EVALUATION OF MYCOLOGICAL QUALITY OF SPICES AND POTENTIAL APPLICATION OF PLANT ESSENTIAL OILS (*Cinnamomum cassia, Syzgium aromaticum,* AND *Illicium verum*) IN INHIBITING GROWTH OF MYCOTOXIGENIC FUNGI

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UNIVERSITI SAINS MALAYSIA

2024

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by

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Thesis submitted in fulfilment of the requirements for the degree of Master of Science

September 2024

ACKNOWLEDGEMENT

Alhamdulillah, all praise and gratitude to Allah, The Creator, The Greatest, The Most Kind and Merciful, who has guided, inspired, and given me the strength to finish my thesis writing. I would like to express my heartfelt gratitude to those who have played a significant role in the completion of this thesis. Their support and encouragement have been invaluable throughout this academic journey.

First and foremost, I am deeply thankful to my supervisor, Dr Norlia Mahror for her guidance, expertise, and unwavering support. This thesis would not have been possible without her guidance. Next, my particular gratitude also goes to the lab assistant, Mr. Ghoni, for his assistance in providing the necessary resources and maintaining a conducive environment for my research.

I am profoundly grateful to my parents for their unending love, encouragement, and sacrifices. Their unwavering support has been my pillar of strength, motivating me to strive for excellence. To my family and friends, thank you for your understanding and encouragement. Their presence has made this journey more enjoyable.

Lastly, I would like to express my gratitude to all those who have directly or indirectly contributed to this thesis. Your support has been crucial in shaping both my academic and personal growth. Thank you all for being a part of this journey. Your contributions have made this achievement possible.

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LIST OF ABBREVIATIONS

ADM	*Aspergillus* Differentiation Medium
AF	Aflatoxin
AFs	Aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2)
ANOVA	Analysis of variance
a_w	Water activity
BPW	Buffered peptone water
CFU	Colony forming unit
CLSI	The Clinical & Laboratory Standards Institute
MEA	Malt extract agar
CYA	Czapek yeast extract agar
DG18	Dichloran-glycerol agar
EU	European Union
EO	Essential oil
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Statistics
FDA	Food and Drug Administration
GRAS	Generally Recognised as Safe
GMP	Good Manufacturing Practices
IARC	International Agency for Research on Cancer
IF	Isolation frequency
MIC	Minimum inhibitory concentration
MFC	Minimum fungicidal concentration
OTA	Ochratoxin A
LC–MS	Liquid chromatography-mass spectrometry
HPLC	High-performance liquid chromatography
NA	Not available
PDA	Potato dextrose agar
UV	Ultraviolet

WHO	World Health Organization

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PENILAIAN KUALITI MIKOLOGI REMPAH DAN POTENSI UNTUK APLIKASI MINYAK PATI TUMBUHAN (*Cinnamomum cassia, Syzgium aromaticum,* DAN *Illicium verum*) DALAM MERENCAT PERTUMBUHAN KULAT BERMIKOTOKSIN

ABSTRAK

Rempah ratus digunakan secara meluas dalam pelbagai masakan di Malaysia untuk meningkatkan rasa dan aroma. Walau bagaimanapun, kecenderungan rempah terhadap jangkitan kulat menimbulkan kebimbangan tentang pencemaran mikotoksin jika keadaan penyimpanan adalah bersesuaian untuk pertumbuhan kulat. Oleh itu, kajian ini bertujuan untuk menentukan kejadian kulat bermikotoksin dalam rempah yang dijual di Pulau Pinang, Malaysia, mengenal pasti kulat bermikotoksin berdasarkan ciri-ciri morfologi, pendekatan molekul, dan penghasilan mikotoksin, serta mengkaji keberkesanan minyak pati tumbuhan terpilih dalam mengawal pertumbuhan kulat bermikotoksin. Sejumlah 110 sampel rempah terdiri daripada jintan, adas, ketumbar, lada (lada hitam dan lada putih), cili (cili kering, pes cili, dan serbuk cili), kayu manis, bunga lawang, cengkih, serbuk kari, dan serbuk kurma dibeli secara rawak dari pasaran runcit di Pulau Pinang. Sampel dianalisis untuk jumlah kiraan kulat (serbuk rempah) dan kejadian jangkitan kulat (rempah keseluruhan). Spesies kulat yang diasingkan dari rempah dikenal pasti berdasarkan pendekatan morfologi dan molekular, dan potensi mikotoksin ditentukan mengguna media agar krim kelapa dan kaedah HPLC. Aktiviti antikulat minyak kayu manis, cengkih, dan bunga lawang disaring menggunakan ujian penyebaran cakera. Kepekatan pengehadan minimum (MIC) dan kepekatan fungisidal minimum (MFC) ditentukan menggunakan kaedah pencairan kaldu dan agar. Minyak pati tumbuhan yang paling berkesan dipilih untuk kajian *in situ* dalam pes cili. Hasil kajian menunjukkan biji ketumbar (rempah serbuk) mencatatkan jumlah kiraan kulat keseluruhan tertinggi (ADM 3.08 log CFU/g; DG18 3.14 log CFU/g), manakala lada hitam (rempah keseluruhan) mencatatkan kejadian jangkitan kulat tertinggi (94%). Menariknya, bunga lawang dan cengkih bebas daripada pencemaran kulat. *Aspergillus flavus* dan *A. niger* adalah kulat bermikotoksin utama dalam kedua-dua rempah serbuk dan keseluruhan. Minyak kayu manis menunjukkan aktiviti antikulat paling kuat terhadap *A. niger*, dengan perencatan sepenuhnya dalam ujian penyebaran cakera, diikuti oleh minyak cengkih (17 ± 3.1 mm) dan minyak bunga lawang (9.3 ± 1.2 mm). Hasil yang serupa diperoleh daripada kaedah pencairan kaldu dan agar. Tambahan pula, minyak kayu manis menunjukkan nilai MIC (0.01–0.03%) dan MFC (0.06–1.0%) yang terendah terhadap kedua-dua kulat tersebut. Penambahan 0.5% minyak kayu manis dalam pes cili secara signifikan mengurangkan bilangan *A. flavus* dan *A. niger* (< 1.0 log CFU/g) pada suhu bilik (30 °C) dan suhu lebih rendah (4 °C) dengan impak minimum terhadap pH, warna, dan aktiviti air semasa tempoh penyimpanan 15 hari. Penemuan ini menunjukkan risiko pendedahan mikotoksin kepada pengguna disebabkan oleh penggunaan rempah yang tinggi dalam masakan tempatan. Sebagai alternatif kepada pengawet kimia, boleh disimpulkan bahawa minyak kayu manis boleh menjadi potensi pengawet semula jadi untuk pes cili.

EVALUATION OF MYCOLOGICAL QUALITY OF SPICES AND POTENTIAL APPLICATION OF PLANT ESSENTIAL OILS (*Cinnamomum cassia, Syzgium aromaticum,* AND *Illicium verum*) IN INHIBITING GROWTH OF MYCOTOXIGENIC FUNGI

ABSTRACT

Spices are widely used in various cuisines in Malaysia to enhance the flavour and aroma. However, the susceptibility of spices to fungal infection raises concerns about potential mycotoxin contamination if the storage conditions are favourable. Thus, this study aimed to determine the prevalence of mycotoxigenic fungi in spices marketed in Penang, Malaysia, to identify and characterize the mycotoxigenic fungi based on the morphological characteristics, molecular approaches and mycotoxin production, and to study the efficacy of selected plant essential oils (EOs) in controlling the growth of mycotoxigenic fungi. A total of 110 spice samples consisting of cumin, fennel, coriander, peppers (black pepper and white pepper), chillies (dried chilli, chilli paste and chilli powder), cinnamon, star anise, cloves, curry powder and korma powder were randomly purchased from Penang's retail markets. The samples were analyzed for the total fungal count (ground spices), and incidence of fungal infection (whole spices). The fungal species isolated from spices were identified based on morphological and molecular approaches, and the mycotoxigenic potential was determined using the Coconut Cream Agar and HPLC method. The antifungal activity of cinnamon, clove and star anise EOs were screened using a disk diffusion assay. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined using broth and agar dilution methods. The most effective EO was selected for *in situ* study in chilli paste. Results showed that coriander seeds (ground) recorded the highest total fungal count (ADM 3.08 log CFU/g; DG18 3.14 log CFU/g), while black pepper (whole) recorded the highest incidence of fungal infection (94%). Interestingly, star anise and cloves were free from fungal contamination. *A. flavus* and *A. niger* were the predominant mycotoxigenic fungi in both ground and whole spices. Cinnamon EO demonstrated the most potent antifungal activity against *A. niger*, with complete inhibition in the disk diffusion assay, followed by clove EO (17 ± 3.1 mm) and star anise EO (9.3 ± 1.2 mm). Similar outcomes were observed from broth dilution and agar dilution methods. Additionally, cinnamon EO exhibited the lowest MIC (0.008–0.03%) and MFC (0.06–1.0%) values against both fungi. Incorporating 0.5% cinnamon EO in chilli paste significantly reduced *A. flavus* and *A. niger* counts (< 1.0 log CFU/g) at ambient (30 ℃) and lower temperature (4 ℃) with minimal impact on pH, colour, and a_w during a 15-day storage period. These findings indicate the risk of mycotoxin exposure to consumers due to the high consumption of spices in local cuisine which often contaminated with mycotoxigenic fungi. As an alternative to chemical preservatives, it can be concluded that cinnamon EO can be a potential natural preservative for chilli paste.

CHAPTER 1

INTRODUCTION

1.1 Background

Spices are agricultural commodities which are derived from various parts of the plants. Historically, spices have been widely used in food and beverages as preservatives, antioxidants and for medicinal purposes (Pickova et al., 2020). Spices are also used as food ingredients to enhance the flavour and aroma of the food. In Malaysia, spices are widely used in various cuisines such as *Nasi lemak, Nasi kandar, rendang,* curry dishes and soup to name a few. Commonly used spices in Malaysia include black pepper, cinnamon, chilli, coriander seeds and white pepper. Each spice has its own distinct flavour and scent profile that contributes to the overall flavour and aroma of the cuisines.

Despite their popularity, spices are very susceptible to fungal contamination from the field to postharvest chains and storage. In Malaysia, the tropical climate with 27 ℃ of temperature and 70% relative humidity is conducive to fungal growth (Afsah-Hejri et al, 2013). Apart from the climate conditions, improper handling, storage, and less hygienic processing can contribute to the proliferation of fungi and subsequent mycotoxin production (Iha and Trucksess, 2019; Pickova et al., 2020). Although spices are used in small amounts, they are considered an important vector of microbial contamination including fungi (Romagnoli et al., 2007; Gnonlonfi et al., 2013; Nguegwouo et al., 2018).

The occurrence of fungi can compromise the safety of the product due to the secondary metabolites produced by some fungi species called mycotoxins (Temu, 2016). Some important mycotoxins in food and feed are aflatoxins (AFs) and ochratoxin A (OTA). Ali et al. (2015) revealed that the occurrence of AFs and OTA in spices marketed in Malaysia ranged from 0.01 to 9.34 µg/kg and from 0.14–20.40 µg/kg, respectively.

These levels surpass the regulatory limit of 5 µg/kg as stipulated by Malaysia Food Regulations (1985). Nevertheless, the study on the prevalence and identification of mycotoxigenic fungi from spices is still lacking. In Malaysia, studies mainly focus on the occurrence of mycotoxins in spices (Ali et al., 2015). However, there is still limited study on the prevalence of mycotoxigenic fungi in spices particularly in Malaysia. The study on mycological quality is crucial since it highlights the significance of inhibiting and controlling fungal growth as a highly effective strategy in preventing mycotoxin contamination. The occurrence of mycotoxins has shown no visible alteration in the commodities. Therefore, mycotoxins have been a significant interest in the food industry owing to their severe effects on humans. *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* are some mycotoxigenic fungi that commonly contaminate dried commodities and produce mycotoxins (Santos et al., 2011). Despite many years of research and implementing best practices in crop production chains, mycotoxins remain a problem. Mycotoxins are unavoidable natural toxicants that pose a significant challenge to food safety. Thus, there is an ongoing need for innovative solutions to reduce fungal contamination in spices.

Despite their undesirable effects and toxicity, chemical additives have been widely used for many years in food. In recent times, the interest and demand in finding natural antimicrobials suitable for food use has increased (Sahab et al., 2014). Essential oils (EOs) are secondary metabolites produced from different parts of plants such as seeds, flowers, buds, leaves, bark and stem (Ribeiro-Santos et al., 2018). EOs are an effective preservative due to the presence of some active components that have antimicrobial activity against bacteria and fungi (Ju et al., 2018). Hu et al. (2019) studied several EOs and found that cinnamon and clove EO showed antifungal activity against *A. niger*, *A. oryzae* and *A.*

ochraceus. Additionally, when tested on naturally contaminated bread, it was noted that these EOs could serve as effective natural antifungal agents (Hu et al., 2019).

1.2 Problem statement and rationale of study

Several studies have reported high levels of mycotoxins in spices from the local market in Malaysia (Afsah-Hejri et al., 2013; Ali et al., 2015). However, studies on the mycological quality of spices in Malaysia are very minimal, especially in Penang. Currently, there is a lack of data on the prevalence of mycotoxigenic fungi in various spices within Penang, which is essential for establishing a baseline to plan for intervention strategies.

Addressing the presence of mycotoxigenic fungi in commodities is crucial, as certain fungi species can produce harmful mycotoxins. Some of these toxins are thermostable and resistant to degradation through high temperatures during cooking or food processing. Notably, the 2016 outbreak in Tanzania, which resulted in 68 reported cases and 20 deaths, due to the consumption of contaminated maize, highlights the severity of this issue (Kamala et al., 2018).

Moreover, identifying mycotoxigenic fungi is challenging due to the high similarities in the morphological characteristics of some species (Zulkifli and Zakaria, 2017). Therefore, to accurately identify the fungal species, a combination of morphological, chemical, and molecular approaches is required.

Chemical preservatives are widely used in foods to extend the shelf life and to ensure the safety and quality of the food products. There is growing interest in utilizing plant EOs as natural preservatives to mitigate the adverse effects of chemical preservatives. Additionally, clean label market is currently growing and piqued interest amongst consumers and producers. Plant EOs contain antifungal properties that can hinder fungal growth in food, thereby reducing the risk of mycotoxin contamination. Research on the efficacy of plant EOs as natural preservatives is reported by many researchers. However, research on the effectiveness of EOs in foods especially in chilli paste is still limited and requires further investigation.

1.3 Objectives

i. To determine the prevalence of fungal contamination in various types of ground and whole spices from the retail market in Penang.

ii. To identify and characterize the fungi from various types of ground and whole spices from the retail market in Penang based on their morphological characteristics, molecular approaches, and mycotoxin production.

iii. To study the effectiveness of selected plant essential oils (*Cinnamomum cassia*, *Syzgium aromaticum*, and *Illicium verum*) in inhibiting the growth of mycotoxigenic fungi in chilli paste.

CHAPTER 2

LITERATURE REVIEW

2.1 Spices

Spices are non-leafy parts of plants such as roots, seeds, buds, leaves, flowers, and stigmas used for flavouring, colouring, taste, fragrance, and other quality attributes of foods . Spice is derived from the Latin word 'species', which means specific kind (Raghavan, 2007). Historically, spices have been used in cooking, medicine, and preservatives since ancient times (Kabak and Dobson, 2017). Constituent compounds, which include alkaloids, flavonoids, isoflavonoids, tannins, coumarins, glycosides, terpenes, and phenolic compounds, not only produce useful in culinary preparations but are also responsible for many of their medicinal and preservative uses (Iha and Trucksess, 2019). These compounds have made them well-known for their unique flavouring and aromatizing characteristics and their preservative, antibacterial, and antioxidant properties.

Spices can be found in the market and are usually marketed as whole, ground and mixed spices. Some commonly used spices worldwide are black pepper, cardamom seed, chilli, cinnamon, clove, coriander seed, cumin seed, fennel seed, ginger, nutmeg, star anise, white pepper and turmeric (Table 2.1) (Kabak and Dobson, 2017). The spices' attributes vary depending on where they are grown, soil conditions and how they are handled after harvest.

Table 2.1 Lists of some commonly used spices.

English name	Family	Scientific name	Part of plant	Malay name
Black pepper	Piperaceae	*Capsicum* spp.	Berries	*Lada hitam*
Cardamom seed	Zingiberaceae	*Piper nigrum*	Fruits	*Buah pelaga*
Chilli	Solanaceae	*Ammomum* spp.	Berries	*Cili*
Cinnamon	Lauraceae	*Cinnamomum* spp.	Barks	*Kayu manis*
Clove	Myrtaceae	*Szygium aromaticum* (L.)	Flower buds	*Bunga cengkih*
Coriander seed	Apiaceae	*Coriandrum sativum* L.	Fruits	*Ketumbar*
Cumin seed	Apiaceae	*Cuminum cyminum* L.	Seeds	*Jintan putih*
Fennel seed	Apiaceae	*Foeniculum vulgare* Mill	Fruits	*Jintan manis*
Ginger	Zingiberaceae	*Allium sativum* L.	Rhizomes	*Halia*
Nutmeg	Myristicaceae	*Myristica fragrans* Houtt.	Seeds	*Buah pala*
Star anise	Illiaceae	*Illicium verum*	Fruits	*Bunga lawang*
Turmeric	Zingibearaceae	*Curcuma longa* L.	Rhizomes	*Kunyit*
White pepper	Piperaceae	*Piper nigrum*	Berries	*Lada putih*

Adapted from: Kabak and Dobson (2017)

2.1.1 Global spice trade and consumption

Spices have been cultivated in a wide range of countries. The global spice trade has been increasing through the years. According to FAOSTAT (2021), the estimated value of the global spice trade is approximately 22.4 billion USD with more than 665,606 tonnes of production. Asia contributed 86% of spice production by region, followed by Africa (12.4%) and America (1.5%). However, the total spice production in Malaysia was only 750 tonnes from 75 hectares of area harvested in 2019 (FAOSTAT, 2019). Malaysia is listed in the top 10 of the biggest importers of spices products with 29,146 tonnes of imported spices worth RM473 million (FAOSTAT, 2021). Spice imports to Malaysia in 2021 have increased tremendously by 106% compared to the data in 2016 (FAOSTAT, 2021).

India, acclaimed as the primary producer of spices, yielded 1.4 M tonnes in 2019 (FAOSTAT, 2019). Additionally, it stands as the foremost consumer and exporter of these aromatic commodities. According to India Brand Equity Foundation (IBEF, 2022), pepper, cardamom, chilli, ginger, turmeric, coriander, cumin, celery, fennel, fenugreek, garlic, nutmeg, and mace, as well as spice oils and oleoresins, are the most produced and exported spices. Notably, chilli, cumin, turmeric, ginger, and coriander collectively contribute to approximately 76% of the total production of these spices (IBEF, 2022).

2.1.2 Use of spices worldwide

Spices are very popular in Malaysia. Malaysian cuisines represent the nation's multi-ethnic people and the cooking traditions and practices that exist here. The majority of Malaysia’s population consists of Malays, Chinese and Indians. Malaysia has diverse types of cuisines. Some of the most commonly used spices in cuisines are black pepper, chilli powder, cinnamon, cumin, ginger, cloves, star anise, nutmeg, smoked paprika, dried oregano and bay leaves. Chilli is one of the most commonly used in Malaysia's kitchens. Chillies are the fruit of the *Capsicum* plants belonging to the genus Solanaceae, which originates from America. Chillies are available in various forms, including fresh, dried, pastes, powder, and flakes.

The chemical in chilli peppers called capsaicin gives them heat and pungent taste (Kabak and Dobson, 2017). Globally, the primary producer of chilli is China followed by Mexico and Turkey. Malaysia mostly imported dried chilli with a total of 68,999 tonnes in 2020 (FAOSTAT, 2020). Chillies have been widely used in many types of cuisine such as soup, salads, stews, curries and many others.

Besides, cinnamon, cardamom, star anise, and cloves are popular spices known as '*rempah empat beradik*' or the four siblings, which are usually found in most Malaysian cuisines. When added to the cuisine, these four spices produce a nice fragrance, flavour and aroma. EOs in these spices, such as cinnamaldehyde in cinnamon, anethole in star anise and eugenol in cloves, are responsible for their great taste and aroma. They are normally combined to make various soups and spicy foods. Cloves serve their best in a whole form because the flavour will disappear once the cloves are ground. It is used in many dishes such as salad dressings, desserts and fruitcakes in the United States. Cloves are used in whole form or ground in garam masala, biryani, meat dishes, and pickles by Sri Lankans and North Indians (Raghavan, 2007). In Malaysia, cloves are usually paired with cinnamon and star anise to make chicken curry and rice.

Black pepper is known as "black gold" and "king of spices" (Zachariah and Parthasarathy, 2008). Pepper fruits are pungent and aromatic. The pungency of black pepper is due to a nonvolatile chemical compound called piperine. The pungent aroma of black pepper is due to its volatile oil, monoterpenes, which consist of sabinene, α-pinene, β-pinene, limonene, and 1,8-cineole (Raghavan, 2007). Black pepper is used to alter the taste of food on the table, added during cooking or used as a marinade. In Southeast Asia including Malaysia, people generally use peppers in soups, curries, and marinating meat and poultry.

On the other hand, spices commonly found on the market are mixed spices. Mixed spice is a blend of different types of spices. Commercially blended spices offer convenience and are easy to use. Complex food recipes often require many spices, making mixed spices a practical choice for consumers to prepare their desired dishes easily. Mixed spices will create a consistent flavour, colour, and texture in the cuisines in culinary establishments. Mixed spices include spices, salt, sugar, monosodium glutamate (MSG), anti-caking agents, and others to enhance overall flavour and quality. Curry and korma mixes are among the popular options available, providing versatility and richness to dishes.

Curry powder is a mixture of a wide range of spices. The essential spices in curry powder are coriander seeds, cumin, turmeric and ginger (Ravindran and Kalluparackal, 2001). Other minor ingredients are fenugreek, celery, black pepper, red pepper, cinnamon, nutmeg, cloves, caraway, fennel, cardamom and salt. Curry powders are widely used in cooking worldwide, especially in South Asian cuisines (Ravindran and Kalluparackal, 2001). Korma mix powder is an instant powder with a mixture of various spices. It consists of coriander seeds, cumin, fennel, white pepper, star anise, cinnamon, *dhall*, dry ginger, cardamom, clove and black pepper. Korma powder is widely used in various Malaysian cuisines.

The global demand for spices increases yearly (FAOSTAT, 2019). However, the susceptibility of spices to fungal contamination and subsequent mycotoxin contamination remains a concern. Thus, proper pre and post-harvest practices are the best mitigation strategy to prevent the occurrence of mycotoxins (Iha and Trucksess, 2019). Besides, the implementation of good agricultural practices (GAP), good storage practices (GSP), and good manufacturing practices (GMP) can mitigate the production of mycotoxin (Kamle et al., 2019).

2.2 Mycological quality of spices

The fungus can be described as a multicellular organism that is composed of long filaments known as hyphae. Despite resembling plants, they are not included in the plant kingdom since they lack chlorophyll and have filamentous structures (Adeyeye, 2016). Some fungal species play an essential role in causing food spoilage. Spoilage fungi in spices can change their organoleptic qualities, thereby reducing their commercial value (Garcia et al., 2018). The spoilage fungi can be classified into two groups: field fungi and storage fungi. Field fungi are those that usually attack the crops in the field, such as *Alternaria*, *Fusarium*, and *Cladosporium* (Hashem and Alamri, 2010). On the other hand, storage fungi such as *Aspergillus* and *Penicillium* are the most common and can grow in stored crops if the moisture content is high (Hashem and Alamri, 2010; Yogendrarajah et al., 2014).

Fungal contamination and mycotoxin production in agricultural products can occur along the food chain from preharvest, and postharvest such as production, processing, transport and storage (Cinar and Onbaşı, 2020). Temperature, relative humidity, fungicides and/or fertilisers, interactions between the colonising toxic fungi species, soil and nutritional elements, geographic location, genetic requirements, and insect infestation are all factors that influence the growth of fungi and the formation of mycotoxin (Cinar and Onbaşı, 2020).

Spices are mainly cultivated in tropical and subtropical countries with high temperatures, humidity, and rainfall, which are suitable conditions for fungal contamination and mycotoxin production (Iha and Trucksess, 2019; Vyhnánek et al., 2018). Contamination can occur depending on where they were cultivated, harvested, and processed (Gnonlonfin et al., 2013; Nguegwouo et al., 2018; Romagnoli et al., 2007). Researchers from different countries have extensively studied the mycological quality of spices (Table 2.2). The authors reported various types of mycotoxigenic fungi such as *A. flavus, A. niger, A. parasiticus, A. chevalieri* from whole and ground spices.

Spices are the main vector of microbial contamination. The fungi can contaminate the spices from the farm to the market. Once the spices are contaminated in the field, the fungi will continue to grow in favourable conditions such as high temperature and moisture (Reddy et al., 2011). The primary causes of fungal contamination in the spices during preharvest include pest and insect infestation (Cinar and Onbaşı, 2020). The pests and insects act as vectors for microorganisms that cause contamination from one commodity to the others.

In addition, the processing of spices is one of the primary sources of contamination. For example, since sun drying is cost-efficient, spices are usually sun-dried, exposed to the environment, and sold in the market without further processing. Improper drying and cleaning are the main causes of fungal contamination during storage. This will cause contamination and deterioration in the quality of the spices (Thanushree et al., 2019). Spices should be dried adequately before storage to avoid fungal growth during storage and, subsequently mycotoxin production.

Table 2.2 Mycological quality of spices from different countries

Country	Type of spices	Total fungal count	Potentially mycotoxigenic fungi	References
Africa	Chilli	3.7 – 5.2 log CFU/g	*Aspergillus, Penicillium, Chaetomium, Fusarium, Rhizopus*	Aasa et al. (2022)
Tunisia	Coriander, cumin, red pepper	<2 log – ≥ 5 log CFU/g	*A. flavus, A. niger, A. versicolor, A. terreus*	Saidi et al. (2021)
Indonesia	Chilli	1×10^3 – 4.08×10^5	*A. flavus, A. niger, A. parasiticus, A. carbonarius, A. japonicus*	Wikandari et al. (2020)
Lebanon	Black pepper, chilli, cinnamon, coriander, cumin, curry, white pepper	Mean for each sample: 10^3 – 4×10^3 CFU/g	*Aspergillus* section *flavi*	Makhlouf et al. (2019)
Italy	Cinnamon, fennel, white pepper, black pepper	Ground spices: 2.55×10^3 – 1.05×10^4 CFU/g Whole spices: 76 -77%	*A. flavus, A. niger, A. parasiticus, A. sydowii,* A. *chevalieri*	Garcia et al. (2018)
Algeria	Aniseed, black pepper, caraway, cinnamon, coriander, cumin, ginger, red pepper, saffron, sweet cumin, and sweet pepper	4.5×10^2 – 2.0×10^3 CFU/g	*Aspergillus* section *flavi*	Azzoune et al. (2016)
China	Cinnamon, cumin fennel, star anise	ND	*A. flavus, A. niger, A. ochraceus*	Kong et al. (2014)

*ND: Not detected

2.2.1 *Aspergillus* spp.

Aspergillus spp. is a diverse genus with significant economic and social impact (Samson et al., 2014). It is generally known as moulds that can contaminate various food and feedstuffs and produce mycotoxins. *Aspergillus* species are xerophilic fungi and can survive at high temperatures, very low a_w or both (Pitt and Hocking, 2009). Along with *Penicillium*, they are predominant fungi contaminants in stored products.

The predominant *Aspergillus* species that can be found in spices including *A. flavus, A. niger, A, ochraceus and A. fumigatus* (Vyhnánek et al., 2018). *A. flavus* is known as AF producer, whereas *A. niger is* known as an OTA producer. Several researchers have reported the occurrence of toxigenic fungi in spices. Data from the studies show that *A. niger* was the most predominant toxigenic fungi isolated from different spices samples, followed by *A. flavus* as shown in Table 2.2.

2.2.2 *Penicillium* spp.

Penicillium spp. is one of the main economic concerns due to its ability to cause food spoilage. *Penicillium* has a wide range of species and habitats; some are known as mycotoxin producers (Pitt and Hocking, 2009). It can grow in various environments, such as soil, air, interior spaces and many food products (Samson et al., 2014). Nevertheless, the conditions that promote fungal growth do not confirm the presence of mycotoxins (Pardo et al., 2006; Sanchis and Magan, 2004). According to the authors, fungi can grow abundantly in certain conditions without producing mycotoxins. *Penicillium* is classified as a storage fungus. It can attack the storage commodities in a warm climate and subsequently produce mycotoxin (Pardo et al., 2006). One of the important mycotoxins produced by *Penicillium* is ochratoxin A (OTA), which has nephrotoxic properties (Pardo et al., 2006). *P. verrucosum* is found to be the only species which can produce OTA. Several researchers have found that the presence of *P. verrucosum* in stored commodities will produce OTA (Pardo et al., 2006).

2.3 Mycotoxins in spices

Mycotoxins are naturally occurring chemical contaminants in food and agricultural commodities. Fungal growth depends on the surrounding factors and can produce toxic secondary metabolites called mycotoxins. Some factors that can contribute to the production of mycotoxins are fungal genetics, nutrient availability, temperature, a_w and humidity. There are more than 400 mycotoxins identified. Among all the identified mycotoxins, AF and ochratoxin A are two of which important mycotoxins that are commonly present in various commodities such as cereals, cereal products, coffee, cocoa, nuts and spices (Schlösser and Prange, 2018). Mycotoxins have high stability and cannot be easily destroyed by heat. The incidence of mycotoxins in spices from various countries has been studied and reported extensively as shown in Table 2.3.

The occurrence of mycotoxins can pose a severe health risk to consumers. For instance, the first major outbreak occurred in Kenya in 1981, resulting in 20 cases and 12 deaths. Studies revealed that the source of aflatoxin exposure was contaminated maize, with maize grains from affected households containing aflatoxin levels as high as 12 mg/kg (Daniel et al., 2011; Ngindu et al., 1982). In a similar outbreak in 2004, the Kenya Ministry of Health identified 317 cases of aflatoxicosis linked to contaminated maize, leading to 125 fatalities (Azziz-Baumgartner et al., 2005). In Malaysia, 17 cases were reported with a 76.5% fatality rate, where 13 children died from aflatoxicosis suspected to be caused by contaminated Loh Su Fun noodles (Chang et al., 2023). The most recent outbreak occurred in Tanzania, with 68 reported cases and 20 deaths, resulting in a 30% fatality rate (Kamala et al., 2018). These incidents highlight the urgent need for effective food safety measures to mitigate the risks posed by mycotoxigenic fungi and to prevent future outbreaks.

Table 2.3 Worldwide occurrence of mycotoxins in spices

Country	Spices	Positive samples (%)	Amount of mycotoxins in samples (μg/kg)	No. of samples exceeding the EU limit (%)	Reference
Ethiopia	Pepper	AFs: 42.00	AFs: 3.10-19.20	AFs: 98.00	Tsehaynesh et al. (2021)
Indonesia	Dried chilli	NA	AFB_1: 39.30–139.50 AFB_2: 2.60–33.30 OTA: 23.70–84.60	NA	Wikandari et al. (2020)
Malaysia	Chilli, peppers, cumin	AFB_1: 93.33	AFB_1: 1.20-3.26	NA	Reddy et al. (2011)
Benin	Pepper, chilli, cumin, cinnamon, anise	AFB_1: 84	AFB_1: 0.46-84.84	40.00	Akpo-Djènontin et al. (2018)
Pakistan	Chilli powder, whole chilli, chilli sauce and crushed chilli	AFs: 56.40 OTA: 40.40	AFs: 12.50-15.16 OTA: 16.68-120.90	AFs: 26.30 OTA: 32.70	Iqbal et al. (2017)
Thailand	Chilli powder	AFB_1: 100.00	AFB_1: 5.32 -11.14	AFB_1: 0.00	Thanaboripat et al., (2016)
Malaysia	Dried chilli, fennel, cumin, black pepper, white pepper, coriander and mixed spices	AFs: 85.00 OTA: 79.00	AFs: 0.01-9.34 OTA: 0.14-20.40	NA	Ali et al. (2015)
India	Black pepper	AFB_1: 45.24 AFs: 76.00 OTA: 78.57	AFB_1: NA AFs: 4.80-26.40 OTA: 4.60-13.80	NA	Jeswal and Kumar (2016)

India	Red chilli	AFB_1: 56.36 AFs: 85.45 OTA: 72.73	AFB_1: NA AFs: 4.30-33.60 OTA: 3.50-8.60	NA	Jeswal and Kumar (2015)
Italy	Peppers, chilli, cinnamon, cloves, coriander, mixed spices	AFs: 15.40 AFB_1: 8.50 OTA: 23.80	AFs: 0.96 AFB_1: 0.31 OTA: 6.18	AFs: NA OTA: 2.30	Prelle et al. (2014)
Qatar	Black pepper	AFs: 41.7	AFs: 1.85-70.33	NA	Hammami et al. (2014)
Malaysia	Dried chilli	AFs: 65.00 OTA: 81.25	AFs: 0.20-79.70 OTA: 0.20-101.20	NA	Jalili and Jinap (2012)
Turkey	Red chilli powder, black pepper, cumin	AFB_1: 43.75 OTA:26.56	AFB_1: 0.13-35.77 OTA: 0.63-98.2	AF: 13.60 OTA: 13.60	Ozbey and Kabak, (2012)
Turkey	Chilli powder	AFs: 63.60 OTA: 54.50	AFs: O.20-35.77 OTA: 0.78-98.20	AFs: 4.50 OTA: 13.60	Ozbey and Kabak (2012)
Korea	Chilli powder, chilli paste	AFs: 13.60	AFs: 0.08-4.66	AFs: 0.00	Cho et al. (2008)
Hungary	Black pepper, mixed spices, chilli	AFB_1: 25.27 OTA: 36.00	AFB_1: 0.16-8.10 OTA:2.1	AFB_1: 20.00 OTA: NA	Fazekas et al. (2005)

NA: Not available
AFs: Aflatoxins (total aflatoxin)
AFB_1: Aflatoxin B_1
AFB_2: Aflatoxin B_2
OTA: Ochratoxin A

2.3.1 Mycotoxin analysis

Generally, the chromatography methods used for mycotoxin analyses are liquid chromatography-mass spectrometry (LC–MS), gas chromatography-mass spectrometry (GC–MS), thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) with UV or fluorescence detector (FLD) (Rahmani et al., 2013). The HPLC method is the most used for mycotoxin analyses since it is more cost effective and has good sensitivity and recovery (Alshannaq and Yu, 2017; Rahmani et al., 2013). Due to limited sensitivity and specificity, other chromatographic methods are rarely used for quantitative analysis of mycotoxin (Alshannaq and Yu, 2017; Anfossiet al., 2016; Pereira et al., 2014).

2.3.2 Aflatoxins

Aflatoxin (AF) contamination of dried commodities is a global food safety issue. AFs commonly occur in peanuts, nuts, figs, corn, rice, spices, and dried fruits (Martinez-Miranda et al., 2019). Toxicity effects of aflatoxins include carcinogenic, mutagenic, teratogenic, and immunosuppressive in animals and humans. AFs are the most potent mycotoxins that are highly toxic and carcinogenic and can cause acute health consequences to humans (Mahato et al., 2019). There are four naturally occurring aflatoxins: AFB_1, AFB_2, AFG_1, and AFG_2 (Kabak and Dobson, 2017). Among these AFs, AFB_1 is the most toxigenic. According to the International Agency for Research on Cancer (IARC, 1993), AFB_1 has been classified as a Class 1 human carcinogen. AFs are one of the most important mycotoxins produced by fungi from the genus *Aspergillus* spp. *A. flavus*, *A. parasiticus*, and *A. nomius* are the most frequent producers of AFs (Ali et al., 2015). The optimum temperature for the survival of *A. flavus* is 30 °C, while the optimum temperature for AF production is between 25 to 30°C (Pitt and Hocking, 2009; Yogendrarajah et al., 2014).

2.3.3 Ochratoxin A

Ochratoxin A (OTA) has been identified as a significant health concern due to its carcinogenic, nephrotoxic, and immunotoxic properties (Wang et al., 2016). The International Agency for Research on Cancer (IARC, 1993) classified OTA as a probable human carcinogen (Group 2B). This mycotoxin is commonly produced by several fungal species, including *A. niger*, *A. ochraceus*, *A. carbonarius*, and *P. verrucosum*. The distribution of these fungi varies, with *A. ochraceus* and *A. carbonarius* predominantly found in tropical climates, while *P. verrucosum* is more prevalent in temperate regions (Richard, 2007; Steyn, 1995; Turner et al., 2009). The optimal temperature range for OTA production is typically between 25 to 30°C, which aligns well with the conditions found in tropical climates. Consequently, tropical regions provide a conducive environment for the growth of OTA-producing fungi and subsequent OTA production (Nguegwouo et al., 2018). Turner et al. (2009) have reported that dried products, including spices, are commonly contaminated with OTA.

2.3.4 Regulatory limits of mycotoxins

Implementing prevention and control measures and establishing regulatory limits are vital to avoiding mycotoxin exposure to consumers. The primary goal is to establish a maximum level of mycotoxins in foods to enable fair trade while preserving consumers' health. The regulatory limits for mycotoxins have been established in over a hundred countries (Van Egmond, 2013). Currently, the regulation limit of mycotoxins in spices ranges from 5–30 μg/kg depending on each country (FAO, 2004; Reddy et al., 2011; Van Egmond et al., 2007). Although each country has its mycotoxin regulations, standardised regulations are yet to be implemented (Iha and Trucksess, 2019). For example, certain countries did not prescribe AFs specifically in spices; therefore, spices fall under "other types of food." Moreover, OTA regulations are only available in coffee and cereal-based foods for infants in most countries.

According to the European Union (EU) standard, the AFB_1 maximum limit in spices like chilli, peppers, nutmeg, ginger, turmeric, and a mixture of spices is 5 μg/ kg. In contrast, the sum of AFB_1, AFB_2, AFG_1, and AFG_2 in spices is 10 μg/kg (European Union, 2010). Meanwhile, the maximum regulated limit of OTA in spices is 15 μg/kg (European Union, 2012). In Malaysia, there is no specific regulation for the spices. However, there is a general maximum limit of total AFs in other food including spices, which is 5 μg/kg as stated in Malaysian Food Regulations (1985). Table 2.4 shows the regulatory limits for mycotoxins in certain countries.

Table 2.4 Regulatory limits for mycotoxins in certain countries

Country	Commodity	Maximum limit (µg/kg)			Reference
		AFB$_1$	AFs	OTA	
European Union	*Piper* spp. (fruits thereof, including white and black pepper), *Myristica fragrans* (nutmeg), *Zingiber officinale* (ginger), *Curcuma longa* (turmeric), mixtures of spices containing one or more of the abovementioned spices,	5	10	15	European Union (2010)
	Capsicum spp. (dried fruits thereof, whole or ground, including chillies, chilli powder, cayenne, and paprika)	10	5	20	
Indonesia	Spice powder	15	20	NA	Codex (2017)
Malaysia	Other foods not specified (including spices)	NA	5	NA	Food Regulations 1985 (1985)
	Chilli	10	15	1	Codex (2017)
Republic of Korea	Curry powder, nutmeg, turmeric, dried chilli, dried paprika, and spices containing nutmeg, turmeric, dried chilli, and dried paprika	10	15	NA	
Thailand	All foods	NA	20	NA	Songsermsakul (2015)
United states	All foods	NA	20	NA	US FDA (2000)

Adapted from: Iha and Trucksess (2019)

2.4 Antifungal compounds of plant essential oils

Essential oils (EOs) are secondary metabolites produced by plants that act as antimicrobial agents (Liu et al., 2017). Most plants produce antimicrobial secondary metabolites, either as part of their normal growth and development program or in response to pathogen attack or stress (Aly et al., 2016). The EOs consist of terpenes, terpenoids and other aromatic and aliphatic constituents with low molecular weight (Gucwa et al., 2018). These compounds are a natural source of antioxidants and biologically active compounds (Sahab et al., 2014). Historically, EOs are commonly used as fragrances and flavouring agents in the perfume and food industries.

Many *in vitro* studies have confirmed that EOs are capable of inhibiting the growth of bacteria, fungi yeasts, viruses, and protozoa (Saad et al., 2013). The potential application of EOs in food products has gained interest from researchers and food processors due to their ability to prevent the growth of pathogenic bacteria and toxigenic fungi (Saeed et al., 2022). EOs have long been regarded as a viable alternative to reducing or replacing the use of synthetic additives, which have been linked to various adverse human health effects (Ribeiro-Santos et al., 2018).

Each species has distinct functional characteristics due to the various bioactive compounds. There are various methods to extract EOs from plants, including steam distillation and hydrodistillation. These two methods are widely used for EO extraction in lab scale (Ribeiro-Santos et al., 2018). The chemical composition of the EO depends on several factors such as part of the plants used and age, soil type, climate conditions, geographic region, harvesting seasons and method of extraction (Ribeiro-Santos et al., 2018).

2.4.1 Cinnamon

Cinnamon spice is the tree's inner bark from the genus *Cinnamomum* derived from the Greek word kinamon (Raghavan, 2007). There are more than 250 varieties of cinnamon were discovered, but only four types are commonly used in the food application including Chinese cinnamon (*Cinnamomum cassia*), Sri Lanka cinnamon (*C. zeylanicum*), Indonesian cinnamon (*C. burmannii*), and Vietnamese cinnamon (*C. loureirii*) (Nabavi et al., 2015). Cinnamon is known as *canelle* in French, *yook-gway* in Chinese, *dal-chini* in Hindi and *kayu manis* in Malaysia (Thomas and Deuthi, 2001).

The major component of EOs in cinnamon bark is cinnamaldehyde (Figure 2.1), which comprises 65-80% of the bark plant (Rao and Gan, 2014). In addition, cinnamon bark also contains 5-10% of eugenol. The cinnamon leaves part consist of 1-5% cinnamaldehyde and 70-95% eugenol. These two compounds are responsible for their spicy-sweet aroma (Rao and Gan, 2014) and act as effective compounds against bacteria and moulds (Tajkarimi et al., 2010). Cinnamon EO is one of the most popular EOs and was recognized as safe by the Food and Drug Administration (FDA) (Valková et al., 2022).

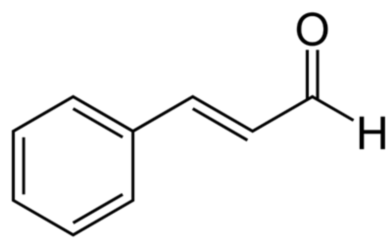

Figure 2.1 Chemical structure of cinnamaldehyde

2.4.2 Cloves

Cloves are dried flower buds of *Syzygium aromaticum* in the family of Myrtaceae (Raghavan, 2007). The name 'clove' is derived from several words; Latin clavus, Spanish clavo, French clou, and German nelke which means 'nail' due to its resemblance to a nail's shape (Raghavan, 2007). The flower buds are picked before opening and sun-dried. After drying, the colour of the cloves turns dark brown with slightly light brown content. Cloves have a strong bitter and woody flavour and release a spicy-sweet aroma.

The volatile oil of clove buds ranges from 5% to 20%. Eugenol, eugenyl acetate, and βcaryophyllene are the main components of the oil. The volatile oil has a significant amount of eugenol, which has biological and antimicrobial properties (Leela and Sapna, 2008). Eugenol volatile oils may inhibit the growth of microorganisms such as *A. niger*, *Saccharomyces cerevisiae*, *Mycoderma* sp., *Lactobacillus acidophilus*, and *Bacillus cereus* to varying degrees (Leela and Sapna, 2008; Meena and Sethi, 1994). The eugenol chemical structure is shown in Figure 2.2 (Kamatou et al., 2012).