Faculty of Resource Science and Technology

Screening of Medicinal Plants against *Aspergillus flavus*

Rammiya A/P Rajasegaran (71367)

Bachelor of Science with Honours

(Resource Biotechnology)

2022

Screening of Medicinal Plants against *Aspergillus flavus*

Rammiya A/P Rajasegaran

A thesis submitted in partial fulfilment of the Requirement of The Degree Bachelor of Science with Honours

(Resource Biotechnology)

SUPERVISOR: DR. ROSMAWATI BT SAAT

Programme of Resource Biotechnology
Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2022

UNIVERSITI MALAYSIA SARAWAK

Grade: ___________

Please tick (√)

Final Year Project Report	√
Masters	
PhD	

DECLARATION OF ORIGINAL WORK

This declaration is made on the 15th day of June 2022.

Student's Declaration:

I Rammiya A/P Rajasegaran, 71367, Faculty of Resource Science and Technology (PLEASE INDICATE STUDENT'S NAME, MATRIC NO. AND FACULTY) hereby declare that the work entitled, Screening of Medicinal Plants against *Aspergillus flavus* is my original work. I have not copied from any other students' work or from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.

15 June 2022
Date submitted

Rammiya A/P Rajasegaran (71367)
Name of the student (Matric No.)

Supervisor's Declaration:

I, Dr Rosmawati Bt Saat (SUPERVISOR'S NAME), hereby certify that the work entitled, Screening of Medicinal Plants against *Aspergillus flavus* (TITLE) was prepared by the above named student, and was submitted to the "FACULTY" as a *partial fulfillment for the conferment of Bachelor of Science with Honours (Resource Biotechnology) (PLEASE INDICATE THE DEGREE), and the aforementioned work, to the best of my knowledge, is the said student's work

Received for examination by: Dr. Rosmawati Bt Saat (Name of the supervisor)   Date: 15 June 2022

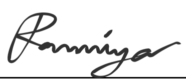
I declare this Project/Thesis is classified as (Please tick (√)):

- [ ] **CONFIDENTIAL** (Contains confidential information under the Official Secret Act 1972)
- [ ] *** RESTRICTED** (Contains restricted information as specified by the organisation where research was done) *****
- [√] **OPEN ACCESS**

Validation of Project/Thesis

I therefore duly affirmed with free consent and willingness declared that this said Project/Thesis shall be placed officially in the Centre for Academic Information Services with the abide interest and rights as follows:

- This Project/Thesis is the sole legal property of Universiti Malaysia Sarawak (UNIMAS).
- The Centre for Academic Information Services has the lawful right to make copies for the purpose of academic and research only and not for other purpose.
- The Centre for Academic Information Services has the lawful right to digitise the content to for the Local Content Database.
- The Centre for Academic Information Services has the lawful right to make copies of the Project/Thesis for academic exchange between Higher Learning Institute.
- No dispute or any claim shall arise from the student himself / herself neither third party on this Project/Thesis once it becomes sole property of UNIMAS.
- This Project/Thesis or any material, data and information related to it shall not be distributed, published or disclosed to any party by the student except with UNIMAS permission.

Student's signature:  (15 June 2022)

Supervisor's signature:  (15 June 2022)

Current Address:

Universiti of Malaysia Sarawak (UNIMAS), Jalan Datuk Mohammad Musa,

94300 Kota Samarahan, Sarawak

Notes: * If the Project/Thesis is **CONFIDENTIAL** or **RESTRICTED**, please attach together as annexure a letter from the organisation with the period and reasons of confidentiality and restriction.

[The instrument was duly prepared by The Centre for Academic Information Services]

ACKNOWLEDGEMENT

First and foremost, all the praises and thanks to God, the Giver of plentiful blessings and opportunities. I am grateful for giving myself strength in both mentally and physically throughout my life and during conducting my research.

Furthermore, I would like to express my sincere gratitude and appreciation to my supervisor, Dr. Rosmawati binti Saat, Lecturer, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak for her continuous support, guidance and encouragement. It is a great honour to work under her supervision. I really appreciate all her contribution regardless the time and ideas she provided to me.

I would like to express my gratitude to my parents, Mr. Rajasegaran Ramasamy and Mrs. Rathika Ramanaidu. The reason why I am very motivated and actively participating in my research is because of their support, continuous love and care.

I also would like to give my gratitude and appreciation to my laboratory postgraduate student, Alexandra Cheryl Anak Dennis and my best friend, Soonali Wasdewa for discussions, suggestions and took care of me during the research period.

Last but not least, I would like to thank all the staffs of Faculty of Resource Science and Technology, Universiti Malaysia Sarawak for all the things that facilitated my research.

Screening of Medicinal Plants against *Aspergillus flavus*

Rammiya A/P Rajasegaran

Resource Biotechnology Programme
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Aspergillus flavus (*A. flavus*) is a pathogenic fungus that proliferates majorly in agricultural crops like corns, nuts, and cotton seeds. Aflatoxin B1 released by *A. flavus* causes economical loss to developing countries as well as creating dreadful health issues to animals and human beings. Researchers have found chemical preservatives and fungicides to hinder the growth of *A. flavus*, but the usage is not only toxic to fungus, but is also hazardous towards humans, animals and the environment. Therefore, the aim of this study is to screen the selected medicinal plants against the fungus in order to identify a suitable disinfectant that can produce stronger antifungal properties in small quantity and also safe. Disc diffusion method was used to determine the effectiveness of the medicinal plant extracts which were rhizomes of turmeric (*C. longa*) and buds of clove (*S. aromaticum*) against *A. flavus* and to evaluate the minimum inhibitory concentration of the medicinal plants against the fungus. The highest concentration (100 mg/mL) had the highest inhibition of fungal growth while lowest concentration (25 mg/mL) had the lowest of inhibition percentage. Eventhough the highest inhibition zone formed by medicinal plant extracts against fungal growth is the concentration of 100 mg/mL, the minimum inhibitory concentration is 25 mg/mL because there is presence of inhibition zone formed by both medicinal plants against the fungus. The colour changes in phytochemical tests were used to indicate the presence of specific phytochemicals such as flavonoid and saponin compounds in both medicinal plant extracts.
Keywords: *Aspergillus flavus*, *Curcuma longa*, *Syzygium aromaticum*, disc diffusion method, phytochemicals

ABSTRAK

Aspergillus flavus (A. flavus) *merupakan satu kulat patogen yang membiak dalam tumbuh-tumbuhan pertanian seperti jagung, kacang, dan biji benih pokok kapas. Pelepasan aflatoksin B1 menyebabkan kerugian dalam ekonomi negara-negara maju dan mewujudkan masalah kesihatan terhadap manusia dan haiwan. Para-para penyelidik telah menemui pengawet kimia dan racun kulat untuk mengelakkan pertumbuhan* Aspergillus flavus, *tetapi penggunaan bahan kimia tersebut bukan sahaja toksik kepada kulat, malah juga berbahaya kepada manusia, haiwan, dan persekitaran. Oleh itu, matlamat kajian ini adalah untuk melaksanakan saringan kepada tumbuhan ubatan terhadap kulat untuk mengenalpasti pembasmi kuman yang boleh menghasilkan sifat antikulat yang lebih sesuai dan kuat dalam kuantiti yang kecil dan juga selamat untuk digunakan. Difusi cakera telah digunakan untuk menentukan keberkesanan ekstrak tumbuhan ubatan iaitu akar kunyit (*C. longa*) dan putik cengkih (*S. aromaticum*) terhadap pertumbuhan kulat dan untuk menilai kepekatan perencatan minima tumbuh-tumbuhan ubatan terhadap kulat. Kepekatan yang tertinggi (100 mg/mL) mempunyai perencatan pertumbuhan kulat yang tertinggi manakala kepekatan yang terendah (25 mg/mL) mempunyai peratus perencatan yang paling rendah. Walaupun zon perencatan yang tertinggi dibentuk oleh ekstrak tumbuh-tumbuhan ubatan terhadap pertumbuhan kulat ialah kepekatan 100 mg/mL, kepekatan perencatan minima ialah 25 mg/mL kerana terdapat zon perencatan yang dibentuk oleh kedua-dua tumbuh-tumbuhan ubatan terhadap kulat. Perubahan warna dalam ujian fitokimia telah digunakan untuk menunjukkan kehadiran fitokimia tertentu seperti flavonoid dan saponin dalam ekstrak tumbuhan ubatan.*
Kata Kunci: Aspergillus flavus, Curcuma longa, Syzygium aromaticum, *kaedah difusi cakera, fitokimia*

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A. flavus	*Aspergillus flavus*
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFM1	Aflatoxin M1
C. longa	*Curcuma longa*
C8	Carbon 8
C9	Carbon 9
DMIs	Demethylation inhibitors
DMSO	Dimethyl sulfoxide
EOs	Essential oils
HCl	Hydrochloric acid
MIC	Minimum inhibitory concentration
NaOH	Sodium chloride
PDA	Potato dextrose agar
PP	Phenyl-pyrroles
S. aromaticum	*Syzygium aromaticum*
SBIs	Sterol biosynthesis inhibitors
SDHIs	Succinate dehydrogenase inhibitors

CHAPTER 1

INTRODUCTION

1.1 Research Background

Aspergillus flavus (*A. flavus*) is a saprophytic soil fungus that can be found mostly in warm soils, and decomposing materials like dead plants and animals. This fungus has been the most common species that causes contamination to stored agricultural productions such as maize seeds, peanuts, cottonseeds, and rice grains (Ramsdam et al., 2021).

A. flavus spreads abundance of spores that contain secondary metabolite known as aflatoxins in hot and dry weather conditions with the help of wind and insects towards plants, animals and humans. The production of aflatoxins is highly toxic to humans, animals and the environment whereby it is correlated to spoilage of food in tropical and subtropical countries, poor quality and the quantity of food and feed material, as well as the development of various risks and diseases towards animals and humans. Researchers have found possibilities to inhibit the growth of *Aspergillus* species on stored food grains using chemical preservatives but this usage has associated to several health conditions towards consumers (Masiello et al., 2019).

Nevertheless, the growth of *Aspergillus* species can be controlled using essential oils of medicinal plants because of the bioactive compounds that contain antifungal properties (Nazzaro et al., 2017). Medicinal plants are high volatile, transient as well as biodegradable in nature that can be used as disinfectant with less residual of toxicity in stored agricultural production. Besides that, medicinal plants are enriched with phytochemicals like flavonoids, saponins, tannins, alkaloids, phenols, steroids, carotenoids, and other secondary metabolites that play an important role in preventive activities such as antifungal, antimicrobial, antiparasitic, anti-inflammatory, and antioxidant. These plants release countless of bioactive compounds to the environment to maintain its normal growth and development as well as the environment (Saxena et al., 2017).

Based on Njoki et al. (2017), six medicinal plants namely *Ocimum lamiifolium* leaves, *Prunus africana* leaves, *Solanum aculeastrum* fruits, *Lippia kituiensis* leaves, *Syzygium cordatum* bark and *Spinacia oleracea* leaves were prepared into organic and aqueous plant extracts to be used for the determination of antifungal activity against the conidia of *A. flavus*. In their research, the presence of bioactive compounds in organic plant extracts expressed stronger antifungal activity compared to aqueous plant extracts due to the respective compounds dissolved better in organic extracts than in aqueous extracts. Moreover, antifungal activity towards *A. flavus* is directly proportional to the plant extract concentrations whereby the larger the inhibition zone form by the fungus, the stronger the plant extract concentration (Njoki et al., 2017).

Aflatoxins B_1 and B_2 produced by *A. flavus* in agricultural crops causes less productivity and poor quality of crop sets which gives no economic value. Humans and livestock exposure to contaminated food materials can generate serious health problems like liver cancer and haemorrhage. As a result, researchers have created chemical preservatives and fungicides to inhibit the proliferation of *A. flavus*. However, despite the fungicides being toxic to the fungi, the fungicides are also hazardous to humans, animals and the environment due to the presence of inorganic compounds. To prevent marketing loss, it is important to identify the way to inhibit the growth of fungus using disinfectant that produces less residual of toxicity.

The determination of the effectiveness and the evaluation of minimum inhibitory concentration of medicinal plant extracts against *A. flavus* was done using a disc diffusion method. This method was used to allow better research on the identification of specific medicinal plant that provides stronger antifungal properties in small quantity that can be used on agricultural production and better treatment towards inhibition of fungal disease.

1.2 Objectives

The objectives of this study were:

i) To analyse and determine the effectiveness of medicinal plants (*C. longa, and S. aromaticum*) in resisting the growth of *Aspergillus flavus*.

ii) To evaluate the minimum inhibitory concentration of plant extracts against *Aspergillus flavus.*

iii) To detect the presence of phytochemical properties in medicinal plants.

CHAPTER 2

LITERATURE REVIEW

2.1 Properties of *Aspergillus flavus*

Moulds are known as the multicellular fungi that exists in various ecological niches and *Aspergillus flavus* is one of them. This pathogen mostly survives in tropical and subtropical regions with temperatures of about 28 °C to 37 °C and with 95 % of relative humidity. The life cycle of *A. flavus* has two ways which are asexual and sexual stages. In the asexual stage, the numerous conidia were produced and dispersed in the form of spores to the environment either by wind or by insects. In the sexual stage, the conidia were germinated into long thin filaments known as hyphae that will branch to form mycelia which eventually produce conidiophores to release more of conidia as shown in Figure 2.1 (Luis et al., 2020).

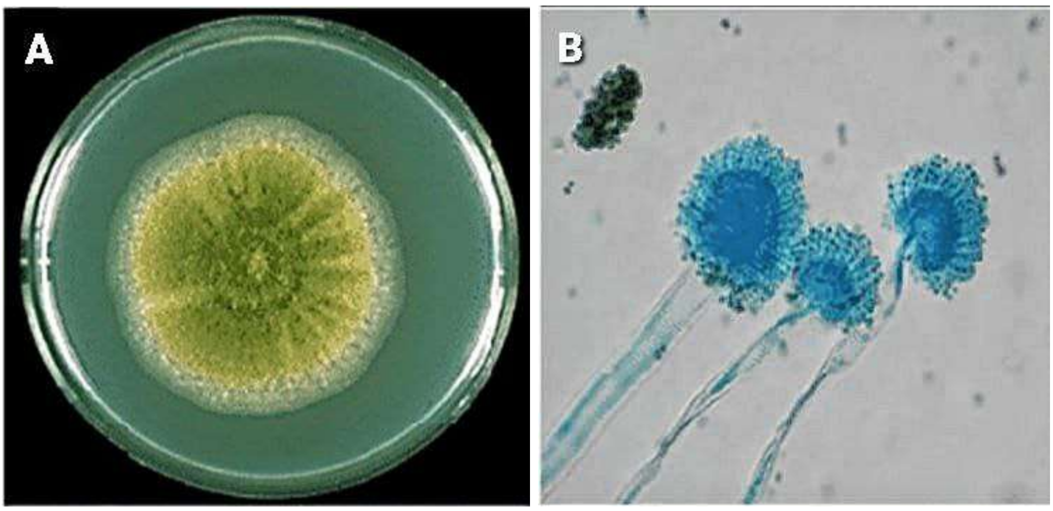


Figure 2.1. *A. flavus* and its conidia. A: *A. flavus* on a PDA plate. B: Microscopic view of conidia of *A. flavus* (Adapted from Rozaliyani et al., 2021).

A. flavus is commonly found proliferating and growing in stored food products like corns, nuts, legumes and other agricultural products. This fungus synthesises and produces abundance of spores containing secondary metabolites like aflatoxin B1 and B2, nitropropionic acid, aspergillic acid, kojic acid and aflam like other fungi. These metabolites functions as a virulent factor during signalling communication, adjustments to environment that favor their existence and for pathogenicity (Okayo et al., 2020). Aflatoxin B1 (AFB1) is the strongest and most commonly produced toxin in any contamination compared to aflatoxin B2 (AFB2) due to the distinct double bond in carbon 8 (C8) and carbon (C9) of the cyclic ring. As a consequence, the heterocyclic compound able to form epoxide, thus, producing more toxic effect compared to AFB2. AFB1 able to spread everywhere in the environment such as air, soil and water and has medium potential that is comparable for bioconcentration in both animal adipose tissue and in aquatic organisms whereby the aflatoxin can easily be absorbed into the stomach and intestines to transmit its toxins as well as can easily hydrolyse in water condition (Lalah et al., 2019).

2.2 Transmission of aflatoxins from plants to humans

The production of aflatoxins by *A. flavus* during agricultural production, pre-harvesting and post-harvesting, storage, and processing of food and feed has become an unavoidable situation towards food contamination. This is because the environment factors like drought stress and temperature favors the production of inoculum such as the capability of microorganisms, insects and wind disperse the spores to the environment and affecting other plants, humans and animals through inhalation, ingestion, or absorption as shown in Figure 2.2 (Khoury et al., 2017). For example, the spores from the soil are transmitted to the surface of the kernels by wind or by insects. The spores can be transmitted via four ways which are either transporting the primary inoculum to the kernels of the corn, moving the inoculum from the silks of corn to the kernels, disseminating the inoculum within the kernels or through facilitating colonisation and infection of the kernels via injuries (Pfliegler et al., 2021).

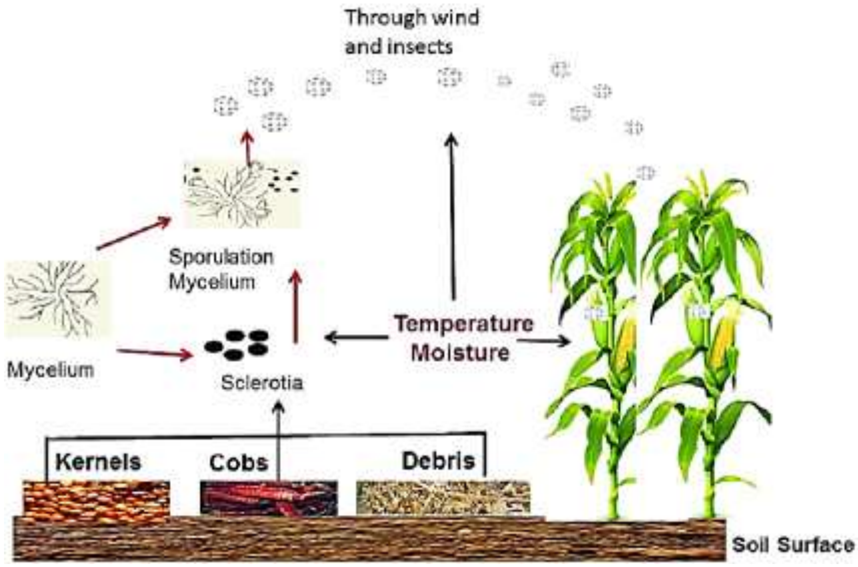


Figure 2.2. The schematic diagram of the transmission route of *A. flavus* spores to the kernels (Adapted from Saleem et al., 2017).

The spreading of *A. flavus* begins from the plants whereby plants are directly in contact with soils that are the main natural habitat of this fungus as shown in Figure 2.3. *A. flavus* not only colonise the injured seeds of the plants, but also invades crop seeds. An example of a plant that most commonly gets invaded is corn. According to Pfliegler et al. (2020), the fungus was mostly found growing in the kernels of corn. These pathogens output the production of inoculum and assisting the fungi to increase the colonisation, thus, accumulates aflatoxins.

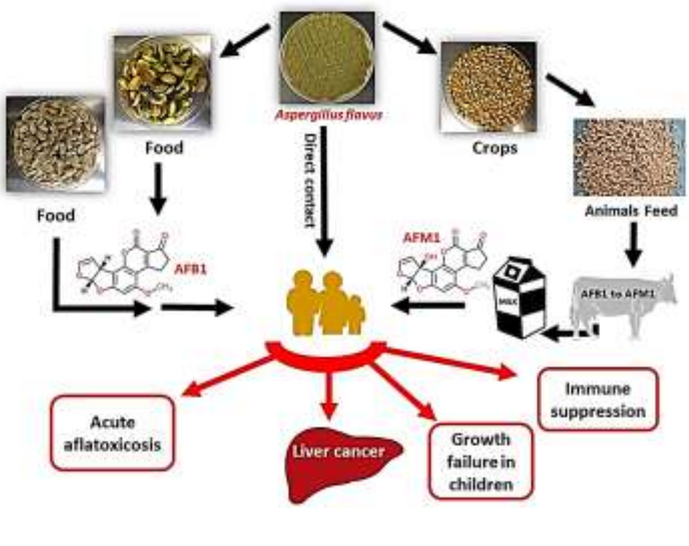


Figure 2.3. The transmission pathway of aflatoxins from *A. flavus* to humans through contamination or exposure routes (Adapted from Alshannaq et al., 2018).

However, without proper agronomic practice to harvest the agricultural crops such as ignoring the usage of fungicide, using hybrid crops that are resistant to fungal infections, or using biocontrol agents such as atoxigenic strains of *A. flavus*, the feeds are given directly to poultry animals like cows. The propagation of toxins in cows are transferred to the products it produces like milks. This processed food is then sold to consumers as an indirect transmission of aflatoxin via consumption of food. Besides that, the transmission of aflatoxin to humans can also be transmitted through the contaminated food itself. Consumption of stored agricultural crops like legumes, nuts, and corns that are contaminated with aflatoxin can also affect the humans' health. Humans and animals can be affected through inhalation of fungal spores that can lead to aspergillosis disease, especially to people who have weakened immune systems or lung diseases and causing problems like respiratory infection, and food poisoning to animals (Sepahvand et al., 2016).

2.3 Chemical reagents used as fungicide and its disadvantages

According to Masiello et al. (2019), the usage of hybrids and biological control of corn borer has contributed to the reduction of the fungus and the toxin contamination. Strategies of biological control have become essential in decreasing and inhibiting mycotoxigenic fungi and its aflatoxin production in the field (Abdallah et al., 2018). Nowadays, chemical control towards the fungal pathogens can be used via different target site fungicides that can cause intolerance towards their mode of action. Succinate dehydrogenase inhibitors (SDHIs), phenyl-pyrroles (PP), sterol biosynthesis inhibitors (SBIs) and demethylation inhibitors (DMIs) fungicides are mostly used as target site fungicides. These fungicides and chemical preservatives can help to lengthen the lifespan of the agricultural crops and secure the safety of food products (Masiello et al., 2019).

However, using acute toxicity of chemical preservatives and fungicides against *A. flavus* can cause long term health effects to humans like irritation to skin, and eyes when in contact, and throat when the spray mist is inhaled. The fungicides are also lethal to animals. According to Ferrigo et al. (2019), the European Parliament and Council has not authorised the usage of fungicides in maize. This is because there is a possibility for commercial fungicide containing prothioconazole and tebuconazole to act against the aflatoxigenic *Aspergillus* at the same time to develop a direct control for more than one pathogenic fungus (Ferrigo et al., 2019).

2.4 Introduction of medicinal plants

Plants produces natural products such as essential oils (Eos) that contain secondary metabolites like flavonoids, tannins, saponins, alkaloids and polysaccharides in various part of the plants has given much attention to researchers mainly due to the presence of antimicrobial, antifungal, antitumor, and anti-inflammatory properties as shown in Table 2.1 (Xi et al., 2018). Eos are known as a complex mixture of high volatile compounds that are extracted from plants and these essential oils are characterised as the low molecular weight of aromatic and aliphatic compounds. The benefits of using antifungal agent have shown the progression of resistance towards drugs. Because of many researchers found strains containing multidrug-resistant in fungus as well as the reduction of drug production allows the researchers to discover more new classes of antifungal properties from the plants including medicinal plants. According to Kumar Mishra et al. (2020), medicinal plants have been used as treatment to human and animal mycoses in traditional systems and this has been considered as one of the most valuable sources for new antifungal drug discovery.

Table 2.1. List of 8 botanical plants that have antifungal activity against toxigenic fungi (Adapted from Mishra et al., 2020)

No.	Botanical name	Family	Parts used	Chemical classes	Microorganism tested
1.	*Eugenia uniflora*	Myrtaceae	Leaves	Sesquiterpenes, Monoterpene, hydrocarbons	*C. albicans, C. dubliniensis, C. glabrata, C. krusci*
2.	*Psidium guajava*	Myrtaceae	Leaves	Methanolic extract	*C. albicans, C. dubliniensis, C. glabrata, C. krusci*
3.	*Curcuma longa*	Zingiberaceae	Rhizome	Turmeric oil	*C. albicans, C. dubliniensis, C. glabrata, C. krusci*
4.	*Schinus terebinthifolius*	Anacardiaceae	Stem bark	Extract	*C. albicans, C. dubliniensis*
5.	*Persea americana*	Lauraceae	Leaves	Chromene	*C. albicans, C. dubliniensis, C. glabrata, C. krusci*
6.	*Parapiptadenia rigida*	Fabaceae	Stem bark	Pyrrolidine amide	*C. albicans*
7.	*Ajania fruticulose*	Asteraceae	Fruits	Guaianolides	*Candida albicans, C. glabrata, A. fumigatus*
8.	*Alibertia macrophylla*	Rubiaceae	Leaves	Extract	*Cladosporium sphacrospermum; C. cladosporioides, A. niger, Colletotrichum glocosporioides*

The bioactive phytochemicals in plants are known as secondary metabolites that are present in abundance gives great chance for livestock improvement, including the diets provided to them. These phytochemicals provided by medicinal plants can be accepted as an alternative source for new and improvised development in antifungal therapy whereby this field is able to give humans and the environment a better treatment towards inhibition of fungal diseases (Kumar Mishra et al., 2020).

2.4.1 *Curcuma longa*

Curcuma longa commonly known as turmeric is one of the members from Zingiberaceae family and have been producing vastly in India and China over 2500 years in medicinal history. The rhizome of *C. longa* has been used as a flavoring and seasoning in food products, as well as pharmacological agents in cosmetics and pharmaceutical industries. This medicinal plant has given much attention to researchers due to the release of many chemical compounds that gives therapeutic properties like antifungal, antioxidant, antimicrobial, antiviral, and antimalarial properties (Chen et al., 2018). An antifungal efficacy of *C. longa* essential oil has been studied against *A. flavus* in both *in vivo* and *in vitro* and researchers have reported that the EO has ability to control the mycelial growth of fungus (Hu et al., 2017).

2.4.2 *Syzygium aromaticum*

Syzygium aromaticum also known as clove, is a dried flower bud that are originated from Maluku islands in Indonesia. These clove buds will be collected in the pre-flowering stage and will be used commercially for medicinal and perfume industry purposes. According to Batiha et al. (2020), clove-derived compounds contain strong antimicrobial and antioxidant properties and has been reviewed as one of the spices that can be used as preservatives in meat processing. The chemical composition and antifungal properties of *S. aromaticum* have been studied against several fungi species that causes contamination towards stored food products and researchers reported that low concentration of essential oil of *S. aromaticum* have been effective against *Aspergillus niger* and *Aspergillus carbonarius* (Sokamte et al., 2016).

2.5 Understanding of phytochemicals and its benefits

Phytochemicals are known as the chemicals which preserves the plant species from microorganisms like fungi, bacteria and viruses. These phytochemicals consist of many compounds that where largely used to give useful affects to humans' health such as vitamin C, folic acid, beta-carotene and vitamin E. Flavonoids are one of the polyphenols that can be found largely among the plants. This phytochemical has benzene rings in its chemical structure that are commonly used as antioxidant, anti-inflammatory, antibacterial, antifungal and as the free radical scavengers to regulate the cellular activity in human body, as well as promote the plant growth in unfavorable conditions. Besides flavonoids, saponins can be found in abundance in plant species as well. These phytochemicals are known to be a specific amphipathic glycoside that produces soap-like foaming which benefits in lowering the saturated fat in humans' body thus, reducing hypertension disease. Saponins also have anticancer, and anti-inflammatory properties that are benefit to human's health (Asao & Asaduzzaman, 2018).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Fresh turmeric rhizomes (*C. longa*), fresh clove buds (*S. aromaticum*), knife, weighing scale, oven, refrigerator, electric blender, sieve conical flasks, schott bottles, 96 % ethanol, shaker, beakers, filter papers, rotary evaporator with round bottomed flask, amber vial, 2 % of dimethyl sulfoxide (DMSO), sterile distilled water, micropipettes and micropipette tips, PDA powder, autoclave, *Aspergillus flavus* from Molecular Biology lab, 6 mm sterile paper discs, laminar flow hood, petri dishes, sterile spatula, sterile forceps, Falcon tubes and test tube rack, 10 % of sodium hydroxide (NaOH), magnetic stirrer, parafilm, ruler