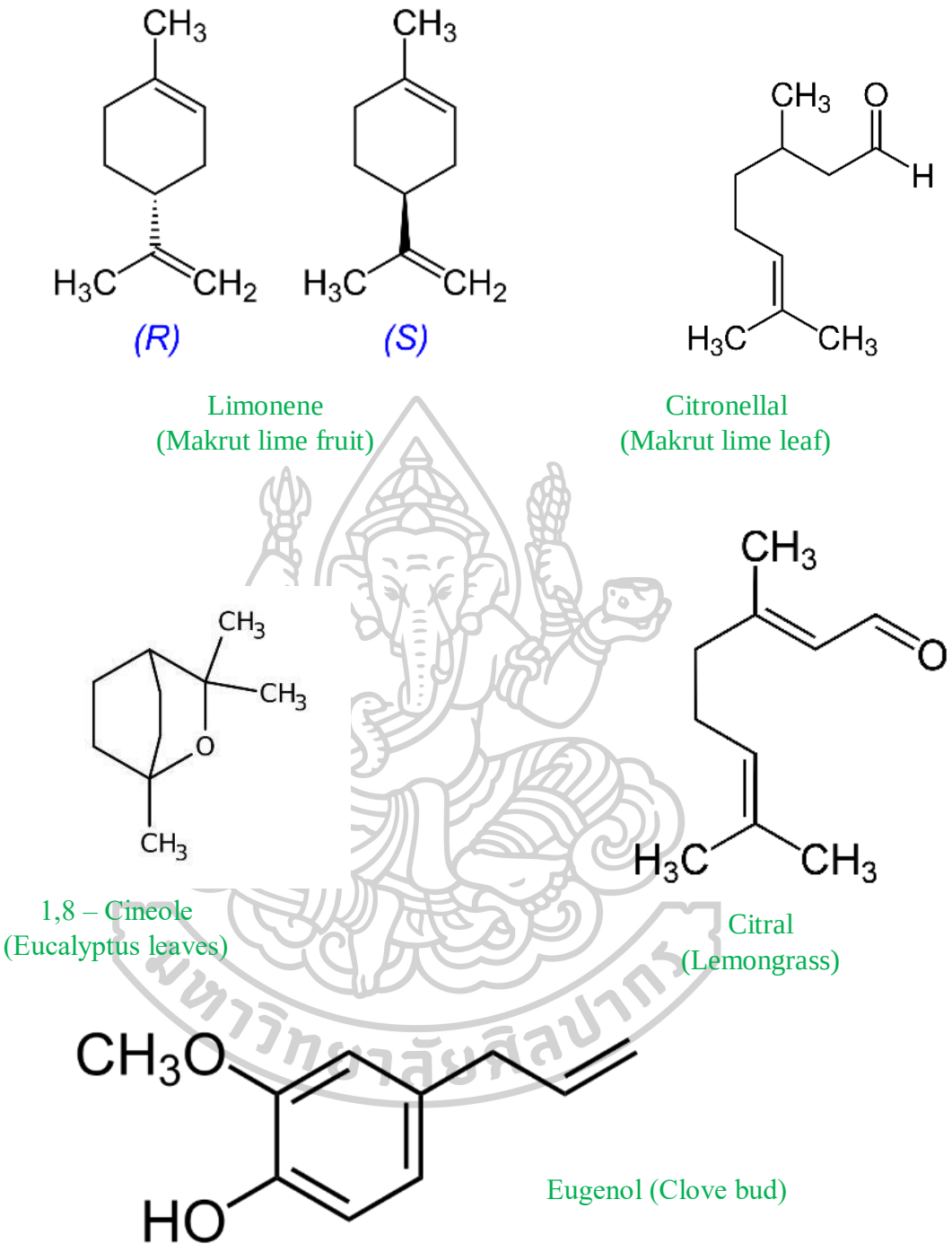


Figure 3 Chemical structures of some phytochemicals from selected plant materials used in this study.

Table 1 The chemical composition of selected essential oils

Plant name	Scientific name	Major chemical constituents	Extraction method	*Identification	References
Lemongrass	*Cymbopogon citratus*	α-Citral (36.2%), β-Citral (26.5%), Nerol (5.1%)	Not mentioned	GC-MS	(Tyagi & Malik, 2010)
		Geranial (42.16%), Neral (31.52%)	Hydrodistillation	GC-MS	(Boukhatem et al., 2014)
Clove	*Syzygium aromaticum*	Eugenol (87.26%) and Eugenyl acetate (10.43%)	Hydrodistillation	GC-MS	(Golmakani et al., 2017)
Makrut lime leaf	*Citrus hystrix*	Citronellal (72.4%), citronellol (6.7%)	Hydrodistillation	GC-MS	(Jantan et al., 1996)
		l-citronellal (61.73%), β-citronellol (13.43%), *l*-limonene (5.90%)	Steam distillation	GC-MS	(Nor, 1999)
		Terpinen-4-ol (13.0%), β-pinene (10.9%), α-terpineol (7.6%), 1,8-Cineole (6.4%) and citronellol (6.0%)	Hydrodistillation	GC-MS	(Waikedre et al., 2010)
Makrut lime peel	*C. hystrix*	Limonene (28.7%), Sabinene (27.5%), citronellal (17.5%)	Steam-distillation	GC-MS	(Hidro, 2013)

		and β-pinene (7.4%)			
		Limonene (38.6%), terpineol (11.7%) and β-pinene (30.5%)			(Kerdchoechuen et al., 2010)
Eucalyptus leaf	*Eucalyptus* sp.	1,8-Cineole	Hydrodistillation	GC-MS	(Elaissi et al., 2011)

*GC-MS = Gas chromatography coupled with mass spectrometry

2.5.3 Mechanism and antimicrobial activity of EOs

The biological activities of EOs have been extensively reviewed (Bakkali et al., 2008). However, their antimicrobial and pesticide efficacies have heralded for their applications in agriculture (Aguiar et al., 2014; Hossain et al., 2016; Isman, 2000; Ju et al., 2018; Kim et al., 2016). Antifungal assays of EOs are majory based on their potential to inhibit fungal mycelial growth (Gakuubi et al., 2017) and sporulation inhibition (Sharma et al., 2017). The mechanism of mycelial growth and spore germination inhibition has been attributed to hyphal and spore morphological deformations (Sharma et al., 2017) and consequently cellular organ failure. EOs are lipophilic thus easily permeate through cell membranes and, in addition to the various phytochemicals, interrupt a number of biological activities in microorganisms. This ease of permeability is highly related to the cytotoxicity of EOs against most deleterious microorganisms such as bacteria (Issa et al., 2019; Nazzaro et al., 2013; O'Bryan et al., 2015) and fungi (Park et al., 2009; Rasooli et al., 2006; Rozwalka et al., 2010; Tao et al., 2013). Caceres et al. (2016) employed Large-Scale q-PCR approach to assess the impact of eugenol on the production of aflatoxins by *A. flavus*. It was revealed that eugenol inhibited expression of 19 genes completely and down regulated eight genes of the total 27 genes that produce aflatoxins in *A. flavus*.

2.5.4 Dispensation of essential oils

Laboratory screening of antifungal efficacy of EOs (*in vitro*) majorly through direct and indirect contact with oil or its compounds (Elhidar et al., 2019; Kohiyama et al., 2015; N. Paster et al., 1995). Comparison of the two methods for effectiveness of EOs were reported (Suhr & Nielsen, 2003; Tullio et al., 2007; Tyagi & Malik,

2011; Velázquez-Nuñez et al., 2013). By direct contact, the EOs are either mixed with the medium (agar dilution or broth dilution) (Delespaul et al., 2000) or loaded into paper discs which are placed directly onto the medium in direct contact with the test pathogen (López et al., 2005). For indirect contact or vapours assay, mainly the method used for *in vitro* assay is disc volatilisation where the EOs are pipetted into a paper disc place separately from the inoculated medium and the oil evaporates into a modified atmosphere (Goni et al., 2009). In the indirect contact method, only the EO vapour produces the efficacy against the test pathogens. Disc volatilisation was preferred over either disc diffusion when cinnamon and clove EOs were exposed to bacteria and fungi (López et al., 2005). While a significant reduction in minimum inhibitory concentration (MIC) was observed when lemongrass (*C. citratus*) was dispensed in vapour phase (MIC : 32.7 mg/L) less than agar or broth dilution (MIC : 288 mg/L) against yeast *Candida albicians* (Tyagi & Malik, 2010). Application of EOs by indirect contact in the field, also known as fumigation, has been sparsely reported but with strong recommendations as a better delivery mechanism of EOs (Boukaew et al., 2017).

2.5.5 Synergism and its benefits in fungicide applications

Synergism is the dispensation of EOs where individual oils act partially (atleast ¼ less than the MIC) to produce a combined antifungal activity greater than for individual oils (Ji et al., 2019). This method of application of EOs has shown significant antimicrobial activity at very low concentration when compared to using individual oils separately (Stević et al., 2014). Synergism of natural plant extracts with antibiotics was reported as a potential for breaking multidrug resistance by many disease causing bacteria (Hemaiswarya et al., 2008). In Iran, the antifungal activity of oils of cinnamon and thyme was found to be significantly higher in combination than for individual oils (Nikkhah et al., 2017). The combination of citronellal and cinnamaldehyde was report to synergistically inhibit growth of *Penicillium digitatum* and control of green leaf mould disease in citrus fruits (OuYang et al., 2020). Clove bud (*S. aromaticum*) EO was synergistic with a fungicide (hymexazol) in controlling *Panax notoginseng* fruit disease (Ma et al., 2019).

The synergism of EO in vapour has been sparsely reported, however, studies have shown that it could be a potential means of antimicrobial efficacy (Goni et al., 2009; Ji et al., 2019). The combination of clove EO and mustard (*Brassica nigra*) EOs in vapour phase was found to be effective *in vitro* and *in vivo* against the *Botrytis cinera* in strawberries (Aguilar-González et al., 2015). Sukatta et al. (2008) reported that the combination of clove and cinnamon EOs in vapour phase was synergistic *in vitro* against six fungi i.e. *Aspergillus niger, Alternaria alternata, Colletotrichum gloeosporioides, Lasiodiplodia theobromae, Phomopsis viticola* and *Rhizopus stolonifera* that cause decay in grape fruits of Thailand.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection and preparation of plant materials

The plant materials were collected from the areas of Cha-Am district, Phetchaburi Province, Thailand during the month of January, 2019. Eight plants (Table 3) were screened for EO extraction. The plant materials were transferred to the laboratory and immediately prepared for EO extraction (Figure 4).

For makrut lime fruits (MP), the fresh fruits were washed under a running tap water and subsequently air dried for 30 minutes at room temperature (26-32 ^{0}C). The green skin of these fruits was peeled off with a cutting knife and cut into small pieces. Approximately 250 g of these green peels were weighed on a digital scale before putting them in the flask extracting EO.

For the other plant materials, the fresh leaves of makrut lime (ML), eucalyptus (EU), indian borage (IB), piper lolot (PL), peppermint (PM) and fresh stems of lemongrass (LG) were individually washed under running tap water and these samples were air dried for 30 minutes at room temperature and they were cut into small pieces. These plant materials were weighed to obtain approximate waits of 350 g (ML), 430 g (EU), 300 g (IB), 135 g (PL), 350 g (PM) and 1 kg (LG) respectively for each plant material using a digital balance.

For clove, the dried clove buds were ground into a particulate powder using a blender and 220 g of this ground clove were weighed for further use.

3.2 Essential oil extraction

The EO extraction was conducted in the laboratory by hydrodistillation technique using Clevenger-type apparatus (Pingret et al., 2014).

The prepared plant materials, as described above, were individually added into a two-neck boiling flask (1 Litre (L) capacity) containing porcelain boiling chips. For each extraction cycle, not more than 250 g of the plant material was added to the flask. Distilled water was added to slightly submerge the plant material. The flask was then placed onto a heater and the entire setup was fitted to a Clevenger-type glass apparatus and a condenser (Figure 4A). The repeated distillation was used to treat the plant materials with heat to a boiling point at a constant temperature of 120 ^{0}C until

the first condensate drop was obtained, after which the temperature was reduced to 95 ^{0}C to prevent the volatile compounds from evaporating (Tandon, 2008). The heating was stopped when there was no more EO that was emitted into the distillate receiver.

The EO layer was separated from aqueous distillate by using a separating funnel into a 25 ml receiver beaker. To dry the EO, 2-5 g of anhydrous sodium sulphate (Na_2SO_4) were added and allowed to settle for about 45 seconds. The pure EO layer was pipetted into pre-weighed glass bottles. The pure EOs were weighed and the percentage of yield was determined on the basis of weight/weight (w/w) (Kapadiya et al., 2018). The pure EOs were kept in a refrigerator at 4-8 ^{0}C until further use.

Table 2 Plant materials and their sources used for extracting essential oils.

Plant	**Scientific name**	**Plant part**	**Source**
Clove	*Syzygium aromaticum*	Buds	Purchased from a local market in HuaHin
Eucalyptus	*Eucalyptus* sp.	Leaves	Collected from the tree at Silpakorn University, Cha-Am
Makrut lime	*Citrus hystrix*	Peels	Collected from a backyard orchard in HuaHin
Makrut lime	*C. hystrix*	Leaves	Collected from a backyard orchard in HuaHin
Lemongrass	*Cymbopogon citratus*	Stems	Provided in-kind from a banana garden in Cha-Am
Lolot	*Piper sarmentosum*	Leaves	Collected from the tree at Silpakorn University, Cha-Am
Peppermint	*Mentha piperita*	Leaves	Provided in-kind from a farmer's garden in Cha-Am
Indian borage	*Plectranthus amboinicus*	Leaves	Provided in-kind from ASAT staff's backyard garden in Cha-Am

Figure 4 Preparation of plant materials for extraction of EO.

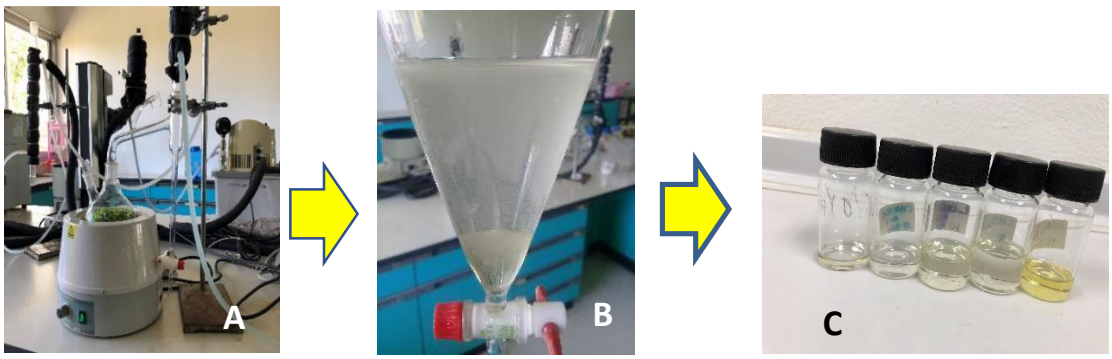


Figure 5 Essential oil extraction A: hydrodistillation with clevenger-type apparatus; B: Distillate decantation with a seperating funnel; C: Pre-weighed essential oils in bottles used for storage

3.3 Isolation techniques and isolates of fungi used in the study

Seven grain samples and one for peanut were used to isolate the fungi used in this experiment (

Table **3**). Blotter paper and agar plating methods were used. Samples one for corn (*Zea mays* L.) and one for rice (Oryza sativa L.) were obtained from the laboratory at the Faculty of Animal Science and Agricultural Technology (ASAT), Silpakorn University (SU), Phetchaburi IT campus (PITC), Cha-Am. Popcorn *(Zea mays* var. *evertta)* and peanut (*Arachis hypogeae*) were bought from Makro Food Store, Huahin Parachuap Kirikhan province. The other samples of corn and one for rice were bought from shops in Cha-Am and Tha-Yang districts of Phetchaburi province, Thailand.

The moisture content (

Table **3**) of the samples was determined by oven drying method (Jittanit et al., 2010) and calculated on a weight loss basis (Thiex, 2009) using the following formula.

Percent Moisture (%MC) = ((IW-FW)/IW) × 100; Where IW = initial weight (g) of the sample before oven drying and FW = final weight (g) after drying.

One hundred fifty grains of each sample were surface sterilized with 1% Sodium hypochlorite (NaOCl) for five minutes with constant stirring. These samples were then washed three times with sterile distilled water for 5 minutes each washing and dried between two layers of sterile tissue paper and used for isolating the storage fungi as described below.

The fungi were isolated by plating each grain sample on PDA (HiMedia, India) and on moistened filter paper (Whatman No. 1; 9 mm in diameter) in sterile glass Petri dishes as described by (Tsedaley & Adugna, 2016). Twenty grains of each sample were plated on PDA and 10 grains on moistened filter per one Petri dish. There were four replications for each grain sample and each isolation technique. The grain samples were incubated for seven days at room temperature (26-32 ^{0}C) with 12 h of light vs darkness.

The fungal growth was detected on a daily basis under a stereo microscope. These fungal isolates were tentatively identified based on their colony appearance and growth rate. After seven days of incubation, fungal characteristics were observed under a compound microscope with a wet-mount technique. The fungi were observed with the compound microscope at x10 and x40 magnifications and the photographs were taken using the Iphone8 Plus camera.

For identification, the fungi (*Aspergillus* sp., *A. niger*, *Fusarium* sp. and *C. lunata*) were named based on colony appearance and morphological characteristics (Albores et al., 2014; Dutta & Hussain, 1959; Nyongesa et al., 2015). The identified fungal isolates were subsequently transferred onto the PDA in Petri dishes. These fungal cultures were incubated on a laboratory bench at room temperature (26-32^{0}C) for seven days. The pure cultures were then stored in a refrigerator at 4^{0}C until further study.

Table 3 Samples used for isolating fungi for this study and the percent moisture content

Sample type	Scientific name	%Moisture content*	Source	Sample name
Corn	*Zea mays* L.	12.4±0.43^{b}	Lab, ASAT	ASAT
Rice	*Oryza sativa*	15.8±0.45^{a}	Shop Tha-Yang	THY
Rice	*O. sativa*	11.3±0.21^{bc}	Lab, ASAT	Asat
Corn	*Z. mays* L.	10.0±0.83^{c}	Shop, Cha-Am	CHM
Corn	*Z. mays* L.	11.6±0.85^{bc}	Shop, Tha-Yang	THY
Corn	*Z. mays* L.	12.0±1.08^{bc}	Shop, Hua-Hin	HH
Popcorn	*Z. mays* var. *evertta*	13.0±0.02^{b}	Makro, HuaHin	PP

Peanut	*Arachis hypogeae*	5.8±0.56[d]	Makro, HuaHin	Pn

*Values are means ± standard deviation; values with different letters are significantly different

3.4 Antifungal assay with individual essential oils

The selected EOs were each tested for their mycelial growth inhibition potential in the vapour phase. Disc volatilisation technique coupled with inverted Petri dish method were used. EOs from either leaves of makrut lime (ML), peels of makrut lime fruits (MP), leaves of eucalyptus (EU), stems of lemongrass (LG) and dry buds of clove (C) were tested for their antifungal activity against *Aspergillus* sp., *Fusarium* sp., *A. niger* and *C. lunata*. The assay was performed as previously described (Boukhatem et al., 2014) with some modifications.

The assay was carried out in sterile plastic cups (capacity 30 mL, circular-shape cup with lid, Aro, Makro, Thailand), which were bought from Makro Food Store, HuaHin, Thailand. Three mL of sterile PDA was put into the cup using a sterile syringe. A mycelial plug (5 mm in diameter) was cut from edge of a 7-day-old fungal colony with a sterile corkborer and transferred onto the solidified PDA using a sterile L-shaped needle.

To deliver the EOs to the fungi, a sterile filter paper disk (Whatman No. 1, 6 mm diameter) was attached underneath the lid of each cup with a sterile double-sticking tape. Using a micropipette, either 5 µL, 10 µL or 20 µL of each EO were individually dispensed to the filter paper discs and the cups were immediately closed with their lids to prevent the volatile compounds from escaping. For the nil control treatment, EO was not added to the paper disc. The cups were incubated upside down for four days at room temperature (26-32 ^{0}C) with 12 h of light versus darkness on a table in the laboratory. The experiment was arranged in a completely randomized design (CRD) with three replications for each treatment.

After four days of incubation (when colony in control had reached the edges of the cup), antifungal activity of each EO was evaluated by measuring colony diameter twice diagonally. Percentage mycelial growth inhibition was calculated using the formula proposed by Ambindei et al. (2017) as follows:

$$\text{Percent Inhibition} = \frac{Mc - Mt}{Mc} \times 100$$

Where Mc = averaged colony diameter (mm) in control, Mt = averaged colony diameter (mm) for treatment.

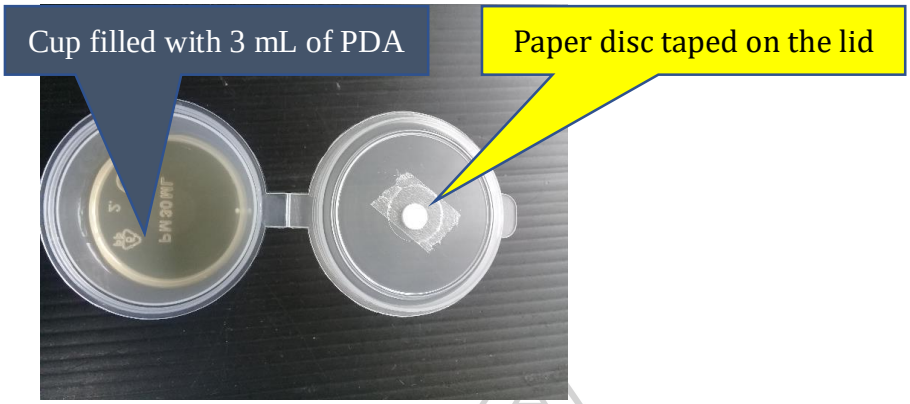


Figure 6 The round sauce plastic cup used for antifungal assay of the essential oils

3.5 Minimum inhibitory concentration (MIC)

The MIC was defined as the smallest concentration of the EO that was able to completely stop mycelial growth after a period of 72 hours of incubation (Ji et al., 2019).

To evaluate the MIC, the smallest inhibitory dose (SID), volume of EO that strongly inhibited fungal mycelial growth, for each EO was further diluted thrice to three quarters, half and quarter by volume (3/4SID, 1/2SID and 1/4SID). The same inoculation and treatment procedure were maintained as described above.

Three mL of PDA was put into the cups using sterile syringe and the respective EO SID dilutions were dispensed on sterile paper discs underneath the lids. The cups were closed with their lids and incubated for 72 hours at room temperature (26-32 ^{0}C) with 12 hours alternating between light and darkness.

After 72 hours of incubation, the mycelial growth for each EO SID dilution was evaluated. The smallest EO SID dilution that showed no visible growth was determined as the minimum inhibitory dose and the MIC was calculated as follows:

MIC = minimum inhibitory dose (µL) ÷ volume of air above the oil in the cup (mL)

Assumption made was that all the EO in the filter paper disc had evaporated into the headspace above PDA in the cup.

3.6 Antifungal assay of EO combinations

Based on the efficacy of MIC, the EOs of LG, EU, MP and ML, were selected for synergism test. Each fungus (*Aspergillus* sp., *Fusarium* sp., *A. niger* and *C. lunata*) was exposed to vapour of two EOs in the same cup.

Two sterile filter paper discs were attached underneath the lid of a cup containing sterile PDA inoculated with the mycelial plug of each fungus. These selected EOs were individually applied to the sterile filter paper discs in combinations such as LG:EU, LG:MP, LG:ML, ML:MP, ML:EU so that two EOs shared the same atmosphere in the cup (Arrebola et al., 2010). The chosen EOs were based on their efficacy, availability and the ease of preparation of the raw plant materials.

This assay was carried out based on the procedure developed by (Ji et al., 2019) with some modifications using the two-dimensional checkerboard arrangement. The treatments as described above (LG:EU, LG:MP, LG:ML, ML:MP and ML:EU) were prepared in the following combined dilutions for each treatment, such as 100:0, 75:25, 50:50, 25:75, and 0:100, with pure EOs. The control was prepared with no EO and there were three replications for each treatment. Each cup that received two discs of EOs as described above was immediately sealed and they were incubated upside down at the room temperature (26-32 ^{0}C) for four days with 12 h light vs darkness.

After four days of incubation, mycelial growth inhibition in each cup was evaluated by measuring the colony diameter twice diagonally, the average colony diameter was determined and percent mycelial growth inhibition was determined using the formula described in the antifungal assay section above. The smallest treatment dose in the cups with no visible mycelial growth were recorded as minimum fractional inhibitory doses of the EOs and were used to calculate the fractional MIC for the combined EOs.

The MIC of each EO in combination (MIC_X) in relation to the MIC of the same EO alone (MIC_Y) were used to calculate the fractional inhibitory concentration (FIC) and then fractional inhibitory concentration index (FICI), using the following formula as previously described (Boukhatem et al., 2014):

For a combination of two EOs A and B,

$FIC_A = MIC_X/MIC_Y$, and $FIC_B = MIC_X/MIC_Y$

$FICI = FIC_A + FIC_B$,

Where MIC_X = MIC of EO in combination, MIC = MIC of EO alone, FIC_A = FIC of essential oil A and FIC_B = FIC of essential oil B.

3.7 Antifungal assay with raw materials

Makrut lime peel and clove powder were assayed for their potential to inhibit mycelial growth of *Aspergillus* sp., *C. lunata* and *F. sacchari* in vapour phase. The materials were weighed to prepare the samples at 0.25g, 0.50g and 1.00g as treatments. Five mL of molten PDA was aseptically added to a sterile flat-bottomed glass conical flask (50 mL capacity) using a sterile syringe. Fungal mycelial discs (six mm in diameter) were excised with a sterile cork borer from edge of fungal colony and transferred to the solidified PDA using a sterile L-needle. Individual plant samples at the weight of either 0.25g, 0.50g or 1.00g were wrapped with three layers of a sterile cotton gauze and each was suspended over PDA that had been inoculated with each fungus in the flask. A sterile aluminium foil was used to hermetically seal the flasks and these flasks were incubated for 4 days at room temperature (26-30 ^{0}C) in the dark. The mycelial growth of each fungus was assessed after four days of incubation by measuring the diameter of fungal colony in each flask twice diagonally. The two measurements were averaged for each colony in every flask. Fungi growing on PDA in the flasks without either clove powder or makrut lime peel were used as nil control.

3.8 Data analysis

The data collected was subjected to a One-way analysis of variance (ANOVA) using R programme (Venables et al., 2009) computer software package. The mean values were compared using the Duncan's Multiple Range Test.

3.9 Aflatoxin isolation and detection

The pure seven-day-old cultures of *Aspergillus* sp, isolates on PDA were sub-cultured onto *Aspergillus flavus* and *parasiticus* agar (AFPA) (20g of yeast extract, 15 g of agar, 10 g Peptone, 0.5 g Iron III chloride, 1.0 mL of 0.2% (w/v) dichloran in ethanol, 2N NaOH and HCl for pH adjustment). The Petri dishes were incubated at room temperature (26-30^{0}C) for 48 hours. Reverse colony characteristics were observed**.** AFPA differentiates *A. flavus* and *A. paraisticus* from other *Aspergillus* sp. by presenting a reverse colony yellow-orange pigmentation (Klich & Pitt, 1988). The

selected isolates were cultivated on coconut milk agar (CMA) which was prepared as described in the next section. To detect the aflatoxigenic isolates, three test were conducted i.e. CMA test, liquid ammonium vapour (LAV) test and thin layer chromatography (TLC), in concurrence.

3.9.2 Coconut milk agar test

Aflatoxin assay with CMA was conducted as previously described (Yazdani et al., 2010). Coconut milk (100% with no preservatives added) was purchased from 7-Eleven, an international convenience store, in Thailand. The CMA medium was prepared by dissolving 200 mL of coconut milk in 600 mL of distilled water, to this 20 g of Agar was added and the mixture was gently mixed with a stirring rod **(**Figure 7A**)**. The mixture was further heated on a heat mantle to homogenise before pouring in autoclavable glass bottles. The pH was measured before and after heating to ensure a recommended range of 6.9-7.1 was maintained using a pH meter (No pH adjustments were needed as the pH before and after heating was in range i.e. 6.8-7.08). The medium was sterilized in an autoclave for 15 minutes at 121^0C with 15 psi pressure in a 2-h cycle. After two hours the medium was removed and poured in sterile glass petri-plates following aseptic precautions (Figure 7B). Inoculation was done by cutting a mycelial plug from the edge of a 7-day-old mycelium.

After 48 hours of incubation, the petri-plates were observed on a daily basis through 7 days under UV light (365 nm) using a simple UV-light torch. The presence or absence of blue-fluorescence was recorded as positive or negative aflatoxin production respectively.

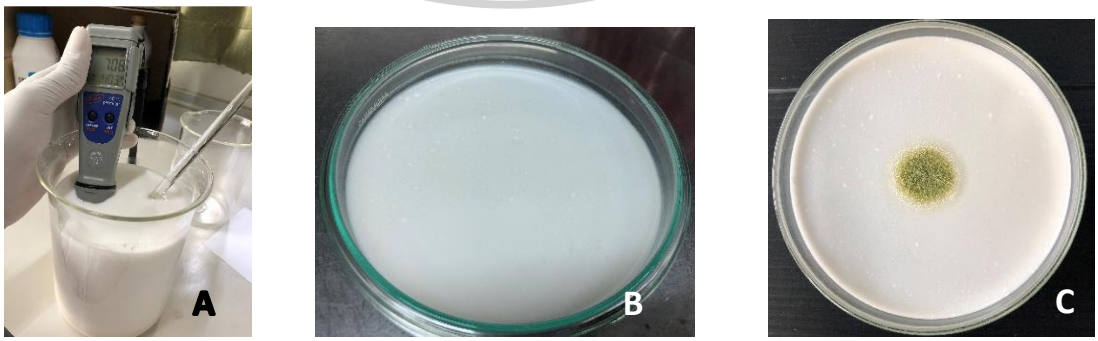


Figure 7 CMA preparation and inoculation with *Aspergillus* sp. A: CMA preparation. B: CMA after aseptically pouring it on petri dishes; C: *Aspergillus* sp. growing on CMA after 3 days of incubation at room temperature (26-34 ^{0}C) in the dark.

3.9.3 Liquid ammonia vapour test

From the CMA test above, the seven-day-old cultures were subjected to liquid ammonia vapour test (Gupta et al., 2017). Briefly, 500 µL of liquid ammonia were pipetted into the centre of the sterile petri-plate lids. The inoculated petri-plates and plastic vials (before EO treatment and after EO treatment respectively) were inverted over the ammonia solution for 5 minutes and the reverse colony colour change was observed and recorded accordingly. Any colour change from yellow to dark yellow, pink or reddish brown was considered as an indicator of positive aflatoxin production. While absence of such colour change was recorded as negative to aflatoxin production. In EO treated vials, this was recorded as either inhibitory or non-inhibitory to aflatoxin production.

3.9.4 Thin layer chromatography test

The TLC test was conducted with reference to previously reported procedures (Yazdani et al., 2010). The test was conducted using silca gel60 20 × 20 cm plates. Aflatoxin was extracted using chloroform while the mobile phase was prepared with chloroform: acetone (9:1 v/v).

Aflatoxin extraction

A five-millimetre mycelia plug was cut from each of the *Aspergillus* isolates (both positive and negative) from the previous tests. The plug was put 1.5 mL Eppendorf tubes and 300 µL of chloroform was added. The tubes were sealed and centrifuged for 5 minutes using the centrifuge. The mixture was filtered through a Whatman No. 1 filter paper fitted in a glass funnel into clean Eppendorf tubes.

TLC plate and mobile phase preparation

TLC plates were cut to pieces of 5 × 10 cm each and marked with HB pencil. The origin and solvent front were marked eight centimetres apart with four lanes marked one centimetre apart. The mobile phase was prepared by adding three millilitres of acetone to 27 mL of chloroform in a 500 mL beaker (500 mL capacity).

From the above chloroform filtrate, 20 µL were pipetted and spotted along the origin, between the lanes to accommodate three spots per piece of the plate. When the spots were dry, the plates were immersed into the mobile phase solvent with the solvent level kept below the origin. The beaker was covered with two layers of foil paper and the mobile phase was allowed to develop up to the solvent front which took

approximately 10 minutes. The plates were allowed one minute to dry and then observed under UV light (365 nm) for blue fluorescence. *A. niger* was maintained as a negative control.

3.10 Anti-aflatoxin efficacy of EOs

The *Aspergillus* sp. isolates that were confirmed as aflatoxin producers, were further studied. Each isolate was subjected to EO vapours of LG, ML, EU and MP as previously described. The potential of selected individual (LG, ML, MP and EU) and mixed (LG : ML) EO vapours to inhibit aflatoxin production was assessed. In both cases of individual and mixed oils, the MICs obtained in antifungal assay were used with each volume making the MIC diluted once and increased once to get three treatments for each EO such as 1/2MIC, MIC and 2×MIC. This was because inhibition for mycelial growth by EOs does not necessarily correspond with aflatoxin inhibition. To assay this, mycelial plugs from edges of colonies in petri-plates that tested positive were cut and regrown on fresh CMA in plastic vials used antifungal assay section above. The inoculum was subjected to EO vapour treatment following a similar procedure described in anti-fungal assay above. The vials were incubated upside down for 4 days under 12 h of light vs darkness at room temperature (26-30 ^{0}C). To the negative control treatment, no EO was added. Three replications were maintained for each treatment.

After four days of incubation, the cups were re-observed under UV-light for presence of blue-fluorescence compared with the control. Presence or absence of blue-fluorescence was recorded as inhibitory or non-inhibitory respectively, by the EOs to aflatoxin production. LAV and TLC tests were repeated on the treated plates following exact procedures described above. Positive and negative aflatoxin tests were recorded as inhibitory and non-inhibitory to aflatoxin production respectively.

3.11 Data analysis

For all the experiments, treatments were arranged in a completely randomized design (CRD). The data collected was subjected to ANOVA using R-program. The mean values were compared using the DMRT.

CHAPTER FOUR

RESULTS

4.1 Extraction of essential oils

Screening for potential of plant materials to yield sufficient EOs was conducted on eight plant parts. Our screening test results (**Table 4**) showed that dried clove buds produced the highest EO yield (4.43%) slightly better than makrut lime peel (3.40%). Indian borage, peppermint and piper lolot produced insufficient amounts of EO and were declared unfit for this experiment. Variation in times of EO extraction were also observed and recorded. It was observed that eucalyptus could produce no more oil after two-hours but had a high rate of production during the first 30-40 minutes. Indian borage and piper lolot produced the recorded EO in initial 30 minutes and the volume was constant till the end of the distillation process. Makrut lime peels, makrut lime leaves, dried clove buds, and lemon grass stems were still producing oil beyond three hours however the quantity reduced with increased distillation time.

Table 4 Percent yield for essential oils extracted by hydrodistillation from different plant materials.

Plant material	Sample weight (g)	Time (minutes)	EO weight (g)	Yield (% w/w)
Lemon grass stem	1000.13	190	5.33	0.53
Eucalyptus leaves	425.7	124	2.81	0.66
Makrut lime leaves	330.8	185	1.61	0.59
Makrut lime fruit epicarps	244.5	193	8.30	3.40
Clove buds	222.3	210	9.86	4.43
Piper lolot leaves	132.3	122	0.12	0.09
Indian borage leaves	295.6	130	0.21	0.07
Pepper mint leaves	345.4	100	negligible	0.00

4.3 Fungi identification

Morphological parameters used in identification of the key pathogens have been presented in Table 5 and Figure 8 (A-D).

***Aspergillus* sp.**: The colonies were cress green to dark green with white edges and a velvety appearance. In some isolates, brown colonies were observed. Septated conidiophores were elongate bearing differently shaped vesicles. The philaids were distinctly biseriate in some isolates and uniseriate in others. Presence of sclerotia was observed. The conidia were round, smooth and rough, greenish yellow in colour.

***Fusarium* sp.:** The colonies were all white with a cotton-like appearance on the inverse view. Cream to pink pigmentation was observed in all isolates. The reverse view showed different colours ranging from orange yellow and Byzantium purple. The hyphae were mainly serrated and branching.

***Curvularia* sp.:** The colony colour ranged from white at initial growth to black at full growth. The spores were olive brown in colour with white base and apex cells, curved toward the basal cell for some, others were straight; the septation was atleast 3 septa. The morphological characteristics were suiting to classify the pathogen as *C. lunata*.

It was observed that *Aspergillus* sp. and *Mucor* sp. were detected in all samples (100%), atleast 50% of the samples were contaminated with *Fusarium* sp. while *C. lunata*, *Rhizopus* sp., *A. niger* and the Unidentified fungi were each detected in atleast 25% of the samples. *P. citrinum* was detected in more than one-third (37.5%) of the samples studied.

Under blotting technique, *Aspergillus* sp., were detected in three-quarters (75%) of the samples. *C. lunata* (25%) *Fusarium* sp. (37.5%), *Rhizopus* sp. (37.5%), and *Mucor* sp. (62.5%) were the other identified fungi from all samples.

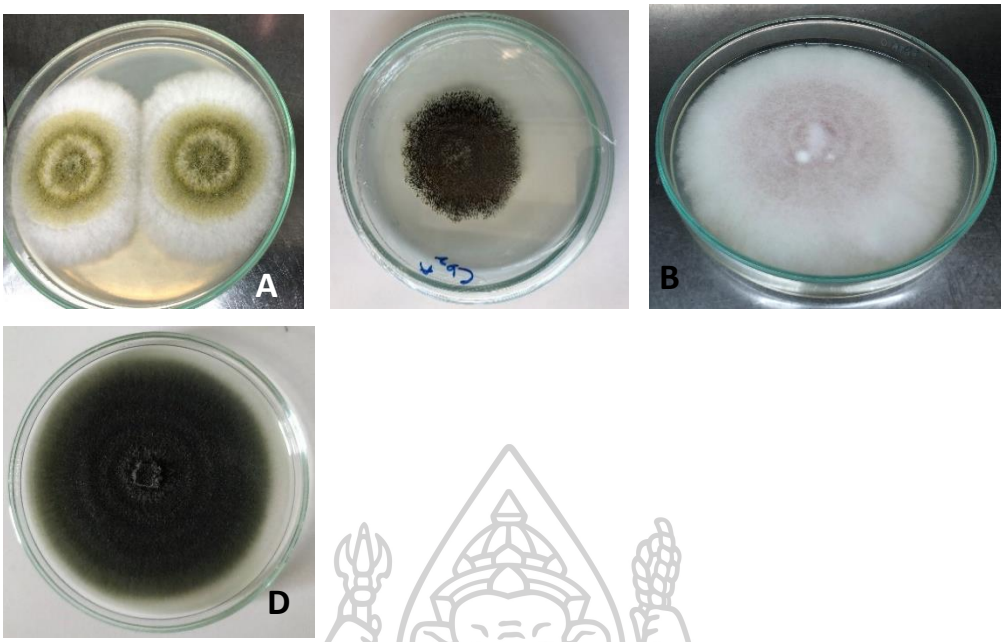


Figure 8 Morphological characteristics of the different isolates of *Aspergillus* sp. (A), *A. niger* (B), *Fusarium* sp. (C) and *C. lunata* (D) after seven days of incubation at room temperature (26-32 ^{0}C) on PDA.

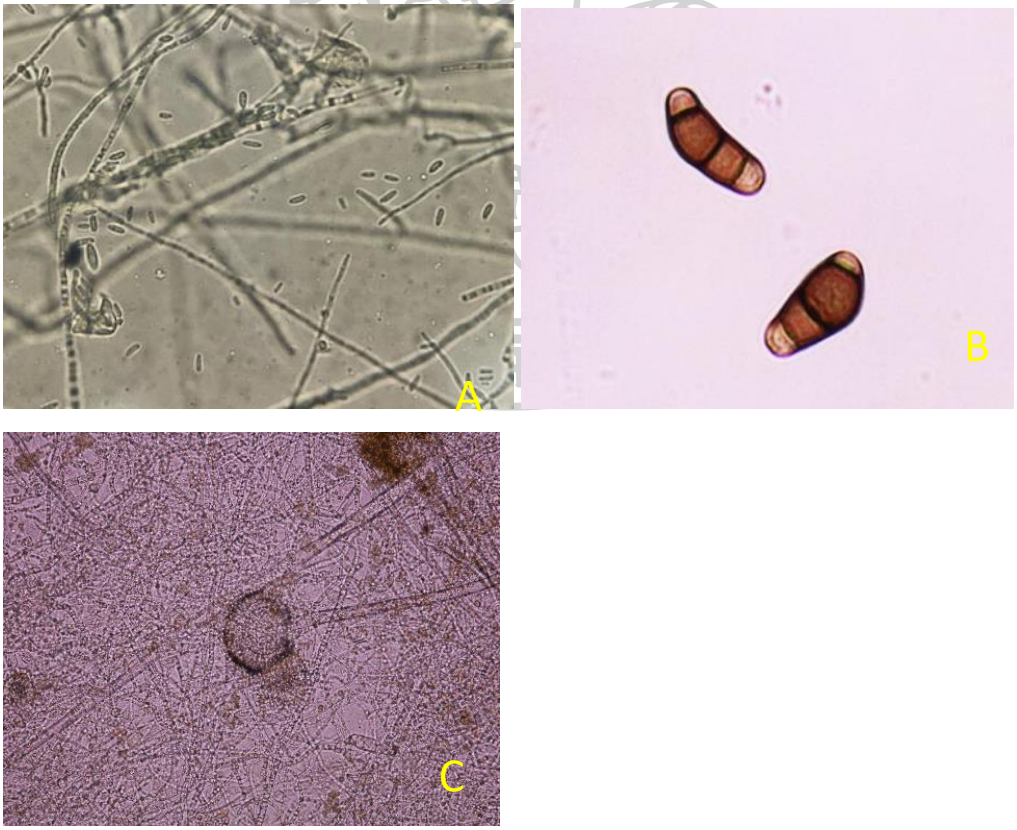


Figure 9 Microscopic identification of (A) *Fusarium* sp., (B) *C. lunata* and (C) Conidiophore and vesicle of *Aspergillus* sp.

Table 5 Morphological characteristics of the different isolates of *Aspergillus* sp., *Fusarium* sp. and *Curvularia* sp.

Sample	Colony		Conidia		Sclerotia/ Chlamydospore	Identity
	Inverse	Reverse	Colour	Shape		
ASAT, HH, CHM	Cress green	Yellow	Greenish yellow	Round	Sclerotia	*Aspergillus* sp.
ASAT	Brown	Brown	Brown	Round	None	*Aspergillus* sp.
Peanut, THY, PP	Dark green	Yellow	Greenish yellow	Round	Sclerotia	*Aspergillus* sp.
Peanut	White	Orange	None	None	None	*Fusarium* sp.
HH	Cream White	Violet		Cylindrical, No septa	Chlamydospore	*Fusarium* sp.
HH, CHM	White Pink	Byzantium pink		Oval, hooked apex, blunt base, 3 septa	Chlamydospore	*Fusarium* sp.
THY, Asat	Black	Black	Olive brown	Oval, curved/straight, ≥3 septa	None	*C. lunata*
HH, ASAT, CHM	Black	Yellow	None		None	*A.niger*

ASAT = maize sample from ASAT Lab, HH; from HuaHin, CHM; from Cha-Am, and THY; from Tha-Yang. PP = Raw popcorn, Asat = Rice sample from ASAT Lab.

4.4 The fungal incidence in the samples

The pathogens isolated from each sample have been presented in **Table 6** below and percentage incidence of each fungus has been presented in Figure 10 for direct plating and blotting techniques. *Aspergillus* sp. and *Mucor* sp. were the most abundant from all the samples. *Penicillium* sp. were only isolated from one sample at 37.5 %.

Incidence of the major pathogens (Table 7) showed that with blotting technique, *Aspergillus* sp. were significantly highest (70%) in unsterilized sample HH compared to 10% in the sterilized. The incidence for THY was unchanged (66.7%) while PP, Asat, ThY were all uninfected. However, direct plating technique showed

that ASAT had the highest infection (83.3%) compared to unsterilized that had 30% incidence. It was observed that *Aspergillus* sp. on sterilized corn HH was higher (40%) than the unsterilized corn (15%) on PDA.

Fusarium sp. were more detected on samples directly plated on PDA than in samples on blotting paper. CHM (66.7%) was the most contaminated while PP (1.7%) was the least contaminated of the unsterilized samples. When sterilised, the highest incidence was recorded in Asat (20%).

C. lunata was not detected in the samples by blotting technique. With direct plating technique, 62% were in the sterilized samples.

Table 6 Percent moisture composition of the samples determined by oven-drying method at 105 ^{0}C for 72 hours and pathogens identified from each sample.

Sample	**Sample Type**	**Identified pathogen**
ASAT	Corn	*Aspergillus* sp., *A. niger*, *Penicillium* sp., *Mucor* sp.
HH	Corn	*Aspergillus* sp., *A. niger*, *Rhizopus* sp., *Mucor* sp. *Fusarium* sp.
PP	Popcorn	*Aspergillus* sp., *Penicillium* sp., *Mucor* sp., *Rhizopus* sp.
THY	Corn	*Aspergillus* sp., *Rhizopus* sp., *Mucor* sp., *Fusarium* sp.
CHM	Corn	*Aspergillus* sp., *A. niger*, *Penicillium* sp., *Mucor* sp., *Rhizopus* sp., *Fusarium* sp.
Asat	Rice	*Aspergillus* sp., *Rhizopus* sp., *Mucor* sp., *C. lunata*, *Fusarium* sp.
Thy	Rice	*Aspergillus* sp., *A. niger*, *Rhizopus* sp., *Mucor* sp., *C. lunata*
Peanut	Peanut	*Aspergillus* sp., *Mucor* sp., *Fusarium* sp., *Rhizopus* sp.

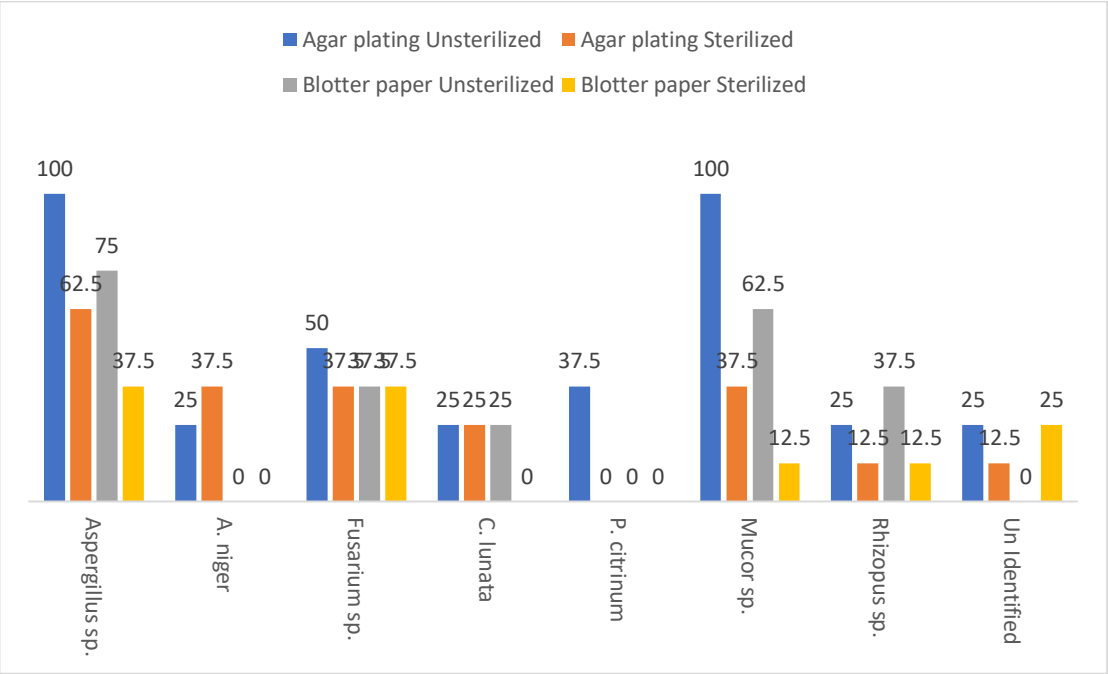


Figure 10 Percent incidence of the detected fungi by different detection methods after 7 days of incubation at room temperature (26-30 ^{0}C) with 12 h of light vs darkness.

Table 7 Frequency of sample infections by the three major fungi detected in both sterilized and unsterilized seeds

Sample	*Aspergillus* sp.				*Fusarium* sp.				*Curvularia lunata*			
	Direct Plating		Blotting		Direct plating		Blotting		Blotting		Direct plating	
	NT***	T***	NT***	T	NT**	T*	NT**	T*	NT*	T*	NT***	T***
ASAT	46.7±25.2ab	0.0±0.0b	83.3±28.9a	30.0±20.0ab	0.0±0.0b	0.0	0.0±0.0c	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0b	0.0±0.0b
HH	70.0±26.5a	10.0±10.0b	15.0±21.8b	40.0±5.0a	36.7±30.5a	23.3±32.1	15.0±21.8bc	10±5	0.0±0.0	0.0±0.0	0.0±0.0b	0.0±0.0b
PP	0.0±0.0	NA	3.3±2.9b	NA	0.0±0.0b	NA	1.7±2.9c	NA	0.0±0.0	NA	0.0±0.0b	NA
THY	66.7±15.3a	66.7±11.5a	31.0±5.3b	5.0±5.0b	10.0±17.3b	36.7±30.6	6.7±7.6bc	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0b	0.0±0.0b
CHM	10.0±10.0b	0.0±0.0b	0.0±0.0b	0.0±0.0b	13.3±15.3ab	1.3±0.6	66.7±57.7a	1.67±2.9	0.0±0.0	0.0±0.0	0.0±0.0b	0.0±0.0b
Asat	0.0±0.0b	0.0±0.0b	0.0±0.0b	13.3±5.7ab	0.0±0.0b	0.0±0.0	46.6±5.8ab	20.0±10.0	0.0±0.0	0.0±0.0	33.3±20.8a	20.0±17.3b
Thy	0.0±0.0b	0.0±0.0b	16.7±12.6b	0.0±0.0b	0.0±0.0b	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	15.0±5.0ab	62.0±5.6a
Peanut	20.0±0.0ab	NA	46.7±15.2ab	NA	0.0±0.0b	NA	0.0±0.0	NA	0.0±0.0	NA	0.0±0.0b	NA

a The samples were not treated with sodium hypochlorite
b Values are average means ± standard deviation
c Values in each column with the same letter are not significantly different
*Values are not significantly different
**Values are significantly different at $p \leq 0.01$
***Values are significantly different at $p \leq 0.001$
NT = Not treated with sodium hypochlorite, T= Treated with sodium hypochlorite, NA = Not applicable (Test was not done)

4.5 Antifungal assay with individual essential oils

Five EO vapours, including ML, MP, EU, LG and C oils were assessed for their mycelial growth inhibitory potential against *Aspergillus* sp., *Fusarium* sp. *A. niger* and *C. lunata.* The results (**Table 8**) showed that lemongrass oil and makrut lime leaf oil were the most effective against all pathogens at all levels of dosage. *Aspergillus* sp. were slightly more resistant than either *Fusarium* sp., *A. niger* or *C. lunata* to the two oils. *C. lunata* was the most sensitive pathogen to all the tested EOs, followed by *Fusarium* sp, and *Aspergillus* sp. The efficacy of the EOs increased with increasing dosage for each EO. Eucalyptus oil vapour was ineffective against *A. niger* with just 45% at maximum dosage (20 µL). *A. niger* was most resistant except when exposed to EO vapour of LG and ML.

The MICs (vol/vol of air) of the EOs were determined and presented in **Table 9**. Lemongrass oil and Makrut leaf oil had the least and similar MICs (0.09-019 µL/mL of Air) while eucalyptus oil (0.37-0.74 µL/mL of Air) for all pathogens. *C. lunata* was observed as the least resistant (0.09-0.37 µL/mL of Air) to all the EOs while *A. niger* was most resistant (0.19-0.74 µL/mL of Air). *Fusarium* sp. were more resistant to eucalyptus oil (about 0.74 µL/mL of Air).

Table 8 Efficacy of essential oils on mycelial growth of *Aspergillus* sp., *A. niger*, *Fusarium* sp., *C. lunata* after 4 days of incubation at 26-32 ^{0}C with 12 h light vs darkness.

Essential oil	Dose (µL)	Percent mycelial growth inhibition			
		Aspergillus sp	*A. niger*	*Fusarium* sp.	*C. lunata*
C	5	42.9±2.7cde	35.0±6.4bc	37.9±3.2c	73.3±1.1b
	10	54.6±4.4cd	55.4±6.1abc	63.3±4.0b	75.4±0.4b
	20	81.7±9.2ab	77.4±11.5ab	100.0±0.0a	100.0±0.0a
EU	5	34.2±4.6de	7.5±0.8c	24.2.5±1.2cd	30.4±4.7c
	10	62.1±3.2bc	9.1±4.4c	35.2±0.8c	60.4±8.1b
	20	100.0±0.0a	45.0±28.0bc	57.8±3.9b	75.0±4.1b
LG	5	89.0±11.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a
	10	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a
	20	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a
ML	5	100.0±0.0a	82.5±15.2ab	35.0±6.9c	100.0±0.0a
	10	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a
	20	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a
MP	5	20.4±5.3e	17.9±11.6c	20.4±1.7cd	40.8±4.1c
	10	52.9±6.0cd	47.9±3.2abc	35.4±7.4c	78.8±8.4b
	20	100.0±0.0a	55.4±3.6abc	85.1±7.6a	100.0±0.0a

Values are means ± standard error (SE)
Values in the same column with the same letter are not significantly different at *P*-value: $p \leq 0.001$ by the Duncan's Multiple Range.

Table 9 Minimum Inhibitory Concentrations for the EO vapours against the fungal mycelium growth after 72 hours of incubation at 26-32 ^{0}C under natural light.

Essential oil	MIC (µL/mL of Air)			
	Aspergillus sp.	*A. niger*	*Fusarium* sp.	*C. lunata*
C	0.37	0.37	0.56	0.37
EU	0.56	>0.74	0.56	0.56
ML	0.19	0.19	0.19	0.09
MP	0.56	0.74	0.56	0.28
LG	0.19	0.19	0.19	0.09

4.6 Antifungal assay with combined essential oils

Synergism was determined for duo mixtures of four EOs based on the MIC values of the proportions in mixture with respect to MICs of the individual oils (Table 9 above). The EO rations of LG:ML (¼ : ¼) that produced 83.3% (Table 10) mycelial growth inhibition against *Aspergillus* sp. was used to determine the FICI. According to results (Table 14), the combination was fully synergistic while ML:EU (¾ : ½) and ML: MP (¾ : ¾) for 100% and 83.3% growth inhibition respectively were evaluated as non-interactive.

Only the combination between the essential oils of ML and LG exerted full synergism (FICI =0.5) against all the tested fungi. The combination of the EOs for LG and MP exhibited full synergistic effect against *Fusarium* sp. and *A. niger*. Oils of LG with EU was fully synergistic against *A. niger*. Oil of ML combined with that of EU had full synergism against *C. lunata* (Table 12) but the oils were non-interactive against the rest of the tested pathogens.

Table 10 Efficacy of vapour phase of essential oils to inhibit the mycelial growth of *Aspergillus* sp. after 4 days of incubation at 26-32 ^{0}C with 12 h light vs darkness.

EO rations	Percent mycelial growth inhibition (%)				
	LG:EU	LG:MP	LG:ML	ML:EU	ML:MP
1	100±0.0^{a}	100±0.0^{a}	100±0.0^{a}	100±0.0^{a}	100±0.0^{a}
1/4:1/4	22.9±9.8^{bc}	43.3±9.1^{c}	**83.3±8.3^{a}**	29.6±16.6^{b}	23.8±4.0^{ab}
1/4:1/2	62.5±5.9^{ab}	50.8±4.6^{c}	90.4±9.6^{a}	38.3±9.8^{ab}	46.3±9.8^{ab}
1/4:3/4	**90.4±9.6^{a}**	**91.7±8.3^{a}**	90±10.0^{a}	40.8±14.6^{ab}	44.2±6.0^{ab}
1/2:1/4	14.2±4.0^{c}	43.3±6.3^{ab}	100±0.0^{a}	44.2±25.5^{ab}	19.2±10.5^{b}
1/2:1/2	72.1±15.9^{a}	59.2±1.5^{bc}	100±0.0^{a}	30±10.8^{b}	47.5±5.9^{ab}
1/2:3/4	100±0.0^{a}	100±0.0^{a}	100±0.0^{a}	37.1±11.3^{ab}	74.2±12.9^{ab}
3/4:1/4	65.8±5.4^{ab}	83.3±8.3^{ab}	100±0.0^{a}	67.1±16.6^{ab}	71.7±3.3^{ab}
3/4:1/2	100±0.0^{a}	100±0.0^{a}	100±0.0^{a}	**100±0.0^{a}**	79.6±10.7^{a}
3/4:3/4	100±0.0^{a}	100±0.0^{a}	100±0.0^{a}	100±0.0^{a}	**83.3±16.7^{a}**
Ctrl	0.0	0.0	0.0	0.0	0.0

Values are means ± SE

Values in the same column with the same letter are not significantly different by DMRT *P*-value: *p<0.001*.

Bold values represent highest inhibition with minimum concentration for each EOs combination

Table 11 Efficacy of vapour phase of essential oils to inhibit the mycelial growth of *A. niger*. after 4 days of incubation at 26-32 ^{0}C with 12 h light vs darkness.

EO rations	Percent mycelial growth inhibition (%)				
	LG:EU	LG:MP	LG:ML	ML:EU	ML:MP
1	100±0.0^{a}	100±0.0^{a}	100±0.0^{a}	100±0.0^{a}	100±0.0^{a}
1/4:1/4	**100±0.0^{a}**	**85.3±14.7^{a}**	75.8±12.2^{b}	12.9±4.0^{c}	39.8±14.4^{c}
1/4:1/2	100±0.0^{a}	100±0.0^{a}	77.5±11.5^{b}	19.1±3.0^{c}	49.8±6.8^{bc}
1/4:3/4	100±0.0^{a}	100±0.0^{a}	**100±0.0^{a}**	29±6.3^{c}	60.2±4.2^{bc}
1/2:1/4	100±0.0^{a}	90.9±9.1^{a}	100±0.0^{a}	25.5±1.2^{c}	51.9±8.5^{bc}
1/2:1/2	100±0.0^{a}	100±0.0^{a}	100±0.0^{a}	29.4±4.1^{c}	49.8±3.1^{bc}
1/2:3/4	100±0.0^{a}	90±10.0a	100±0.0^{a}	48.5±15.2^{bc}	36.4±7.2^{c}
3/4:1/4	100±0.0^{a}	100±0.0^{a}	100±0.0^{a}	53.2±4.6^{bc}	57.6±5.8^{bc}
3/4:1/2	100±0.0^{a}	100±0.0^{a}	100±0.0^{a}	84.4±15.6^{ab}	73.2±15.0^{ab}
3/4:3/4	100±0.0^{a}	100±0.0^{a}	100±0.0^{a}	**100±0.0^{a}**	**91.3±8.6^{a}**
Ctrl	0.0	0.0	0.0	0.0	0.0

Values are means ± SE

Values in the same column with the same letter are not significantly different by DMRT *P*-value: *p<0.001*.

Bold values represent highest inhibition with minimum concentration for each EOs combination

Table 12 Efficacy of vapour phase of essential oils to inhibit the mycelial growth of *Fusarium* sp. after 4 days of incubation at 26-32 ^{0}C with 12 h light vs darkness.

EO rations	Percent mycelial growth inhibition (%)				
	LG:EU	LG:MP	LG:ML	ML:EU	ML:MP
1	100±0.0a	100±0.0a	100±0.0a	100±0.0a	100±0.0a
1/4:1/4	55.8±4.8b	**100±0.0a**	**100±0.0a**	56.6±6.5b	59.6±7.0b
1/4:1/2	**100±0.0a**	91.7±8.3a	100±0.0a	71.3±2.6ab	**100±0.0a**
1/4:3/4	100±0.0a	82.5±8.8a	100±0.0a	72.5±1.4ab	100±0.0a
1/2:1/4	82.1±9.0a	100±0.0a	100±0.0a	61.6±10.4b	66.7±4.7b
1/2:1/2	100±0.0a	100±0.0a	100±0.0a	85.4±14.6ab	100±0.0a
1/2:3/4	100±0.0a	100±0.0a	100±0.0a	76.7±11.9ab	100±0.0a
3/4:1/4	82.9±8.5a	100±0.0a	100±0.0a	62.9±18.6b	91.3±8.8a
3/4:1/2	100±0.0a	100±0.0a	100±0.0a	**100±0.0a**	100±0.0a
3/4:3/4	100±0.0a	100±0.0a	100±0.0a	100±0.0a	100±0.0a
Ctrl	0.0	0.0	0.0	0.0	0.0

Values are means ± SE
Values in the same column with the same letter are not significantly different by DMRT *P*-value: *p<0.001*.
Bold values represent highest inhibition with minimum concentration for each EOs combination

Table 13 Efficacy of vapour phase of essential oils to inhibit the mycelial growth of *Curvularia* sp. after 4 days of incubation at 26-32 ^{0}C with 12 h light vs darkness.

EO rations	Percent mycelial growth inhibition (%)				
	LG:EU	LG:MP	LG:ML	ML:EU	ML:MP
1	100±0.0^a	100±0.0^a	100±0.0^a	100±0.0^a	100±0.0^a
1/4:1/4	77.5±1.4^b	83.3±8.4^{ab}	**100±0.0^a**	**100±0.0^a**	**87.9±12.1^a**
1/4:1/2	85±7.8^{ab}	84.6±7.8^{ab}	100±0.0^a	100±0.0^a	100±0.0^a
1/4:3/4	85±7.8^{ab}	**100±0.0^a**	100±0.0^a	100±0.0^a	100±0.0^a
1/2:1/4	75.8±1.5^b	75.8±1.5^{ab}	100±0.0^a	100±0.0^a	80±10.4^a
1/2:1/2	93.3±6.6^{ab}	100±0.0^a	100±0.0^a	100±0.0^a	100±0.0^a
1/2:3/4	86.7±6.7^{ab}	100±0.0^a	100±0.0^a	100±0.0^a	100±0.0^a
3/4:1/4	93.3±6.6^{ab}	100±0.0^a	100±0.0^a	100±0.0^a	85±15.0^a
3/4:1/2	**100±0.0^a**	89.2±10.9^{ab}	100±0.0^a	100±0.0^a	100±0.0^a
3/4:3/4	100±0.0^a	100±0.0^a	100±0.0^a	100±0.0^a	100±0.0^a
Ctrl	0.0	0.0	0.0	0.0	0.0

Values are means ± SE
Values in the same column with the same letter are not significantly different by DMRT *P*-value: *p<0.001*.
Bold values represent highest inhibition with minimum concentration for each EOs combination

Table 14 Fractional Inhibitory Concentration Indices for the EO vapours against the study fungi calculated from the MICs of each oil and each oil fraction.

EOs		*Aspergillus* sp.			*Fusarium* sp.			*A. niger*			*C. lunata*		
A	B	FIC A	FIC B	FIC I	FIC A	FIC B	FIC I	FIC A	FIC B	FIC I	FIC A	FIC B	FIC I
LG	EU	0.25	0.75	1	0.25	0.5	0.75	0.25	0.25	0.5	0.75	0.5	1.25
LG	MP	0.25	0.75	1	0.25	0.25	0.5	0.25	0.25	0.5	0.25	0.75	1
LG	ML	0.25	0.25	0.5	0.25	0.25	0.5	0.25	0.25	0.5	0.25	0.25	0.5
ML	EU	0.75	0.5	1.25	0.75	0.5	1.25	0.75	0.75	1.5	0.25	0.25	0.5
ML	MP	0.75	0.5	1.25	0.25	0.5	0.75	0.75	0.75	1.5	0.25	0.25	0.5

Interpretation: synergistic FICI ≤0.5, partially synergistic FICI ≥0.5≤1 and no interaction FICI >1

The summary of sections 4.5 and 4.5 have been presented in Appendix 1.

4.7 Antifungal assay with raw materials volatile oils

The efficacy of volatile oils from clove powder and makrut lime peel was evaluated against mycelial growth of *Aspergillus* sp., *F. sacchari* and *C. lunata* after four days of incubation. Results (Table 15) showed that CP completely inhibited fungal growth at all levels of treatments (0.25g, 0.50g and 1.00 g). *C. lunata* was least sensitive (45.15% growth inhibition at 1.00g) to volatile oils of MP while *Aspergillus* sp., and *F. sacchari* were significantly inhibited only at 1.00 g of the plant material.

Table 15 Percentage inhibition of the mycelial growth of the fungi by clove powder and makrut lime peel after 4 days of incubation at room temperature (26-30 ^{0}C).

Fungi	Mycelial growth inhibition (%)					
	Clove powder			Makrut lime peel		
	0.25g	0.50g	1.00g	0.25g	0.50g	1.00g
Aspergillus sp.	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	42.9±2.4 e	48.4±5.0 d	73.8±2.4b
F. sacchari	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	30.4±2.6 f	49.1±1.9 d	66.3±1.3c
C. lunata	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a	18.6±3.2 g	25.7±4.4 f	45.2±3.2d e

Values are means ± standard error. Within each row, means with the same letter are not significantly different (P < 0.01).

4.8 Aflatoxin isolation and detection

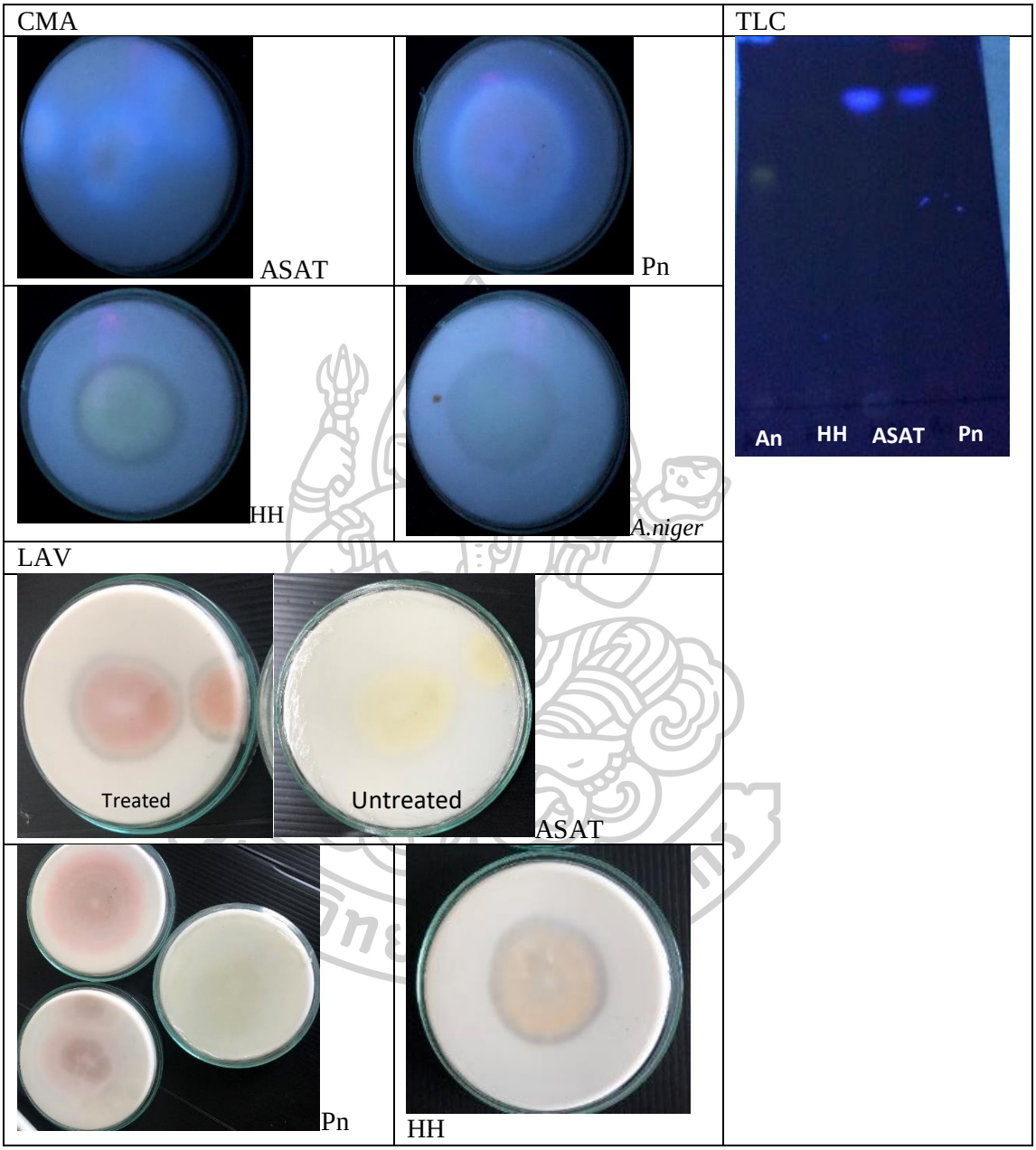
The results for aflatoxin isolation and detection (Table 16) showed that of the seven tested sample isolates, only *Aspergillus* sp. isolated from corn (ASAT) and Peanut tested positive with the three detection methods.

In CMA test, a blue fluorescence was first observed on the third day for both positive isolates. The fluorescence was visibly stronger in ASAT isolate than Peanut isolate. This blue fluorescence was stronger on the fourth day but was observed to decline on the following days in both isolates.

Liquid ammonia vapour test was consistent with the CMA test. After 7 days of incubation at room temperature (26-32 ^{0}C) under natural light, a reddish pink reverse colony colour was observed in both ASAT and Peanut isolates. The rest of the isolates maintained pale yellow reverse-colony colour and were recorded as negative. The control isolate, *A. niger* also showed no observable changes in reverse colony colour after being subjected to ammonia vapour for atleast five minutes.

A TLC test was conducted using the *A. niger* isolate as control, HH, ASAT, and Peanut isolates. The blue fluorescence was used as the indicator of positive sample while no blue fluorescence was a negative for aflatoxin. The results showed that *A. niger* and HH isolates were negative while ASAT and Peanut were positive. The distance moved by the aflatoxin spot up the plate for both ASAT and Peanut was the same as observed under UV light (365 nm).

Table 16 Illustrations of the aflatoxin detection from seven-day-old *Aspergillus* sp. cultures on using blue fluorescence (CMA and TLC) and reverse colony colour under LAV test.



Pn = Peanut, An = *A. niger*,

4.9 Anti-aflatoxin efficacy of EO vapours

The EOs of ML and LG were strongly inhibitory against the five *Aspergillus* sp. isolates (Table 17). The LG oil was slightly stronger in efficacy than ML at 5 µL. Results for the inhibitory effect of EO vapours against aflatoxin production are displayed in Table 18 below. Aflatoxin inhibition was dose dependent with higher

doses inhibiting strongly than lower doses of the EOs. None of the tested EO vapours, at any dose level, completely inhibited mycelial growth after the 4 days of incubation period at room temperature (26-30 ^{0}C). At a dose of 10 µL, ML and LG oils completely inhibited aflatoxin production by *Aspergillus* sp. The combination of lemongrass with makrut leaf oils showed a synergistic activity against aflatoxin production. However, makrut peel oil was unable to inhibit aflatoxin production at any of the tested doses. It was observed that CMA and Ammonia vapour tests were not conclusive to confirm the inhibitory capacity of the EO as evidenced by the TLC tests.

Table 17 Fungal mycelial growth inhibition by EOs on PDA incubated at room temperature (26-30 ^{0}C) for 4 days with 12 h light vs darkness.

EOs	Dose (µL)	Pn	ASAT	HH	THY	CHM
LG	5	85.4±13.9^a	89.0±19.1^a	84.2±19.4^{abc}	79.1±18.1^{ab}	94.3±9.8^a
MIC = 0.19 FICI = 0.5	10	94.7±9.2^a	100.0±0.0^a	87.5±21.6^{abc}	89.3±18.5^{ab}	100.0±0.0^a
	20	100.0±0.0^a	100.0±0.0^a	100.0±0.0^a	100.0±0.0^a	100.0±0.0^a
ML	5	89.7±6.1^a	100.0±0.0^a	58.3±39.6^{abcde}	65.8±8.5^{bc}	97.2±4.9^a
MIC = 0.19 FICI = 0.5	10	98.3±2.8^a	100.0±0.0^a	91.7±14.4^{ab}	100.0±0.0^a	100.0±0.0^a
	20	100.0±0.0^a	100.0±0.0^a	100.0±0.0^a	100±0.0^a	98.0±3.4^a

Table 18 Effect of EOs on aflatoxin production by *Aspergillus* sp under three different tests.

Essential oils	Dose (µL)	CMA	LAV	TLC
ML	2.5	NI	NI	NI
	5	I	I	PI
	10	I	I	I
LG	2.5	NI	NI	NI
	5	I	I	PI
	10	I	I	I
LG:ML	2.5:2.5	I	I	I
	2.5:1.25	PI	PI	PI
	1.25:2.5	PI	PI	PI
	1.25:1.25	NI	NI	NI

CMA= Coconut milk agar, TLC = Thin Layer Chromatography, LAV = Liquid ammonia vapour

I = Complete inhibition, PI = Partial inhibition and NI = No inhibition

CHAPTER FIVE

DISCUSSION

5.1 Isolation and identification of fungi for the study

Peanuts and corn grains have been reported as suitable substrates for fungal colonisation with *Aspergillus* sp. (Giorni et al., 2007; Nyongesa et al., 2015). *Fusarium* sp. and *C. lunata* has been severally isolated from infected maize and rice grains. A higher incidence of infection was observed with direct plating than with blotting technique. Direct plating has been used previously for isolation of *Aspergillus* sp. from coffee beans (Perrone et al., 2007). Surface sterilisation reveals that mostly these fungal pathogens are seedborne. In our study, surface sterilisation had effect on fungal incidence of *Aspergillus* sp., which is an opportunistic fungus. The incidence of *Fusarium* sp. and *C. lunata* was not altered by surface sterilization of the grain kernels. Our study revealed that *Aspergillus* sp. had the highest incidence in all samples (Figure 5) in both techniques for the seeds were surface sterilized or not. In Thailand, major fungi in corn included *Fusarium moniliforme, Aspergillus flavus, Penicillium citrinum, A. niger, Lasiodiplodia theobromae* and *Fusarium semitectum* (Pitt et al., 1994), in which some of these fungi (such as *A. flavus, A. niger* and *F. graminearum*) produce toxins that pose a hazard to health of people in Thailand (Suttajit, 2007). *Curvularia lunata* was one of the causal agents of grain discoloration of rice (CABI, 2020) and this fungus was reported to associate with seeds of various rice varieties (Imolehin, 1983). Recently, Seephueak et al. (2019) reported that *C. lunata* was among the most frequently isolated fungi from rice in Thailand. The presence of this fungus inside rice seeds may complicate the implementation of disease control should the fungus is pathogenic to rice seedlings.

5.2 Antifungal assay with individual essential oils

Extensive reviews for the antifungal activity of EOs (Nazzaro et al., 2017) as well as their dispensation by vapour contact (Laird & Phillips, 2012) have been published. Several reports have indicated that EOs vapour phase achieves a greater antifungal activity as compared to other methods of antifungal assay (Avila-Sosa et al., 2012; Romina Bluma et al., 2009; Caccioni et al., 1998; Inouye et al., 2006).

Lemongrass oil was completely inhibitory to the mycelial growth of all tested fungi (Table 7), although percent yield of the essential oil of plant was quite low (Table 3). Lemongrass oil contained approximately 65-85% citral and this substance was inhibitory to *Candida* spp., the causal agents of the superficial mycoses of the skin in human (Silva et al., 2008). Wang et al. (2019) reported that citral completely suppressed mycelial growth of *Alternaria alternata* as the minimum inhibitory concentration (MIC) at 0.25 μL/mL and inhibited more than 97% of the mycotoxin that was produced by this fungus. Plant pathogenic fungi in peanut (*Arachis hypogaea*), such as *Alternaria alternata*, *A. flavus*, *C. lunata*, *Fusarium moniliforme*, *F. pallidoroseum*, and *Phoma sorghina*, were completely inhibited by citral in paper disc agar diffusion assays (Kishore et al., 2007). Tang et al. (2018) also found that citral was also suppressive to the growth of *Aspergillus flavus* and *A. ochraceus*, two common grain pathogens. Cell wall and plasma membrane damage, and Ca^{2+}, K^{+} and Mg^{2+} leakage were observed when *A. niger* and *A. flavus* hypha were exposed to *C. citratus* EO by fumigation method (Helal et al., 2007). Citral is the major chemical compound of lemongrass (Boukhatem et al., 2014; Tyagi & Malik, 2010). It was reported that the vapours of geraniol and citral were the most effective compounds among others including eugenol isolated from clove, against the mycelial growth of *A. flavus* and *A. ochraceus* (Tang et al., 2018).

The chemical compounds of makrut lime leaves were investigated to have various biological activities (Dertyasasa & Tunjung, 2017). According to various investigations, the major chemical composition of makrut leaf essential oils is citronellal (Raksakantong et al., 2012; Setiyoningrum et al., 2018), while citronellol, sabinene, citronellyl acetate and linalool were among minor compounds (Setiyoningrum et al., 2018; Warsito et al., 2017). *Corymbia citriodora* essential oil (61.78% citronellal and citronellol) inhibited the mycelial growth of *Aspergillus* sp. in vapour phase by 66% (Aguiar et al., 2014). Srisukh et al. (2012) reported that citronellal was the major component (at about 80%) in makrut leaf oil based on the analysis using gas chromatography-mass spectrometry. This makrut leaf oil had the lowest MIC against the bacteria causing disease to respiratory tract of human. Citronellal, extracted from *Cymbopogon nardus*, completely inhibited the growth of *Aspergillus* spp., *Penicillium* spp. and *Eurotium* spp. (Nakahara et al., 2013).

Clove oil, from the floral bud of *S. aromaticum*, was widely reported as a potential antimicrobial, anti-mycotic and antioxidant agent (Cortés-Rojas et al., 2014; Hu et al., 2018). Chemical analysis using gas chromatography coupled with mass spectrometry (GC-MS) revealed that clove oil contained eugenol (62.52%), eugenol acetate (23.62%) and β-caryophyllene (9.77%) and α-caryophyllene (1.39%) (Rodríguez et al., 2018). Tullio and colleagues reported that vapour phase of EOs of clove (*Eugenia caryophyllata* Thunb) and other herbs was more effective than broth microdilution based on the values of the lower minimum inhibitory concentrations (MICs) as percent volume/volume (% v/v) (Tullio et al., 2007). The antifungal activity of clove oil in our study is similar to a previous study which reported complete inhibition of both *A. flavus* and *F. moniliforme* at 5 μL and 10 μL pure clove oil respectively (Massoud et al., 2012). The analysis of clove essential oil in liquid and vapour phase revealed that eugenol the major chemical component of clove oil was less concentrated in vapour phase (57.78%) than it was in liquid (74.25%) moreover the number of compounds were also fewer in liquid phase than in vapour phase (Prates et al., 2019). Although clove bud had percent yield of the EO more than the other plant materials (Table 4), the efficacy of its EO vapour to inhibit the mycelial growth of *Aspergillus* sp., *A. niger*, *Fusarium* sp., *C. lunata* was not as good as that of lemongrass stem and makrut lime leaf (Table 7). Different plant parts, from the same plant species, have different both percent yield of the EO and its efficacy to inhibit the fungi (Table 4 and Table 7). Even though makrut lime peel had percent yield of higher than makrut lime leaf (Table 4), the peel of makrut lime contained the EO that was less effective than that of the makrut lime leaf in suppressing the mycelial growth of the fungi (Table 7). The oil from makrut lime leaf completely inhibited the mycelial growth of all four tested fungi at all doses (except *A. niger* and *Fusarium* sp., both at 5 μL).

The major chemical composition of the makrut lime peel oil is β-pinene, limonene, citronellal, and sabinene (Chanthaphon et al., 2008). The EO of eucalyptus (*Eucalyptus globulus*) was demonstrated to contain limonene (29.9%) which was the second most abundant chemical constituent and when this EO was used against a range of microorganisms, the vapour completely inhibited *A. flavus* at 60 μL (Tyagi & Malik, 2011). Orange peel oil which contained more than 95% limonene in vapour

phase was more effective than that in solution in the medium to inhibit *A. flavus* (Velázquez-Nuñez et al., 2013).

5.3 Antifungal assay with combined essential oils

Vapour phase synergism of EOs cominations against fungal pathogens was previously investigated (Ji et al., 2019). The combination of lemongrass (fresh stem and leaves) and makrut lime EOs in a nanoemulsion (4% VCO; 17.3% Tween 80; 8.7% PEG 400; 70% water) showed that composition of the major volatiles compounds was not changed (Juniatik et al., 2017). This implies that the individual compounds act independently to cause effect on the same pathogen as observed in our study.

The initial treatment doses (Table 7) and the MIC values (Table 8), required to show the suppressive effect to the mycelial growth of all tested fungi of the essential oils of both makrut lime leaf and lemongrass stem, indicated that they had potential to use to control the storage fungi. The combined effect of the vapor phase of these oils had full synergistic effect to suppress *Aspergillus* sp., *Fusarium* sp. *A. niger* and *C. lunata* (Table 7). The synergism between these EOs of these two plants (Table 6), in conjunction with their potency against the tested fungi (Table 4 and Table 5), has enhanced their efficacy to control the storage fungi. This is because the active ingredient in each EO of these two plants (possibly citral in lemongrass stem and citronellal in makrut lime leaf) will differ in its mode of action against the target fungi. However, the antifungal mechanism of the active compounds in EOs remains poorly understood, although the cell membrane is the possible target of bioactive volatile compounds because the essential oils are mixtures of compounds that are lipid soluble.

5.4 Aflatoxin isolation, detection and inhibition by essential oils

Wu et al. (2016) suggested that the antifungal activity of citronellal against *Penicilium digitatum* was due to its potential to disrupt the integrity of the cell membrane. This can accelerate the disintegration of the fungal cell wall as other compounds can penetrate the cell wall and synergistically suppress the fungal growth. In addition to disintegrations of cell membrane permeability, citral and geraniol reduced the expression of *afl*R gene in *A. flavus* (Tang et al., 2018). The *afl*R gene

plays a significant role in regulation of aflatoxin production by *Aspergillus* sp. (Caceres et al., 2020). Lemongrass EO applied by fumigation severely damaged the cell membrane in *A. flavus*, leaking Ca^{2+}, K^{+} and Mg^{2+}, and inhibition of aflatoxin B_1 production at sub lethal dose (Helal et al., 2007). Citrus EOs from lime (*C. limon* L.) (85 % limonene, 2.2% citral), bergamot (*C. bergamia* Risso) (40% limonene, 8% linalool, 0.5% citral) and bitter orange (*C. aurantium*) (93% limonene, 0.12% citral) significantly inhibited growth of and aflatoxin B_1 production by *A. flavus* (Restuccia et al., 2019).

The high antifungal activity in vapour phase may be because the active ingredients in the vapour phase of these compounds are more concentrated than the aqueous phase resulting in increased potency against the tested fungi. The EOs applied as vapour phase should also have higher efficacy to inhibit the fungal growth than those applied in a medium (Edris & Farrag, 2003). This is because EOs applied in the medium need to move in the matrix causing the delay of EOs in acting against the fungi. Physiologically, fungal mycelia are lipophilic which renders them highly absorbent to the EOs

The use of hermetic storage system and airtight silos have been promoted and implemented by most governments among small scale farmers. These storage methods are suitable for application of volatile compounds and therefore EOs can easily be adopted by the small holder farmers who grow rice and corn to use to protect their crops against postharvest loss from fungal infection. In our study, the EOs were extracted from the plants, which are abundant and readily available in the backyard orchards in Thailand. The vapor phase of these oils has been used in the antifungal assays and, both lemongrass and makrut lime are plants that have potential to use for controlling postharvest diseases of stored grains caused by *Aspergillus* sp., *Fusarium* sp. and *C. lunata*.

CONCLUSIONS

This study revealed the efficacy of lemongrass and makrut lime leaf essential oils against the tested fungi when applied by vapour contact. Both EOs completely inhibited mycelial of all the tested fungi. The combination of the two oils yielded a synergistic antifungal activity against all the tested fungi. Lemongrass and makrut lime leaf EOs showed a reduction in aflatoxin detected by TLC, both individually and in combination. Eucalyptus essential oil vapour was the lowest mycelial growth inhibitor for all the tested fungi, however was able to inhibit aflatoxin production by *A. flavus* at sublethal dose of 20 µL. The EO concentration was reduced by more than half which reduces the side effects of these oils when used in food materials or food packaging or food storage. Lemongrass, makrut lime are readily available herbs in many parts of the world, and these could provide a source of biofungicides with high efficacy against some notorious food pathogens like *Aspergillus* sp. This was the first study for combination of lemongrass and makrut lime leaf oils in vapour phase. Further research is needed to test the potential of these oils to be used in storage.

APPENDIX

Appendix 1 Summary of the antifungal assay

EO	Initial treatment (Doses) for all EOs: 5 µL, 10 µL, and 20 µL. Incubation 4 days, 26-30 ^{0}C	SID (µL)				SID dilutions. ¾, ½ and ¼ MID = ¾ SID or ½ SID or ¼ SID	MID (µL)			
		As	An	Fu	Cu		As	An	Fu	Cu
C		20	20	20	20		10	10	15	10
EU		20	30	20	20		15	20	15	15
LG		5	5	5	5		5	5	5	2.5
ML		5	10	10	5		5	5	5	2.5
MP		20	30	20	10		15	20	15	7.5

SID = smallest inhibitory dose (the smallest volume of initial treatment that significantly or completely inhibited the growth of the test fungi.)

MID = Mininimum Inhibitory Dose (the smallest volume of SID dilution that completely inhibited fungal growth)

MIC = MID/27 mL 27 mL is volume of air above the EO in the closed cup

Fraction MIC (FMIC) = the minimum inhibitory concentration of each EO in combination

	MIC (µL/mL)			
	s	An	F	C
	.37	0.37 0	0.55 6	0.37 0
	0.55 6	0.74 1	0.55 6	0.55 6
	0.18 5	0.18 5	0.18 5	0.09 3
	0.18 5	0.18 5	0.18 5	0.09 3
	0.55 6	0.74 1	0.55 6	0.27 8

EO scombined	Fractional MICs (µL/mL)			
	As	An	Fu	Cu
LG:EU	0.046:0.41 7	0.046:0.18 5	0.046:0.27 8	0.069:0.27 8
LG:ML	0.046:0.04 6	0.046:0.13 8	0.046:0.04 6	0.023:0.02 3
LG:MP	0.046:0.41 7	0.046:0.18 5	0.046:0.13 9	0.023:0.20 8
ML:EU	0.139:0.27 8	0.139:0.37 0	0.139:0.27 8	0.023:0.13 9
ML:MP	0.139:041 7	0.139:0.55 6	0.046:0.27 8	0.023:0.13 9

FIC = FMIC ÷ MIC

FICI = summation of FIC of individual oils in combination

EO combined	FICs (µL/mL)				FICI (µL/mL)			
	As	An	Fu	Cu	As	An	Fu	Cu
LG:EU	0.25:0.75	0.25:0.25	0.25:0.5	0.75:0.5	1	0.5	0.75	1.25
LG:ML	0.25:0.25	0.25:0.5	0.25:0.25	0.25:0.25	0.5	0.75	0.5	0.5
LG:MP	0.25:0.75	0.25:0.25	0.25:0.25	0.25:0.75	1	0.5	0.5	0.5
ML:EU	0.75:0.5	0.75:0.50	0.75:0.5	0.25:0.25	1.25	1.25	1.25	0.5
ML:MP	0.75:0.75	0.75:0.75	0.25:0.5	0.25:0.5	1.5	1.5	0.75	0.75

REFERENCES

Abbas, H. K., Wilkinson, J. R., Zablotowicz, R. M., Accinelli, C., Abel, C. A., Bruns, H. A., & Weaver, M. A. (2009). Ecology of Aspergillus flavus, regulation of aflatoxin production, and management strategies to reduce aflatoxin contamination of corn. *Toxin Reviews, 28*(2-3), 142-153. doi:10.1080/15569540903081590

Abbaszadeh, S., Sharifzadeh, A., Shokri, H., Khosravi, A., & Abbaszadeh, A. (2014). Antifungal efficacy of thymol, carvacrol, eugenol and menthol as alternative agents to control the growth of food-relevant fungi. *Journal de mycologie medicale, 24*(2), e51-e56.

Aguiar, R. W. d. S., Ootani, M. A., Ascencio, S. D., Ferreira, T. P. S., Santos, M. M. d., & Santos, G. R. d. (2014). Fumigant Antifungal Activity ofCorymbia citriodoraandCymbopogon nardusEssential Oils and Citronellal against Three Fungal Species. *The Scientific World Journal, 2014*, 1-8. doi:10.1155/2014/492138

Aguilar-González, A. E., Palou, E., & López-Malo, A. (2015). Antifungal activity of essential oils of clove (Syzygium aromaticum) and/or mustard (Brassica nigra) in vapor phase against gray mold (Botrytis cinerea) in strawberries. *Innovative food science & emerging technologies, 32*, 181-185.

Albores, L. C., Baños, S. B., Herrera, J. M., Necha, L. B., López, M. H., & Hernández, A. C. (2014). Morphological and molecular characterization of pathogenic isolates of Fusarium spp. obtained from gladiolus corms and their sensitivity to Jatropha curcas L. oil. *Afr. J. Microbiol. Res, 8*(8), 724-733.

Allen, S., Wild, C., Wheeler, J., Riley, E., Montesano, R., Bennett, S., . . . Greenwood, B. (1992). Aflatoxin exposure, malaria and hepatitis B infection in rural Gambian children. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 86*(4), 426-430.

Ambindei, W. A., Jazet, P. M. D., Tatsadjieu, L. N., Priya, P., Manilal, V., Krishnakumar, B., & Zollo, P. H. A. J. A. J. o. B. (2017). Effect of the essential oils of Thymus vulgaris, Cinnamomum zeylanicum and Mentha piperita on fungal growth and morphology. *African Journal of Biotechnology, 16*(9), 388-399. doi:10.5897/ajb2017.15881

Arendrup, M., Guinea, J., Cuenca-Estrella, M., Meletiadis, J., Mouton, J., Lagrou, K., & Howard, S. (2015). EUCAST DEFINITIVE DOCUMENT E. DEF 9.3. *Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds*. Retrieved from https://www.aspergillus.org.uk/sites/default/files/pictures/Lab_protocols/EUCAST_E_Def_9_3_Mould_testing_definitive_0.pdf.

Arrebola, E., Sivakumar, D., Bacigalupo, R., & Korsten, L. (2010). Combined application of antagonist Bacillus amyloliquefaciens and essential oils for the control of peach postharvest diseases. *Crop protection, 29*(4), 369-377. doi:10.1016/j.cropro.2009.08.001

Arshad, H. M. I., Khan, J. A., Naz, S., Khan, S. N., & Akram, M. (2009). Grain discoloration disease complex: a new threat for rice crop and its management. *Pak. J. Phytopathol, 21*(1), 31-36.

Arya, S. S., Salve, A. R., & Chauhan, S. (2016). Peanuts as functional food: a review.

Journal of food science and technology, 53(1), 31-41. doi:10.1007/s13197-015-2007-9

Atehnkeng, J., Ojiambo, P., Ikotun, T., Sikora, R., Cotty, P., & Bandyopadhyay, R. (2008). Evaluation of atoxigenic isolates of Aspergillus flavus as potential biocontrol agents for aflatoxin in maize. *Food additives and Contaminants, 25*(10), 1264-1271.

Avila-Sosa, R., Palou, E., Munguía, M. T. J., Nevárez-Moorillón, G. V., Cruz, A. R. N., & López-Malo, A. J. I. j. o. f. m. (2012). Antifungal activity by vapor contact of essential oils added to amaranth, chitosan, or starch edible films. *Int J Food Microbiol, 153*(1-2), 66-72. doi:10.1016/j.ijfoodmicro.2011.10.017

Babiah, P., Upreti, D., & John, S. (2015). Assessment of fungicidal potential of lichen Heterodermia leucomelos (L.) Poelt against pathogenic fungi. *Current Research in Environmental & Applied Mycology, 5*(2), 92-100.

Bakkali, F., Averbeck, S., Averbeck, D., & Idaomar, M. (2008). Biological effects of essential oils–a review. *Food and chemical toxicology, 46*(2), 446-475.

Baser, K. H. C., & Buchbauer, G. (2015). *Handbook of essential oils: science, technology, and applications*: CRC press.

Başer, K. H. C., & Demirci, F. (2007). Chemistry of essential oils. *Flavours and Fragrances: Chemistry, Bioprocessing and Sustainability, edited by Berger RG. New York: Springer*. 43-86.

Bellio, A., Bianchi, D., Gramaglia, M., Loria, A., Nucera, D., Gallina, S., . . . Decastelli, L. (2016). Aflatoxin M1 in Cow's Milk: Method Validation for Milk Sampled in Northern Italy. *Toxins, 8*(3). doi:10.3390/toxins8030057

Benkerroum, N. (2020). Chronic and Acute Toxicities of Aflatoxins: Mechanisms of Action. *International Journal of Environmental Research and Public Health, 17*(2), 423.

Berkow, E. L., Lockhart, S. R., & Ostrosky-Zeichner, L. (2020). Antifungal Susceptibility Testing: Current Approaches. *Clin Microbiol Rev, 33*(3). doi:10.1128/cmr.00069-19

Bluma, R., Amaiden, M. R., & Etcheverry, M. (2008). Screening of Argentine plant extracts: Impact on growth parameters and aflatoxin B1 accumulation by Aspergillus section Flavi. *International Journal of Food Microbiology, 122*(1-2), 114-125.

Bluma, R., Landa, M. F., & Etcheverry, M. (2009). Impact of volatile compounds generated by essential oils on Aspergillus section Flavi growth parameters and aflatoxin accumulation. *Journal of the Science of Food and Agriculture, 89*(9), 1473-1480. doi:10.1016/j.fm.2007.10.004

Boukaew, S., Prasertsan, P., & Sattayasamitsathit, S. (2017). Evaluation of antifungal activity of essential oils against aflatoxigenic Aspergillus flavus and their allelopathic activity from fumigation to protect maize seeds during storage. *Industrial crops and products, 97*, 558-566.

Boukhatem, M. N., Ferhat, M. A., Kameli, A., Saidi, F., & Kebir, H. T. (2014). Lemon grass (Cymbopogon citratus) essential oil as a potent anti-inflammatory and antifungal drugs. *Libyan J Med, 9*, 25431. doi:10.3402/ljm.v9.25431

Bressac, B., Puisieux, A., Kew, M., Volkmann, M., Bozcall, S., Mura, J. B., . . . Wands, J. (1991). p53 mutation in hepatocellular carcinoma after aflatoxin exposure. *The Lancet, 338*(8779), 1356-1359.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods--a review. *Int J Food Microbiol, 94*(3), 223-253. doi:10.1016/j.ijfoodmicro.2004.03.022

CABI. (2020). Curvularia (black kernel). In: Invasive Species Compendium (Data Sheet). Retrieved from https://www.cabi.org/isc/datasheet/17046. Retrieved 30 Jan 2020, from CAB International https://www.cabi.org/isc/datasheet/17046

Caccioni, D. R., Guizzardi, M., Biondi, D. M., Renda, A., & Ruberto, G. J. I. j. o. f. m. (1998). Relationship between volatile components of citrus fruit essential oils and antimicrobial action on Penicillium digitatum and Penicillium italicum. *Int J Food Microbiol, 43*(1-2), 73-79. doi:10.1016/s0168-1605(98)00099-3

Caceres, I., El Khoury, R., Medina, Á., Lippi, Y., Naylies, C., Atoui, A., . . . Puel, O. (2016). Deciphering the anti-aflatoxinogenic properties of eugenol using a large-scale q-PCR approach. *Toxins, 8*(5), 123.

Caceres, I., Khoury, A. A., Khoury, R. E., Lorber, S., Oswald, I. P., Khoury, A. E., . . . Bailly, J.-D. (2020). Aflatoxin Biosynthesis and Genetic Regulation: A Review. *Toxins, 12*(3), 150.

Camiletti, B. X., Torrico, A. K., Maurino, M. F., Cristos, D., Magnoli, C., Lucini, E. I., & Pecci, M. d. l. P. G. (2017). Fungal screening and aflatoxin production by Aspergillus section Flavi isolated from pre-harvest maize ears grown in two Argentine regions. *Crop protection, 92*, 41-48.

CDC. (2004). *Outbreak of aflatoxin poisoning--eastern and central provinces, Kenya, January-July 2004* (1545-861X). Retrieved from

Chain, E. P. o. C. i. t. F., Schrenk, D., Bignami, M., Bodin, L., Chipman, J. K., del Mazo, J., . . . Leblanc, J. C. (2020). Risk assessment of aflatoxins in food. *EFSA Journal, 18*(3), e06040.

Chanthaphon, S., Chanthachum, S., & Hongpattarakere, T. (2008). Antimicrobial activities of essential oils and crude extracts from tropical Citrus spp. against food-related microorganisms. *30*.

Chegere, M. J. (2017). *Post-harvest losses, intimate partner violence and food security in Tanzania*.

Chulze, S. (2010). Strategies to reduce mycotoxin levels in maize during storage: a review. *Food additives and Contaminants, 27*(5), 651-657.

Clarke, S. (2008). Chapter 3-Families of compounds that occur in essential oils. *Essential Chemistry for Aromatherapy*. 41-77.

Clinical Laboratory Standards Institute. (2008). Reference method for broth dilution antifungal susceptibility testing of yeasts. *Approved Standard-Third Edition M27-A3, 28*.

Coppock, R. W., Christian, R. G., & Jacobsen, B. J. (2018). Aflatoxins. In *Veterinary toxicology* (pp. 983-994): Elsevier.

Corsini, E., Birindelli, S., Fustinoni, S., De Paschale, G., Mammone, T., Visentin, S., . . . Colosio, C. (2005). Immunomodulatory effects of the fungicide Mancozeb in agricultural workers. *Toxicology and applied pharmacology, 208*(2), 178-185.

Cortés-Rojas, D. F., de Souza, C. R. F., & Oliveira, W. P. (2014). Clove (Syzygium aromaticum): a precious spice. *Asian Pacific journal of tropical biomedicine, 4*(2), 90-96.

Danh, L. T., Han, L. N., Triet, N. D. A., Zhao, J., Mammucari, R., & Foster, N. (2013). Comparison of Chemical Composition, Antioxidant and Antimicrobial Activity

of Lavender (Lavandula angustifolia L.) Essential Oils Extracted by Supercritical CO2, Hexane and Hydrodistillation. *Food and Bioprocess Technology, 6*(12), 3481-3489. doi:10.1007/s11947-012-1026-z

Das, S. K., Roy, A., & Barman, H. (2016). Fungi toxic efficiency of some plant volatile essential oils against plant pathogenic fungi. *African Journal of Microbiology Research, 10*(37), 1581-1585.

Davis, J. S., & Lubulwa, G. (1994). *Evaluation of postharvest research: results for an application to tropical fruit research projects and some further Methodological issues*. Retrieved from

Delespaul, Q., de Billerbeck, V. G., Roques, C. G., Michel, G., Marquier-Viñuales, C., & Bessière, J.-M. (2000). The antifungal activity of essential oils as determined by different screening methods. *Journal of essential oil research, 12*(2), 256-266.

Dertyasasa, E. D., & Tunjung, W. A. S. (2017). Volatile Organic Compounds of Kaffir Lime (Citrus Hystrix DC.) Leaves Fractions and Their Potency as Traditional Medicine. *Biosciences Biotechnology Research Asia, 14*(4), 1235-1250.

Dhananjayan, V., & Ravichandran, B. (2018). Occupational health risk of farmers exposed to pesticides in agricultural activities. *Current Opinion in Environmental Science & Health, 4*, 31-37.

Dobre, A. A., Gagiu, V., & Petru, N. (2011). Antimicrobial activity of essential oils against food-borne bacteria evaluated by two preliminary methods. *Romanian Biotechnological Letters, 16*(6), 119-125.

Dorner, J. W., Cole, R. J., & Blankenship, P. D. (1998). Effect of inoculum rate of biological control agents on preharvest aflatoxin contamination of peanuts. *Biological Control, 12*(3), 171-176.

Douglas, W. A., & Tullis, E. C. (1950). *Insects and fungi as causes for pecky rice*: US Dept. of Agriculture.

Dubale, B., Solomon, A., Geremew, B., Sethumadhava, R., Waktole, S. J. A. j. o. f., agriculture, nutrition, & development. (2014). Mycoflora of grain maize (Zea mays L.) stored in traditional storage containers (Gombisa and sacks) in selected woredas of Jimma zone, Ethiopia. *African journal of food, agriculture, nutrition development, 14*(2), 8676-8694. Retrieved from https://www.ajol.info/index.php/ajfand/article/view/104748

Dutta, A., & Hussain, S. (1959). Leaf spot of Rose caused by Curvularia lunata (Wakker) Boedijn. *Current Science, 28*(10).

Edris, A. E., & Farrag, E. S. (2003). Antifungal activity of peppermint and sweet basil essential oils and their major aroma constituents on some plant pathogenic fungi from the vapor phase. *Nahrung/Food, 47*(2), 117-121. doi:10.1002/food.200390021

Eeckhout, M., Landschoot, S., Deschuyffeleer, N., De Laethauwer, S., & Haesaert, G. (2013). Guidelines for prevention and control of mould growth and mycotoxin production in cereals. *Mycohunt, synagra. be/Download. ashx*.

Elaissi, A., Salah, K. H., Mabrouk, S., Larbi, K. M., Chemli, R., & Harzallah-Skhiri, F. (2011). Antibacterial activity and chemical composition of 20 Eucalyptus species' essential oils. *Food Chemistry, 129*(4), 1427-1434.

Elhidar, N., Nafis, A., Kasrati, A., Goehler, A., Bohnert, J. A., Abbad, A., . . . Mezrioui, N.-E. (2019). Chemical composition, antimicrobial activities and synergistic

effects of essential oil from Senecio anteuphorbium, a Moroccan endemic plant. *Industrial crops and products, 130*, 310-315. doi:10.1016/j.indcrop.2018.12.097

Ellis, W., Smith, J., Simpson, B., Oldham, J., & Scott, P. M. (1991). Aflatoxins in food: occurrence, biosynthesis, effects on organisms, detection, and methods of control. *Critical Reviews in Food Science & Nutrition, 30*(4), 403-439.

Elshafie, H. S., & Camele, I. (2017). An overview of the biological effects of some mediterranean essential oils on human health. *BioMed research international, 2017*.

Esper, R. H., Goncalez, E., Marques, M. O., Felicio, R. C., & Felicio, J. D. (2014). Potential of essential oils for protection of grains contaminated by aflatoxin produced by Aspergillus flavus. *Front Microbiol, 5*, 269. doi:10.3389/fmicb.2014.00269

Feyaerts, A. F., Mathé, L., Luyten, W., De Graeve, S., Van Dyck, K., Broekx, L., & Van Dijck, P. (2018). Essential oils and their components are a class of antifungals with potent vapour-phase-mediated anti-Candida activity. *Scientific reports, 8*(1), 1-10.

Gakuubi, M. M., Maina, A. W., & Wagacha, J. M. (2017). Antifungal activity of essential oil of Eucalyptus camaldulensis dehnh. against selected Fusarium spp. *International journal of microbiology, 2017*.

Ganjewala, D., & Luthra, R. (2010). Essential Oil Biosynthesis and Regulation in the Genus Cymbopogon. *Natural Product Communications, 5*(1), 1934578X1000500137. doi:10.1177/1934578x1000500137

Giorni, P., Magan, N., Pietri, A., Bertuzzi, T., & Battilani, P. (2007). Studies on Aspergillus section Flavi isolated from maize in northern Italy. *Int J Food Microbiol, 113*(3), 330-338.

Golmakani, M.-T., Zare, M., & Razzaghi, S. (2017). Eugenol enrichment of clove bud essential oil using different microwave-assisted distillation methods. *Food Science and Technology Research, 23*(3), 385-394.

Goni, P., López, P., Sánchez, C., Gómez-Lus, R., Becerril, R., & Nerín, C. J. F. c. (2009). Antimicrobial activity in the vapour phase of a combination of cinnamon and clove essential oils. *Food Chem, 116*(4), 982-989. doi:10.1016/j.foodchem.2009.03.058

Guenther, E., & Althausen, D. (1948). *The essential oils* (Vol. 1): Van Nostrand New York.

Gunes, H., Gulen, D., Mutlu, R., Gumus, A., Tas, T., & Topkaya, A. E. (2016). Antibacterial effects of curcumin: an in vitro minimum inhibitory concentration study. *Toxicology and industrial health, 32*(2), 246-250.

Hashemi, S. M. B., Khaneghah, A. M., & de Souza Sant'Ana, A. (2017). *Essential oils in food processing: chemistry, safety and applications* (Retrieved from https://books.google.co.th/books?id=PK45DwAAQBAJ&pg=PT464&lpg=PT464&dq=safety+of+essentials+safety+in+food+JECFA&source=bl&ots=VIn7m36rHG&sig=ACfU3U2PBwmLUBtW8u181Cd_uKJE9r8mug&hl=en&sa=X&ved=2ahUKEwjokpDV5OLpAhUkjuYKHRUiDXUQ6AEwCXoECAoQAQ#v=onepage&q=safety%20of%20essentials%20safety%20in%20food%20JECFA&f=false): John Wiley & Sons.

Helal, G., Sarhan, M., Abu Shahla, A., & Abou El-Khair, E. (2007). Effects of Cymbopogon citratus L. essential oil on the growth, morphogenesis and

aflatoxin production of Aspergillus flavus ML2-strain. *Journal of Basic Microbiology, 47*(1), 5-15.

Hell, K., & Mutegi, C. (2011). Aflatoxin control and prevention strategies in key crops of Sub-Saharan Africa.

Hell, K., Mutegi, C., & Fandohan, P. (2010). Aflatoxin control and prevention strategies in maize for Sub-Saharan Africa. *Julius-Kühn-Archiv*. (425), 534.

Hemaiswarya, S., Kruthiventi, A. K., & Doble, M. (2008). Synergism between natural products and antibiotics against infectious diseases. *Phytomedicine, 15*(8), 639-652.

Herrero, M., Mendiola, J. A., Cifuentes, A., & Ibáñez, E. (2010). Supercritical fluid extraction: Recent advances and applications. *Journal of Chromatography A, 1217*(16), 2495-2511.

Hidro, P. (2013). Effect of temperature on kaffir lime oil by using Hydro-diffusion steam distillation system. *Malaysian Journal of Analytical Sciences, 17*(2), 326-339.

Hossain, F., Follett, P., Vu, K. D., Harich, M., Salmieri, S., & Lacroix, M. J. F. m. (2016). Evidence for synergistic activity of plant-derived essential oils against fungal pathogens of food. *Food Microbiology, 53*, 24-30. doi:10.1016/j.fm.2015.08.006

Hu, Q., Zhou, M., & wei, S. (2018). Progress on the antimicrobial activity research of clove oil and eugenol in the food antisepsis field. *Journal of food science, 83*(6), 1476-1483.

Hua, H., Xing, F., Selvaraj, J. N., Wang, Y., Zhao, Y., Zhou, L., . . . Liu, Y. (2014). Inhibitory effect of essential oils on Aspergillus ochraceus growth and ochratoxin A production. *PloS one, 9*(9).

IARC. (2002). *IARC Working Group on the Evaluation of Carcinogenic Risk to Humans. Some Traditional Herbal Medicines, Some Mycotoxins, Naphthalene and Styrene*. (Vol. 100). Lyon, France: International Agency for Research on Cancer.

IARC. (2012). *Chemical agents and related occupations / IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (2009: Lyon, France)* (2012/12/01 ed. Vol. 100). Lyon, France.

Imolehin, E. (1983). Rice seedborne fungi and their effect on seed germination. *Plant disease, 67*(12), 1334-1336.

Inouye, S., Nishiyama, Y., Uchida, K., Hasumi, Y., Yamaguchi, H., & Abe, S. (2006). The vapor activity of oregano, perilla, tea tree, lavender, clove, and geranium oils against a Trichophyton mentagrophytes in a closed box. *J Infect Chemother, 12*(6), 349-354. doi:10.1007/s10156-006-0474-7

Irshad, M., Subhani, M. A., Ali, S., & Hussain, A. (2019). Biological Importance of Essential Oils. In *Essential Oils-Oils of Nature*: IntechOpen.

Ismaiel, A. A., & Papenbrock, J. (2015). Mycotoxins: producing fungi and mechanisms of phytotoxicity. *Agriculture, 5*(3), 492-537.

Isman, M. B. (2000). Plant essential oils for pest and disease management. *Crop protection, 19*(8-10), 603-608.

Issa, D., Najjar, A., Greige-Gerges, H., & Nehme, H. (2019). Screening of Some Essential Oil Constituents as Potential Inhibitors of the ATP Synthase of Escherichia coli. *Journal of food science, 84*(1), 138-146.

Jamdagni, P., Khatri, P., & Rana, J. (2018). *Biogenic synthesis of silver nanoparticles from leaf extract of Elettaria cardamomum and their antifungal activity against phytopathogens.* Paper presented at the Adv. Mater. Proc.

Jantan, I., Ahmad, A. S., Ahmad, A. R., Ali, N. A. M., & Ayop, N. J. J. o. E. O. R. (1996). Chemical composition of some Citrus oils from Malaysia. *8*(6), 627-632.

JECFA. (2016). *Joint FAO/WHO Expert Committee on Food Additives* (JECFA/83/SC). Retrieved from Rome: http://www.fao.org/3/a-bq821e.pdf

Ji, H., Kim, H., Beuchat, L. R., & Ryu, J.-H. (2019). Synergistic antimicrobial activities of essential oil vapours against Penicillium corylophilum on a laboratory medium and beef jerky. *International Journal of Food Microbiology, 291*, 104-110. doi:10.1016/j.ijfoodmicro.2018.11.023

Jittanit, W., Srzednicki, G., & Driscoll, R. (2010). Corn, rice, and wheat seed drying by two-stage concept. *Drying Technology*, *28*(6), 807-815.

Jiumpanyarach, W. (2018). The impact of social trends: teenagers' attitudes for organic food market in Thailand. *International Journal of Social Economics, 45*(4), 682-699. doi:10.1108/ijse-01-2017-0004

Johnson, E. M., Szekely, A., & Warnock, D. W. (1998). In-vitro activity of voriconazole, itraconazole and amphotericin B against filamentous fungi. *Journal of Antimicrobial Chemotherapy, 42*(6), 741-745. doi:10.1093/jac/42.6.741

Ju, J., Xu, X., Xie, Y., Guo, Y., Cheng, Y., Qian, H., & Yao, W. (2018). Inhibitory effects of cinnamon and clove essential oils on mold growth on baked foods. *Food Chem, 240*, 850-855. doi:10.1016/j.foodchem.2017.07.120

Juniatik, M., Hida, K., Wulandari, F. P., Pangestuti, N., Munawaroh, N. m., Martien, R., & Pratiwi, S. U. T. (2017). FORMULATION OF NANOEMULSION MOUTHWASH COMBINATION OF LEMONGRASS OIL (Cymbopogon citratus) AND KAFFIR LIME OIL (Citrus hystrix) FOR ANTICANDIDIASIS AGAINST Candida albicans ATCC 10231. *Majalah Obat Tradisional, 22*(1), 7-15.

Kamle, M., Mahato, D. K., Devi, S., Lee, K. E., Kang, S. G., & Kumar, P. (2019). Fumonisins: impact on agriculture, food, and human health and their management strategies. *Toxins, 11*(6), 328.

Kanjanamaneesathian, M., & Nimanong, W. (2019). based inoculum of Bacillus megaterium and its practical application for simple culture preparation. *New Zealand Plant Protection, 72*, 195-204.

Kapadiya, S. M., Parikh, J., & Desai, M. A. (2018). A greener approach towards isolating clove oil from buds of Syzygium aromaticum using microwave radiation. *Industrial crops and products, 112*, 626-632. doi:10.1016/j.indcrop.2017.12.060

Karaca, G., Bilginturan, M., & Olgunsoy, P. (2017). Effects of some plant essential oils against fungi on wheat seeds. *Indian Journal of Pharmaceutical Education and Research, 3*, S385-S388.

Kerdchoechuen, O., Laohakunjit, N., Singkornard, S., & Matta, F. B. (2010). Essential oils from six herbal plants for biocontrol of the maize weevil. *HortScience, 45*(4), 592-598.

Kim, S. W., Lee, H. R., Jang, M. J., Jung, C. S., & Park, I. K. (2016). Fumigant Toxicity of Lamiaceae Plant Essential Oils and Blends of Their Constituents against

Adult Rice Weevil Sitophilus oryzae. *Molecules, 21*(3), 361. doi:10.3390/molecules21030361

Kishore, G. K., Pande, S., & Harish, S. (2007). Evaluation of essential oils and their components for broad-spectrum antifungal activity and control of late leaf spot and crown rot diseases in peanut. *Plant disease, 91*(4), 375-379.

Klich, M., & Pitt, J. (1988). Differentiation of Aspergillus flavus from A. parasiticus and other closely related species. *Transactions of the British Mycological Society, 91*(1), 99-108.

Kohiyama, C. Y., Yamamoto Ribeiro, M. M., Mossini, S. A. G., Bando, E., Bomfim, N. d. S., Nerilo, S. B., . . . Machinski, M. (2015). Antifungal properties and inhibitory effects upon aflatoxin production of Thymus vulgaris L. by Aspergillus flavus Link. *Food Chemistry, 173*, 1006-1010. doi:10.1016/j.foodchem.2014.10.135

Kongsom, C., & Panyakul, V. (2016). Production and Market of Certified Organic Products in Thailand. *International Journal of Social, Behavioural, Educational, Economic, Business and Industrial Engineering, 10*(8), 2671-2675.

Koutsaviti, A., Antonopoulou, V., Vlassi, A., Antonatos, S., Michaelakis, A., Papachristos, D. P., & Tzakou, O. (2018). Chemical composition and fumigant activity of essential oils from six plant families against Sitophilus oryzae (Col: Curculionidae). *Journal of pest science, 91*(2), 873-886.

Krishnamachari, K., Bhat, R., Nagarajan, V., & Tilak, T. (1975). Investigations into an outbreak of hepatitis in parts of western India. *The Indian journal of medical research, 63*(7), 1036.

Kumar, D., & Kalita, P. (2017). Reducing Postharvest Losses during Storage of Grain Crops to Strengthen Food Security in Developing Countries. *Foods, 6*(1), 8. doi:10.3390/foods6010008

Laird, K., & Phillips, C. (2012). Vapour phase: a potential future use for essential oils as antimicrobials? *Letters in Applied Microbiology, 54*(3), 169-174.

Liu, J., Wang, Q.-C., Han, J., Xiong, B., & Sun, S.-C. (2015). Aflatoxin B1 is toxic to porcine oocyte maturation. *Mutagenesis, 30*(4), 527-535.

López, P., Sánchez, C., Batlle, R., & Nerín, C. (2005). Solid- and Vapor-Phase Antimicrobial Activities of Six Essential Oils: Susceptibility of Selected Foodborne Bacterial and Fungal Strains. *Journal of Agricultural and Food Chemistry, 53*(17), 6939-6946. doi:10.1021/jf050709v

Ma, Y.-N., Xu, F.-R., Chen, C.-J., Li, Q.-Q., Wang, M.-Z., Cheng, Y.-X., & Dong, X. (2019). The beneficial use of essential oils from buds and fruit of Syzygium aromaticum to combat pathogenic fungi of Panax notoginseng. *Industrial crops and products, 133*, 185-192.

Mahmoud, S. S., & Croteau, R. B. (2003). Menthofuran regulates essential oil biosynthesis in peppermint by controlling a downstream monoterpene reductase. *Proceedings of the National Academy of Sciences, 100*(24), 14481-14486. doi:10.1073/pnas.2436325100

Manandhar, A., Milindi, P., & Shah, A. (2018). An Overview of the Post-Harvest Grain Storage Practices of Smallholder Farmers in Developing Countries. *Agriculture, 8*(4). doi:10.3390/agriculture8040057

Manssouri, M., Znini, M., El Harrak, A., & Majidi, L. (2016). Antifungal activity of essential oil from the fruits of Ammodaucus leucotrichus Coss. & Dur., in liquid

and vapour phase against postharvest phytopathogenic fungi in apples. *Journal of Applied Pharmaceutical Science, 6*(5), 131-136.

Mary, V. S., Arias, S. L., Otaiza, S. N., Velez, P. A., Rubinstein, H. R., & Theumer, M. G. (2017). The aflatoxin B1-fumonisin B1 toxicity in BRL-3A hepatocytes is associated to induction of cytochrome P450 activity and arachidonic acid metabolism. *Environmental toxicology, 32*(6), 1711-1724.

Massoud, M., Saad, A., Soliman, E., & El-Moghazy, A. (2012). Antifungal activity of some essential oils applied as fumigants against two stored grains fungi. *J. Adv. Agric. Res., 17*(2), 296-306.

Mateo, E. M., Gómez, J. V., Gimeno-Adelantado, J. V., Romera, D., Mateo-Castro, R., & Jiménez, M. (2017). Assessment of azole fungicides as a tool to control growth of Aspergillus flavus and aflatoxin B1 and B2 production in maize. *Food Additives & Contaminants: Part A, 34*(6), 1039-1051.

McLean, M. (1995). The phytotoxicity of selected mycotoxins on mature, germinatingZea mays embryos. *Mycopathologia, 132*(3), 173-183.

McLean, M., Berjak, P., Watt, M., & Dutton, M. (1992). The effects of aflatoxin B 1 on immature germinating maize (Zea mays) embryos. *Mycopathologia, 119*(3), 181-190.

Memarzadeh, S. M., Pirbalouti, A. G., & AdibNejad, M. (2015). Chemical composition and yield of essential oils from Bakhtiari savory (Satureja bachtiarica Bunge.) under different extraction methods. *Industrial crops and products, 76*, 809-816.

Mendoza, J., Sabillón, L., Martinez, W., Campabadal, C., Hallen-Adams, H., & Bianchini, A. (2017). Traditional maize post-harvest management practices amongst smallholder farmers in Guatemala. *Stored Products Research, 71*, 14-21. doi:10.1016/j.jspr.2016.12.007

Nakahara, K., Alzoreky, N. S., Yoshihashi, T., Nguyen, H. T., & Trakoontivakorn, G. (2013). Chemical composition and antifungal activity of essential oil from Cymbopogon nardus (citronella grass). *Japan Agricultural Research Quarterly: JARQ, 37*(4), 249-252.

Nazzaro, F., Fratianni, F., Coppola, R., & Feo, V. D. (2017). Essential oils and antifungal activity. *Pharmaceuticals, 10*(4), 86.

Nazzaro, F., Fratianni, F., De Martino, L., Coppola, R., & De Feo, V. (2013). Effect of essential oils on pathogenic bacteria. *Pharmaceuticals, 6*(12), 1451-1474.

Nekoei, M., & Mohammadhosseini, M. (2016). Chemical compositions of the essential oils from the aerial parts of Achillea wilhelmsii using traditional Hydrodistillation, microwave assisted hydro-distillation and solvent-free microwave extraction methods: comparison with the volatile compounds obtained by headspace solid-phase microextraction. *Journal of Essential Oil Bearing Plants, 19*(1), 59-75.

Ngindu, A., Kenya, P., Ocheng, D., Omondi, T., Ngare, W., Gatei, D., . . . Jansen, A. (1982). Outbreak of acute hepatitis caused by aflatoxin poisoning in Kenya. *The Lancet, 319*(8285), 1346-1348.

Nguefack, J., Leth, V., Lekagne Dongmo, J., Torp, J., Amvam Zollo, P., & Nyasse, S. (2008). Use of three essential oils as seed treatments against seed-borne fungi of rice (Oryza sativa L.). *American-Eurasian. Journal of Agricultural and Environmental Science, New work, 4*(5), 554-560.

Nikkhah, M., Hashemi, M., Habibi Najafi, M. B., & Farhoosh, R. (2017). Synergistic

effects of some essential oils against fungal spoilage on pear fruit. *International Journal of Food Microbiology, 257*, 285-294. doi:10.1016/j.ijfoodmicro.2017.06.021

Nor, O. M. (1999). Volatile aroma compounds in Citrus hystrix oil. *J. Trop. Agric. Food Sci, 27*, 225-229.

Norlia, M., Jinap, S., Nor-Khaizura, M. A. R., Radu, S., Chin, C. K., Samsudin, N. I. P., & Farawahida, A. H. (2019). Molecular characterisation of aflatoxigenic and non-aflatoxigenic strains of Aspergillus section Flavi isolated from imported peanuts along the supply chain in Malaysia. *Toxins, 11*(9), 501.

Nyongesa, B. W., Okoth, S., & Ayugi, V. (2015). Identification key for Aspergillus species isolated from maize and soil of Nandi County, Kenya. *Advances in Microbiology, 5*(04), 205-229. doi:10.4236/aim.2015.54020

O'Bryan, C. A., Pendleton, S. J., Crandall, P. G., & Ricke, S. C. (2015). Potential of plant essential oils and their components in animal agriculture–in vitro studies on antibacterial mode of action. *Frontiers in veterinary science, 2*, 35.

OECD/FAO. (2019). *OECD-FAO Agricultural Outlook 2019-2028* (ISBN 978-92-64-31245-6). Retrieved from Rome: https://doi.org/10.1787/agr_outlook-2019-en

Okonkwo, E., & Okoye, W. (1996). The efficacy of four seed powders and the essential oils as protectants of cowpea and maize grains against infestation by Callosobruchus maculatus (Fabricus)(Coleoptera: Bruchidae) and Sitophilus zeamais (Motschulsky)(Coleoptera: Curculionidae) in Nigeria. *International Journal of Pest Management, 42*(3), 143-146.

Okoye, J., Gugnani, H., & Okeke, C. (1989). Pulmonary Infections Due to Aspergillus flavus in Turkey Poults and Goslings: Lungeninfektionen durch Aspergillus flavus bei Puten-und Gänsenküken. *Mycoses, 32*(7), 336-339.

Oliveira, M. S. d., Costa, W. A. d., Bezerra, P. N., Filho, A. P. d. S. S., & Junior, R. N. d. C. (2018). Potentially Phytotoxic of Chemical Compounds Present in Essential Oil for Invasive Plants Control: A Mini-Review. In *Biological Approaches for Controlling Weeds* (10.5772/intechopen.74346).

Oluwafemi, F., Odebiyi, T., & Kolapo, A. (2012). Occupational aflatoxin exposure among feed mill workers in Nigeria. *World Mycotoxin Journal, 5*(4), 385-389. doi:10.3920/wmj2012.1399

Otsuki, T., Wilson, J. S., & Sewadeh, M. (2001). Saving two in a billion:: quantifying the trade effect of European food safety standards on African exports. *Food Policy, 26*(5), 495-514. doi:https://doi.org/10.1016/S0306-9192(01)00018-5

OuYang, Q., Okwong, R. O., Chen, Y., & Tao, N. (2020). Synergistic activity of cinnamaldehyde and citronellal against green mold in citrus fruit. *Postharvest Biology and Technology, 162*, 111095.

Panuwet, P., Prapamontol, T., Chantara, S., Thavornyuthikarn, P., Montesano, M. A., Whitehead Jr, R. D., & Barr, D. B. (2008). Concentrations of urinary pesticide metabolites in small-scale farmers in Chiang Mai Province, Thailand. *Science of the total environment, 407*(1), 655-668.

Panuwet, P., Siriwong, W., Prapamontol, T., Ryan, P. B., Fiedler, N., Robson, M. G., & Barr, D. B. (2012). Agricultural pesticide management in Thailand: status and population health risk. *Environmental science & policy, 17*, 72-81.

Park, M., Gwak, K., Yang, I., Kim, K., Jeung, E., Chang, J., & Choi, I. (2009). Effect of citral, eugenol, nerolidol and α-terpineol on the ultrastructural changes of

Trichophyton mentagrophytes. *Fitoterapia*, *80*(5), 290-296.

Paster, N., Menasherov, M., Ravid, U., & Juven, B. (1995). Antifungal Activity of Oregano and Thyme Essential Oils Applied as Fumigants Against Fungi Attacking Stored Grain. *J Food Prot*, *58*(1), 81-85. doi:10.4315/0362-028x-58.1.81

Paster, N., Menasherov, M., Ravid, U., & Juven, B. (1995). Antifungal activity of oregano and thyme essential oils applied as fumigants against fungi attacking stored grain. *Journal of food protection*, *58*(1), 81-85.

Patel, R. M., & Jasrai, Y. T. (2013). Evaluation of fungitoxic potency of Piper betle L.(Mysore variety) leaf extracts against eleven phyto-pathogenic fungal strains. *CIBTech Journal of Bio-Protocols*, *2*(2), 21-28.

Peers, F., Bosch, X., Kaldor, J., Linsell, A., & Pluijmen, M. (1987). Aflatoxin exposure, hepatitis B virus infection and liver cancer in Swaziland. *international Journal of Cancer*, *39*(5), 545-553.

Perrone, G., Susca, A., Cozzi, G., Ehrlich, K., Varga, J., Frisvad, J. C., . . . Samson, R. A. (2007). Biodiversity of Aspergillus species in some important agricultural products. *Studies in mycology*, *59*, 53-66.

Pingali, P. (2015). Agricultural policy and nutrition outcomes – getting beyond the preoccupation with staple grains. *Food Security*, *7*(3), 583-591. doi:10.1007/s12571-015-0461-x

Pingret, D., Fabiano-Tixier, A.-S., & Chemat, F. (2014). An improved ultrasound Clevenger for extraction of essential oils. *Food analytical methods*, *7*(1), 9-12.

Pitt, J. I. (2000). Toxigenic fungi and mycotoxins. *British medical bulletin*, *56*(1), 184-192.

Pitt, J. I., & Hocking, A. D. (1997). Methods for isolation, enumeration and identification. In *Fungi and food spoilage* (pp. 21-57): Springer.

Pitt, J. I., Hocking, A. D., Bhudhasamai, K., Miscamble, B. F., Wheeler, K. A., & Tanboon-Ek, P. (1994). The normal mycoflora of commodities from Thailand. 2. Beans, rice, small grains and other commodities. *International Journal of Food Microbiology*, *23*(1), 35-53.

Powers, C. N., Osier, J. L., McFeeters, R. L., Brazell, C. B., Olsen, E. L., Moriarity, D. M., . . . Setzer, W. N. (2018). Antifungal and Cytotoxic Activities of Sixty Commercially-Available Essential Oils. *Molecules*, *23*(7). doi:10.3390/molecules23071549

Prasertsri, P. (2019). Thailand Grain and Feed Annual. In (Retrieved from https://apps.fas.usda.gov/newgainapi/api/report/downloadreportbyfilename?filename=Grain%20and%20Feed%20Annual_Bangkok_Thailand_3-14-2019.pdf): GAIN Report TH9031, US Department of Agriculture, Foreign Agricultural Service.

Prates, L. H. F., Faroni, L. R. D. A., Heleno, F. F., de Queiroz, M. E. L. R., de Sousa, A. H., & de Assis Silva, M. V. (2019). Eugenol diffusion coefficient and its potential to control Sitophilus zeamais in rice. *Scientific reports*, *9*(1), 1-11.

Raksakantong, P., Siriamornpun, S., & Meeso, N. (2012). Effect of drying methods on volatile compounds, fatty acids and antioxidant property of Thai kaffir lime (Citrus hystrix DC). *International journal of food science & technology*, *47*(3), 603-612.

Rasooli, I., Rezaei, M. B., & Allameh, A. (2006). Growth inhibition and morphological

alterations of Aspergillus niger by essential oils from Thymus eriocalyx and Thymus x-porlock. *Food Control, 17*(5), 359-364.

Restuccia, C., Conti, G. O., Zuccarello, P., Parafati, L., Cristaldi, A., & Ferrante, M. (2019). Efficacy of different citrus essential oils to inhibit the growth and B1 aflatoxin biosynthesis of Aspergillus flavus. *Environmental Science and Pollution Research, 26*(30), 31263-31272.

Riccioni, L., Orzali, L., Romani, M., Annicchiarico, P., & Pecetti, L. (2019). Organic seed treatments with essential oils to control ascochyta blight in pea. *European Journal of Plant Pathology, 155*(3), 831-840.

Richard, J. L. (2008). Discovery of aflatoxins and significant historical features. *Toxin Reviews, 27*(3-4), 171-201.

Richter, J., & Schellenberg, I. (2007). Comparison of different extraction methods for the determination of essential oils and related compounds from aromatic plants and optimization of solid-phase microextraction/gas chromatography. *Analytical and bioanalytical chemistry, 387*(6), 2207-2217.

Rodrigues, P., Venâncio, A., Kozakiewicz, Z., & Lima, N. (2009). A polyphasic approach to the identification of aflatoxigenic and non-aflatoxigenic strains of Aspergillus section Flavi isolated from Portuguese almonds. *International Journal of Food Microbiology, 129*(2), 187-193.

Rodríguez, J. D. W., Peyron, S., Rigou, P., & Chalier, P. (2018). Rapid quantification of clove (Syzygium aromaticum) and spearmint (Mentha spicata) essential oils encapsulated in a complex organic matrix using an ATR-FTIR spectroscopic method. *PloS one, 13*(11), e0207401. doi:10.1371/journal.pone.0207401

Rodriquez-Tudela, J., Donnelly, J., & Arendrup, M. (2008). EUCAST definitive document EDef 7.1: method for the determination of broth dilution MICs of antifungal agents for fermentative yeasts. *Clinical Microbiology and Infection, 14*(4), 398-405.

Rozwalka, L. C., Alves, E., & do Amaral, D. C. (2010). Ultrastructural study of conidia of Colletotrichum gloeosporioides and Colletotrichum musae treated with essential oils. *Interciencia, 35*(12), 912-915.

Safaralie, A., Fatemi, S., & Salimi, A. (2010). Experimental design on supercritical extraction of essential oil from valerian roots and study of optimal conditions. *Food and Bioproducts Processing, 88*(2-3), 312-318.

Samson, R. A., Visagie, C. M., Houbraken, J., Hong, S.-B., Hubka, V., Klaassen, C. H., . . . Tanney, J. B. (2014). Phylogeny, identification and nomenclature of the genus Aspergillus. *Studies in mycology, 78*, 141-173.

Seephueak, P., Preecha, C., & Seephueak, W. (2019). The diversity of fungi associated with rice (Oryza sativa L.) from Nakhon Si Thammarat, Thailand. *International Journal of Agricultural Technology, 15*(3), 485-500.

Serra, B., J., Ventura, C., F., & Ruiz Martínez, J. A. (2016). Residues of essential oils in honey after treatments to control Varroa destructor. *Journal of essential oil research, 28*(1), 22-28.

Setiyoningrum, F., Lioe, H., Apriyantono, A., & Abbas, A. (2018). Drying and pulverization processes affect the physico-chemical properties of kaffir lime leaves (Citrus hystrix DC). *International Food Research Journal, 25*(6).

Sharma, A., Rajendran, S., Srivastava, A., Sharma, S., & Kundu, B. (2017). Antifungal activities of selected essential oils against Fusarium oxysporum f. sp. lycopersici

1322, with emphasis on Syzygium aromaticum essential oil. *J Biosci Bioeng, 123*(3), 308-313. doi:10.1016/j.jbiosc.2016.09.011

Shiferaw, B., Smale, M., Braun, H.-J., Duveiller, E., Reynolds, M., & Muricho, G. (2013). Crops that feed the world 10. Past successes and future challenges to the role played by wheat in global food security. *Food Security, 5*(3), 291-317. doi:10.1007/s12571-013-0263-y

Shu, X., Livingston III, D. P., Franks, R. G., Boston, R. S., Woloshuk, C. P., & Payne, G. A. (2015). Tissue-specific gene expression in maize seeds during colonization by A spergillus flavus and F usarium verticillioides. *Molecular plant pathology, 16*(7), 662-674.

Silva, C. d. B. d., Guterres, S. S., Weisheimer, V., & Schapoval, E. E. (2008). Antifungal activity of the lemongrass oil and citral against Candida spp. *Brazilian Journal of Infectious Diseases, 12*(1), 63-66.

Sinha, K., & Prasad, G. (1996). Effects of citrinin on pigment, protein and nucleic acid contents in maize seeds. *Biologia plantarum, 38*(2), 317.

Sirma, A. J., Makita, K., Grace Randolph, D., Senerwa, D., & Lindahl, J. F. (2019). Aflatoxin exposure from milk in rural Kenya and the contribution to the risk of liver cancer. *Toxins, 11*(8), 469.

Soares, C., Morales, H., Faria, J., Figueiredo, A. C., Pedro, L. G., & Venâncio, A. (2016). Inhibitory effect of essential oils on growth and on aflatoxins production by Aspergillus parasiticus. *World Mycotoxin Journal, 9*(4), 525-534. doi:10.3920/wmj2015.1987

Somda, I., Leth, V., & Sereme, P. (2007). Antifungal effect of Cymbopogon citratus, Eucalyptus camaldulensis and Azadirachta indica oil extracts on sorghum seed-borne fungi. *Asian Journal of Plant Sciences, 6*(8), 1182-1189.

Songsamoe, S., Matan, N., & Matan, N. (2017). Antifungal activity of Michelia alba oil in the vapor phase and the synergistic effect of major essential oil components against Aspergillus flavus on brown rice. *Food Control, 77*, 150-157. doi:10.1016/j.foodcont.2017.02.010

Soylu, E. M., Kurt, Ş., & Soylu, S. (2010). In vitro and in vivo antifungal activities of the essential oils of various plants against tomato grey mould disease agent Botrytis cinerea. *International Journal of Food Microbiology, 143*(3), 183-189.

Srisukh, V., Tribuddharat, C., Nukoolkarn, V., Bunyapraphatsara, N., Chokephaibulkit, K., Phoomniyom, S., . . . Srifuengfung, S. (2012). Antibacterial activity of essential oils from Citrus hystrix (makrut lime) against respiratory tract pathogens. *ScienceAsia, 38*(2), 212. doi:10.2306/scienceasia1513-1874.2012.38.212

Stefanello, M. É. A., Pascoal, A. C., & Salvador, M. J. (2011). Essential oils from neotropical Myrtaceae: chemical diversity and biological properties. *Chemistry & biodiversity, 8*(1), 73-94.

Stević, T., Berić, T., Šavikin, K., Soković, M., Gođevac, D., Dimkić, I., & Stanković, S. (2014). Antifungal activity of selected essential oils against fungi isolated from medicinal plant. *Industrial crops and products, 55*, 116-122. doi:10.1016/j.indcrop.2014.02.011

Suhr, K. I., & Nielsen, P. V. (2003). Antifungal activity of essential oils evaluated by two different application techniques against rye bread spoilage fungi. *Journal of Applied Microbiology, 94*(4), 665-674.

Sukatta, U., Haruthaithanasan, V., Chantarapanont, W., Dilokkunanant, U., & Suppakul, P. J. K. J. N. S. (2008). Antifungal activity of clove and cinnamon oil and their synergistic against postharvest decay fungi of grape in vitro. *42*, 169-174.

Suleiman, R., Rosentrater, K., & Bern, C. (2013). Effects of deterioration parameters on storage of maize: A review. *Journal of Natural Sciences Research, 3*(9), 147-165.

Sun, L.-H., Lei, M.-y., Zhang, N.-Y., Gao, X., Li, C., Krumm, C. S., & Qi, D.-S. (2015). Individual and combined cytotoxic effects of aflatoxin B1, zearalenone, deoxynivalenol and fumonisin B1 on BRL 3A rat liver cells. *Toxicon, 95*, 6-12.

Suttajit, M. (2007). A perspective review of aflatoxin research in Thailand: prevention and control. *JSM Mycotoxins, 2006*(Suppl4), 86-96.

Tandon, S. (2008). 7 Distillation Technology for Essential Oils. *Extraction technologies for medicinal and aromatic plants*. 115.

Tang, X., Shao, Y.-L., Tang, Y.-J., & Zhou, W.-W. (2018). Antifungal activity of essential oil compounds (geraniol and citral) and inhibitory mechanisms on grain pathogens (Aspergillus flavus and Aspergillus ochraceus). *Molecules, 23*(9), 2108.

Tao, C., Wei, Q., Yin, Z.-Q., Zhou, L.-J., Jia, R.-Y., Xu, J., . . . Deng, Y.-X. (2013). Antifungal activity of essential oil from Cinnamomum longepaniculatum leaves against three dermatophytes in vitro. *Afr. J. Pharm. Pharmcol, 7*, 1148-1152.

Tawatsin, A. (2015). Pesticides used in Thailand and toxic effects to human health. *Medical Research Archives*. (3).

Thiex, N. (2009). Evaluation of analytical methods for the determination of moisture, crude protein, crude fat, and crude fiber in distillers dried grains with solubles. *Journal of AOAC International, 92*(1), 61-73.

Thobunluepop, P., Pawelzik, E., & Vearasilp, S. (2008). Possibility of biological seed coating application on direct-seed rice production: Emphasis on plant productivity and environment awareness. *Agric. Sci. J, 39*(3 Suppl), 449-452.

Thundiyil, J. G., Stober, J., Besbelli, N., & Pronczuk, J. (2008). Acute pesticide poisoning: a proposed classification tool. *Bulletin of the World Health Organization, 86*, 205-209.

Tola, M., & Kebede, B. (2016). Occurrence, importance and control of mycotoxins: A review. *Cogent Food & Agriculture, 2*(1), 1191103. doi:10.1080/23311932.2016.1191103

Tongnuanchan, P., & Benjakul, S. (2014). Essential Oils: Extraction, Bioactivities, and Their Uses for Food Preservation. *Journal of food science, 79*(7), R1231-R1249. doi:10.1111/1750-3841.12492

Tsedaley, B., & Adugna, G. (2016). Detection of Fungi Infecting Maize (Zea mays L.) Seeds in Different Storages Around Jimma, Southwestern Ethiopia. *Journal of Plant Pathology & Microbiology, 07*(03), 1-6. doi:10.4172/2157-7471.1000338

Tullio, V., Nostro, A., Mandras, N., Dugo, P., Banche, G., Cannatelli, M. A., . . . Carlone, N. A. (2007). Antifungal activity of essential oils against filamentous fungi determined by broth microdilution and vapour contact methods. *J Appl Microbiol, 102*(6), 1544-1550. doi:10.1111/j.1365-2672.2006.03191.x

Tyagi, A. K., & Malik, A. (2010). Liquid and vapour-phase antifungal activities of selected essential oils against Candida albicans: microscopic observations and chemical characterization of Cymbopogon citratus. *BMC Complement Altern*

Med, 10, 65. doi:10.1186/1472-6882-10-65

Tyagi, A. K., & Malik, A. (2011). Antimicrobial potential and chemical composition of Eucalyptus globulus oil in liquid and vapour phase against food spoilage microorganisms. *Food Chem, 126*(1), 228-235. doi:10.1016/j.foodchem.2010.11.002

Valentine, H. (2016). The role of peanuts in global food security. In *Peanuts* (pp. 447-461): Elsevier.

Velázquez-Nuñez, M. J., Avila-Sosa, R., Palou, E., & López-Malo, A. (2013). Antifungal activity of orange (Citrus sinensis var. Valencia) peel essential oil applied by direct addition or vapor contact. *Food Control 31*(1), 1-4. doi:10.1016/j.foodcont.2012.09.029

Venables, W. N., Smith, D. M., & Team, R. D. C. (2009). An introduction to R. In: Citeseer.

Waikedre, J., Dugay, A., Barrachina, I., Herrenknecht, C., Cabalion, P., & Fournet, A. (2010). Chemical composition and antimicrobial activity of the essential oils from New Caledonian Citrus macroptera and Citrus hystrix. *Chemistry & biodiversity, 7*(4), 871-877.

Wang, L., Jiang, N., Wang, D., & Wang, M. (2019). Effects of Essential Oil Citral on the Growth, Mycotoxin Biosynthesis and Transcriptomic Profile of Alternaria alternata. *Toxins, 11*(10), 553.

Warsito, W., Palungan, M. H., & Utomo, E. P. (2017). Profiling study of the major and minor components of kaffir lime oil (Citrus hystrix DC.) in the fractional distillation process. *The Pan African medical journal, 27*.

Wesołowska, A., Grzeszczuk, M., & Jadczak, D. (2014). Comparison of chemical compositions of essential oils isolated by hydrodistillation from wild thyme (Thymus serpyllum L.) with use of Deryng and Clevenger apparatus. *Herba Polonica, 60*(2), 7-17.

Williams, R. J., & McDonald, D. (1983). Grain molds in the tropics: problems and importance. *Annual Review of Phytopathology, 21*(1), 153-178. doi:10.1146/annurev.py.21.090183.001101

Wogan, G. N. (1966). Chemical nature and biological effects of the aflatoxins. *Bacteriological reviews, 30*(2), 460.

Wu, Y., OuYang, Q., & Tao, N. (2016). Plasma membrane damage contributes to antifungal activity of citronellal against Penicillium digitatum. *Journal of food science and technology, 53*(10), 3853-3858.

Yazdani, D., Zainal, A. M., Tan, Y., & Kamaruzaman, S. (2010). Evaluation of the detection techniques of toxigenic Aspergillus isolates. *African Journal of Biotechnology, 9*(45), 7654-7659. Retrieved from https://www.ajol.info/index.php/ajb/article/view/130415

Zorya, S., Morgan, N., Diaz Rios, L., Hodges, R., Bennett, B., Stathers, T., . . . Lamb, J. (2011). Missing food: the case of postharvest grain losses in sub-Saharan Africa.

Zulkifli, N. A., & Zakaria, L. (2017). Morphological and molecular diversity of Aspergillus from corn grain used as livestock feed. *HAYATI Journal of Biosciences, 24*(1), 26-34.

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