ANTAGONISTIC EFFECTS OF ESSENTIAL OILS FROM SELECTED MALAYSIAN HERBS AND SPICES AGAINST SPOILAGE AND MYCOTOXIGENIC FUNGI ON FOOD PACKAGING APPLICATION

By

MAZLIZA BINTI RAMLI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Chair : Nik Iskandar Putra Samsudin, PhD
Faculty : Food Science and Technology

Essential oils (EO) have a long history and wide application. Although EO of various herbs and spices from other parts of the world have shown antifungal effects, those from Malaysian herbs and spices remain underreported; thus, they can be further utilised in the search for novel bioactive compounds as natural antifungals to fulfil the consumers' demand for greener, safer, healthier, and higher-quality foods with longer shelf life. Therefore, the objectives of the present work were (i) to investigate the fungicidal and fungistatic potentials of commercial EO from selected Malaysian herbs and spices against common spoilage and mycotoxigenic fungi; and (ii) to examine mycotoxin prevention and reduction in peanut through the incorporation of the most potent EO in food packaging system. Essential oils from ten herbs and spices (betel, cinnamon, clove, coriander, galangal, ginger, lemongrass, lime, nutmeg, turmeric) were analysed against five common foodborne spoilage and mycotoxigenic fungi; which were *Aspergillus flavus, A. parasiticus, A. niger, Fusarium verticillioides*, and *F. incarnatum*. Fungal isolates were obtained from common household food products (maize and peanuts), and isolated by the culture method. Molecular identification of the fungal isolates was performed using primer pair of the Internal Transcribed Spacer (ITS) region. The mycotoxigenic potential of fungal isolates was determined by high performance liquid chromatography with a fluorescence detector (HPLC-FLD). The EO bioactive compounds were identified by gas chromatography and mass spectrometry (GC-MS). Preliminary *in vitro* screening was conducted with the agar disk diffusion and agar well diffusion methods on Potato Dextrose Agar (PDA), and microwell assay method on Potato Dextrose Broth (PDB). Following this, three most potent EO were selected for their Minimal Inhibitory Concentration (MIC), Minimal Fungicidal Concentration (MFC), and fungal spore germination test. Molecular identification confirmed the fungal isolates' identity with existing species in the GenBank. Based on HPLC-FLD results, all the fungal isolates were confirmed to be

mycotoxigenic based on their ability to produce mycotoxins. Based on GC-MS results, a total of 120 bioactive compounds were detected from the EO; which were 26.25% anethole (betel), 63.39% cinnamaldehyde (cinnamon), 87.16% eugenol (clove), 54.79% linalool (coriander), 29.56% propenoic acid (galangal), 26.32% α-zingiberene (ginger), 42.61% geranial (lemongrass), 39.84% limonene (lime), 27.80% β-phellandrene (nutmeg), and 41.81% ar-turmerone (turmeric). Based on preliminary *in vitro* screening, cinnamon, clove, and lemongrass EO yielded the highest fungal growth inhibition. Further, cinnamon EO yielded the lowest MIC, MFC, and spore germination, hence were selected to be applied in food packaging system. The inhibitory effects of cinnamon EO were found comparable to the commercial antifungal, cycloheximide. Sachet including inclusion complexes (ICs; a combination of cinnamon EO and β-cyclodextrin) by kneading method was prepared, and the fungal growth inhibition *in vitro* was determined under different temperature (25°C, 50°C, and 75°C) and pH values (5.5, 7.4, and 13.0) to see its stability in peanut. The ICs' antifungal activity in peanuts was also determined in terms of aflatoxin reduction (peanut was selected since it is the most commonly colonised food crop commodity by *Aspergillus* section *Flavi*). The prepared ICs showed excellent fungal growth inhibition *in vitro* and aflatoxin reduction in peanuts as compared to control sample. Various temperature levels and pH values did not affect the ICs' activity.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KESAN ANTAGONISTIK MINYAK PATI DARI HERBA DAN REMPAH TERPILIH DI MALAYSIA TERHADAP KULAT PEROSAK DAN MIKOTOKSIGENIK DALAM APLIKASI PEMBUNGKUSAN MAKANAN

Oleh

MAZLIZA BINTI RAMLI

April 2022

Pengerusi : Nik Iskandar Putra Samsudin, PhD
Fakulti : Sains dan Teknologi Makanan

Minyak pati mempunyai sejarah yang panjang dan penggunaan yang meluas. Walaupun minyak pati pelbagai herba dan rempah dari tempat lain di dunia telah menunjukkan kesan antikulat, minyak pati herba dan rempah dari Malaysia masih kurang dilaporkan; justeru, boleh digunakan dalam pencarian sebatian bioaktif baru antikulat semulajadi untuk memenuhi permintaan pengguna dalam penghasilan makanan yang lebih selamat, sihat, dan berkualiti tinggi dengan jangka hayat yang lebih lama. Oleh itu, objektif kajian ini adalah (i) untuk menyiasat potensi fungisidal dan fungistatik minyak pati komersial herba dan rempah terpilih dari Malaysia terhadap kulat perosak dan kulat mikotoksigenik yang lazim; dan (ii) untuk mengkaji pencegahan dan pengurangan mikotoksin dalam kacang tanah melalui penggabungan minyak pati paling berkesan dalam sistem pembungkusan makanan. Minyak pati daripada 10 herba dan rempah (sirih, kayu manis, cengkih, ketumbar, lengkuas, halia, serai, limau nipis, buah pala, kunyit) telah dianalisa terhadap lima kulat perosak makanan dan kulat mikotoksigenik yang lazim dijumpai; iaitu *Aspergillus flavus, A. parasiticus, A. niger, Fusarium verticillioides*, dan *F. incarnatum*. Isolat kulat telah diperolehi daripada produk makanan lazim (jagung dan kacang), dan dipencil menggunakan kaedah kultur. Pengenalpastian molekul isolat kulat telah dilakukan, menggunakan pasangan primer bahagian Internal Transcribed Spacer (ITS). Potensi mikotoksigenik isolat kulat telah ditentukan oleh kromatografi cecair berprestasi tinggi dengan pengesan pendarfluor (HPLC-FLD). Sebatian bioaktif minyak pati telah dikenalpasti melalui kromatografi gas dan spektrometri jisim (GC-MS). Saringan awal *in vitro* telah dijalankan dengan kaedah resapan cakera agar dan resapan telaga agar menggunakan agar kentang dekstrosa, dan kaedah asai telaga mikro menggunakan kaldu kentang dekstrosa. Berikutan itu, tiga minyak pati paling berkesan telah dipilih untuk ujian Kepekatan Perencatan Minimum (MIC), Kepekatan Fungisidal Minimum (MFC), dan ujian percambahan spora kulat. Pengenalpastian molekul mengesahkan identiti isolat kulat dengan identiti spesis sedia ada di dalam GenBank. Berdasarkan keputusan HPLC-FLD, kesemua isolat kulat telah disahkan sebagai mikotoksigenik berdasarkan kebolehan menghasilkan mikotoksin. Berdasarkan keputusan GC-MS, sejumlah 120 sebatian bioaktif telah dikesan daripada minyak pati; 26.25% anethole (sirih), 63.39% cinnamaldehyde (kayu manis), 87.16% eugenol (cengkih), 54.79% linalool (ketumbar), 29.56% asid propenoik (lengkuas), 26.32% α-zingiberene (halia), 42.61% geranial (serai), 39.84% limonene (limau nipis), 27.80% β-phellandrene (buah pala), dan 41.81% ar-turmerone (kunyit). Berdasarkan saringan awal *in vitro*, minyak pati kayu manis, cengkih, dan serai menghasilkan perencatan pertumbuhan kulat tertinggi. Selanjutnya, minyak pati kayu manis menghasilkan perencatan kulat terbaik dalam MIC, MFC, dan percambahan spora, justeru dipilih untuk digunakan dalam sistem pembungkusan makanan. Kesan perencatan minyak pati kayu manis didapati setanding dengan antikulat komersial, sikloheksimida. Pembungkus yang mengandungi kompleks inklusi (IC; gabungan minyak pati kayu manis dan β- siklodekstrin) dengan kaedah kneading telah disediakan, dan perencatan pertumbuhan kulat secara *in vitro* ditentukan di bawah suhu (25°C, 50°C, dan 75°C) dan nilai pH (5.5, 7.4, dan 13.0) yang berbeza untuk melihat kestabilannya di bawah sistem makanan yang berbeza. Aktiviti antikulat IC dalam kacang tanah juga ditentukan dari segi pengurangan aflatoksin (kacang tanah dipilih kerana ia adalah komoditi tanaman makanan yang paling lazim ditumbuhi oleh *Aspergillus* section *Flavi*). Kompleks inklusi yang disediakan menunjukkan perencatan pertumbuhan kulat yang sangat baik secara *in vitro* dan pengurangan aflatoksin dalam kacang tanah berbanding dengan sampel kawalan. Suhu dan nilai pH yang pelbagai tidak menjejaskan aktiviti IC.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Nik Iskandar Putra bin Samsudin, PhD
Senior Lecturer
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Chairman)

Nur Hanani binti Zainal Abedin, PhD
Associate Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Member)

____
ZALILAH MOHD SHARIFF, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 11 August 2022

Declaration by Graduate Student

I hereby confirm that:

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LIST OF ABBREVIATIONS

α	Significance level of hypothesis test
β-CD	Beta-cyclodextrin
ACN	Acetonitrile
AF	Aflatoxin
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
CLSI	Clinical and Laboratory Standards Institute
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOA	Department of Agriculture
e.g.	*exempli gratia* (Latin for "for example")
et al.	*et alia* (Latin for "and others")
E	European
EC	European Commission
EFSA	European Food Safety Authority
EO	Essential oils
EU	European Union
eV	Electronvolt
F	Fumonisin
FDA	Food and Drug Administration
FLD	Fluorescence detector
GC-MS	Gas chromatography-mass spectrometry
HPLC	High Performance Liquid Chromatography
HCl	Hydrochloric acid

IAC	Immuno-affinity column
IARC	International Agency for Research on Cancer
i.e.	*id est* (Latin for “in other words”)
ITS	Internal Transcribed Spacer
IUPAC	International Union of Pure and Applied Chemistry
LOD	Limit of Detection
LOQ	Limit of Quantification
MeOH	Methanol
n	Number of samples
NaCl	Sodium chloride
NIST	National Institute of Standards and Technology
nm	nanometre
OPA	*Ortho*-phthalaldehyde
OTA	Ochratoxin A
p	Probability
PCR	Polymerase chain reaction
PDA	Potato Dextrose agar
PDB	Potato Dextrose broth
pH	Potential hydrogen
PHRED	Photochemical reactor for enhanced detection
ppb	Parts per billion
r	Correlation coefficient
R^2	Regression coefficient
RSD	Relative Standard Deviation
SE	Standard Error
sp.	Species (singular)

spp.	Species (plural)
UK	United Kingdom
UN	United Nations
UPM	Universiti Putra Malaysia
USA	United States of America
UV	Ultraviolet
v/v	Volume per volume
WHO	World Health Organization
ZEN	Zearalenone

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CHAPTER 1

INTRODUCTION

1.1 General introduction

Spoilage fungi in foods pose a risk to human health by degrading nutrients, shortening food shelf life, and affecting overall food quality. Spoilage fungi also contribute significantly to pre- and post-harvest wastes (Chen *et al.*, 2008). Certain spoilage fungi could also produce mycotoxins, and are called mycotoxigenic fungi. *Aspergillus*, *Fusarium*, and *Penicillium* are the most common mycotoxin-producing genera that can produce mycotoxins such as aflatoxins, ochratoxin A, patulin, fumonisins, trichothecenes (nivalenol, deoxynivalenol, T-2, HT-2), and zearalenone. Mycotoxins are a public health concern due to their widespread occurrence in nature, high toxicity, and potential carcinogenicity (Samson *et al.*, 2004). On humans and animals, mycotoxins can exert neurotoxicity, nephrotoxicity, and hepatotoxicity effects, as well as gastrointestinal and reproductive disorders when consumed at sufficient dose (Abbaszadeh *et al.*, 2014). What is worse, mycotoxins are chemically and thermally stable, and could persist in foods and feeds even after further processing (Bryden, 2007), thus making their control a significant challenge.

Due to the ubiquitous nature of fungal contaminants, and the fact that Malaysia's climate is extremely conducive for fungal proliferation, foodborne fungal contamination is a problem to both the food industry and consumers. While many antifungal chemical preservatives are being used by the food industry, their widespread use may increase the risk of toxic residues in foods while also contributing to fungal resistance, with some of them have already been banned by international regulatory authorities from usage in foods (Chen *et al.*, 2008). As a result, scientific interests and commercial applications are moving towards safer and more natural compounds to combat fungal contamination of foods. Generally, most of these compounds are plant-based, famous among which are the essential oils (EO).

In the past, EO were initially, and presently still are, used as flavouring agents in foods, in medicines, and in cosmetics (Bhargava *et al.*, 2015; Oussalah *et al.*, 2007). In recent years, EO have been used as an alternative to chemical preservatives in controlling bacterial contamination in foods, and huge successes of EO against spoilage and pathogenic bacteria in foods have been documented (Calo *et al.*, 2015; Deans and Ritchie, 1987). However, despite the widespread use of EO as antibacterials, their antifungal actions and mechanisms are not fully explored, and have been relatively less documented.

In addition, EO are highly volatile and prone to oxidative damage when exposed to light, oxygen, and temperature (Turek and Stintzing, 2013), thus making their efficacious applications in foods quite challenging. Furthermore, due to their strong odour, the application of EO in foods may be limited since undesirable changes in natural food aroma and taste may occur (Tao *et al.*, 2014).

The applications of EO via the micro-encapsulation technology as an antifungal for the protection of food products and commodities during storage, and for flavour stabilisation in the food industry have been previously described (Burt, 2004). Cyclodextrin coatings are capable of protecting the EO from environmental conditions, and of promoting oil diffusion, thus controlling the EO release on the food products on which they are applied (Capelezzo *et al.*, 2018). Falguera *et al.* (2011) reported that food packaging materials containing EO-based antifungal compounds were used as one of the most advantageous active packaging systems for extending the shelf life of food and preserving its safety, quality, and stability during storage.

Although the EO of various herbs and spices from other parts of the world (*e.g.*, thyme, rosemary, etc.) have shown antibacterial and antifungal effects, those from Malaysian herbs and spices remain underreported. Being a reservoir for various herbs and spices, Malaysia provides excellent opportunity for the exploration of novel bioactive antifungal agents against spoilage and mycotoxigenic fungal colonisation in foods and agricultural commodities pre- and postharvest (DOA, 2018) to fulfil the consumers' demand for greener, safer, healthier, and higher-quality foods with longer shelf life. In the present work, commercial EO of ten selected Malaysian herbs (betel leaf, cinnamon, clove, coriander, galangal, ginger, lime, lemongrass, nutmeg and turmeric) were tested against five common selected spoilage and mycotoxigenic fungi; *Aspergillus flavus* and *A. parasiticus* (producers of aflatoxins), *A. niger* (producer of ochratoxins), *Fusarium verticillioides* (producer of fumonisins), and *F. incarnatum* (producer of zearalenone) *in vitro* and in peanut. Peanut was selected since it is the most commonly contaminated food substrate by *Aspergillus* section *Flavi*.

1.2 Problem statement

The presence of spoilage and mycotoxigenic fungi in foods presents potential hazards to human health such as nutrient and quality degradation (by spoilage fungi), mycotoxicoses (acute effect of ingesting mycotoxins by mycotoxigenic fungi), and cancers (chronic effect upon prolonged consumption of mycotoxins) (de Saeger, 2011). Economically, fungal colonisation and mycotoxin contamination often lead to trade rejections and profit losses. To mitigate this, the food industry often turn to synthetic antifungals. However, the indiscriminate and widespread use of synthetic antifungals can result in the development of fungal resistance and residues in foods (Chen *et al.*, 2008). Furthermore, new information and data on the toxic effects of certain commonly used synthetic antifungals, such as sodium sorbate (E201; sodium salt of sorbic acid), are also continuously being compiled by the relevant international regulatory authorities (EFSA, 2015). To make things worse, certain fungal species have also shown the ability to neutralise or denature the commonly used synthetic antifungals, thus decreasing their intended protective effects on foods (Kinderlerer and Hatton, 1990).

Essential oils have long been safely used in foods, medicines, and cosmetics. Having shown considerable success in inhibiting spoilage and pathogenic bacteria in foods, EO are also increasingly being recognised as safe and natural substances to consumers and the environment in controlling fungal propagation and colonisation in foods. The use of EO offers antimicrobial effects, and when applied in active food packaging, can limit the use of chemical antimicrobials. However, EO is highly volatile and susceptible to oxidative damage when exposed to light, oxygen, and temperature (Turek and Stintzing, 2013). Thus, to overcome these issues, the selection of appropriate packaging materials and techniques for the EO is extremely important (Capelezzo *et al.*, 2018).

1.2 Research objectives

1.3.1 General objective

Generally, the present work aimed to evaluate the antagonistic effects of essential oils from selected Malaysian herbs and spices against spoilage and mycotoxigenic fungi.

1.3.2 Specific objectives

The specific objectives of the present work were as follows:

1) To investigate the fungicidal and fungistatic potential of commercial essential oils from selected Malaysian herbs and spices against common spoilage and mycotoxigenic fungi, and
2) To examine the mycotoxin prevention or reduction *in vitro* and in peanut through the incorporation of the most potent essential oil in food packaging system.

1.4 Research questions

1) Which essential oil from the selected Malaysian herbs and spices would exhibit the most potent fungicidal and fungistatic effect against common spoilage and mycotoxigenic fungi *in vitro*?
2) Which factor(s) significantly affect(s) the release of antifungal volatiles from the most potent essential oil *in vitro* and in peanut through its incorporation in a food packaging system?

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