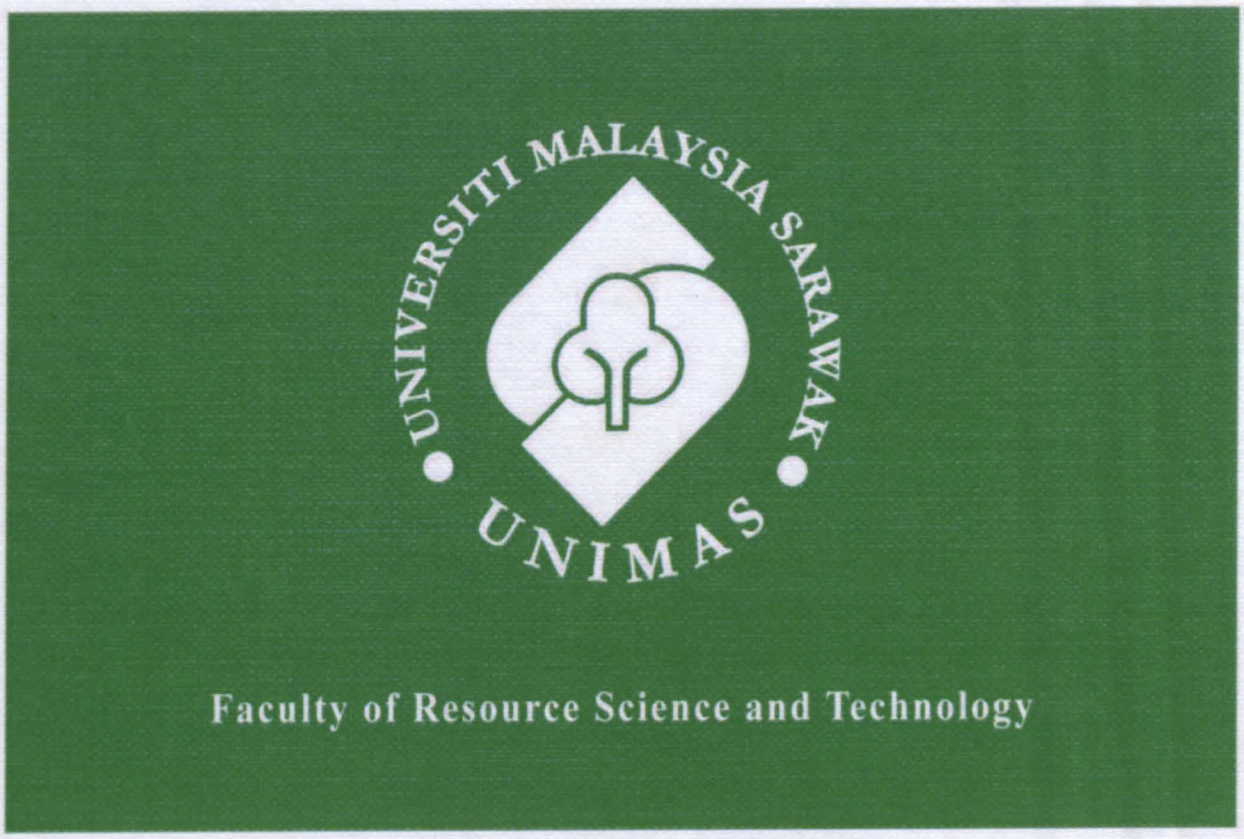

SCREENING OF LOCAL HERBS AGAINST *Aspergillus flavus*

Shubashini a/p Baktha Vachil
(55036)

Bachelor of Science with Honours
(Resource Biotechnology)
2018

UNIVERSITI MALAYSIA SARAWAK

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Final Year Project Report ☑

Masters ☐

PhD ☐

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This declaration is made on the 10 day of JUNE year 2018

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Screening of Local Herbs against *Aspergillus flavus*.

SHUBASHINI A/P BAKTHA VACHIL (55036)

A project is submitted in partial fulfilment of the

Final Year Project 2 (STF 3015) course

Supervisor: Miss Rosmawati Bt Saat

Co-Supervisor: Dr Razip Bin Asaruddin

Resource Biotechnology

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

2018

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I hereby declare that the study entitled "Screening of Local Herbs against *Aspergillus flavus*" submitted to the Faculty of Resource Science and Technology, University Malaysia Sarawak (UNIMAS) is my original work. All sources that I have quoted and cited have been acknowledged to the authors who own the original work of the papers. Acknowledging and giving credits to the owners have been made by means of complete references in this paper.

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Shubashini a/p Baktha Vachil (55036)

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University Malaysia Sarawak.

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Screening of Local Herbs against *Aspergillus Flavus.*

SHUBASHINI A/P BAKTHA VACHIL (55036)

Resource Biotechnology

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak.

ABSTRACT

This study focused on screening of local herbs against *Aspergillus flavus.* Three plant extracts were used in this study. They were *Carica papaya* (papaya), *Piper betle* (betel) and *Hibiscus rosasinensis* (hibiscus) leaves. Methanol extracts leaves of these three plants was screened for their antifungal activity against *A. flavus.* For antifungal assay, poisoned food technique and disc diffusion before or after growth of *A. flavus* was applied. Different concentrations (50, 75 and 100 mg/ml) were prepared from the plant extracts. *C. papaya* leaf showed the highest percentage of inhibition of fungi growth at 100 mg/ml of methanol leaf extract followed by 75 and 100 mg/ml of *P. betle* leaf and 50mg/ml of *H. rosasinensis* leaf extract. In disc diffusion before the growth of *A. flavus*, the zone of inhibition was not formed in all concentrations of the methanol leaf extracts. For disc diffusion after growth of *A. flavus* there was partial zone of inhibition at 100mg/ml of *C. papaya* leaf, 75 and 100 mg/ml for *P. betle* leaf and for *H. rosasinensis* leaf was at 50 mg/ml of leaf extract. Using this poisoned food technique, the different concentrations of methanol leaf extracts have shown inhibition against *A. flavus* and for disc diffusion after growth of *A. flavus* have shown partial zones of inhibition. The highest percentage of inhibition for antifungal activity was observed in poisoned food technique.

Key words: *Aspergillus flavus,* poisoned food technique, Methanol extract and Disc diffusion.

ABSTRAK

Kajian ini memberi tumpuan kepada pengujian kesan herba tempatan terhadap Aspergillus flavus. *Tiga ekstrak tumbuhan digunakan dalam kajian ini. Mereka adalah daun betik* (Carica papaya), *sirih* (Piper betle) *dan daun bunga raya* (Hibiscus rosasinensis). *Ekstrak daun metanol ketiga- tiga tumbuhan ini telah disaring untuk aktiviti antikulat mereka terhadap* A. flavus. *Untuk ujian antikulat, teknik makanan beracun dan penyebaran cakera sebelum atau selepas pertumbuhan* A. flavus *digunakan. Kepekatan yang berbeza (50, 75 dan 100 mg/ml) disediakan dari ekstrak tumbuhan. Bagi daun betik,* A. flavus *memperlihatkan perencatan tertinggi pertumbuhan kulat pada 100 mg/ml ekstrak daun metanol diikuti oleh 75 dan 100 mg / ml ekstrak daun sirih dan bagi daun bunga raya pada 50 mg/ml ekstrak daun. Penyebaran cakera sebelum pertumbuhan* A. flavus, *zon perencatan tidak terbentuk dalam semua kepekatan daun ekstrak metanol. Bagi penyebaran cakera selepas pertumbuhan* A. flavus *terdapat zon sekatan parsial pada 100 mg/ml daun betik, 75 dan 100 mg/ml untuk daun sirih dan daun bunga raya pada 50 mg/ml. Menggunakan teknik makanan beracun ini, kepekatan ekstrak daun methanol yang berbeza telah menunjukkan penghambatan terhadap* A. flavus *dan untuk penyebaran cakera selepas pertumbuhan* A. flavus *telah menunjukkan zon parsial separa. Peratusan tertinggi perencatan aktiviti antikulat diperhatikan dalam teknik makanan beracun.*

Kata kunci: Aspergillus flavus, *Aktiviti antikulat, Teknik makanan beracun, Ekstrak methanol dan Teknik penyebaran cakera.*

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LIST OF ABBREVIATIONS

A. flavus	*Aspergillus flavus*
C. papaya	*Carica papaya L.*
H. rosa-sinesis	*Hibiscus rosa-sinesis L.*
P. betle	*Piper betle L.*
µl	Microlitre
cm	Centimetre
DMSO	Dimethyl sulfoxide
°C	Degree celcius
%	Percentage
G	Grams
ml	Millilitre
Hr	Hour
PDA	Potato Dextrose Agar
mm	Millimetre
mg/ml	Miligram per mililitre

1.0 INTRODUCTION

Over the past years, *Aspergillus* infections have been increasing tremendously in human and animals (Pema, 2006). *Aspergillus* is a mold or fungi. It can cause aspergillosis. These fungi present in outdoors and indoors. If the person's immune system is weak then they could get sinus or lung infection where then it will spread to several parts of the body but if their immune system is at a strong condition, then *Aspergillus* will not harm the person. *Aspergillus fumigatus* is the genus which is the pervasive species that causes *Aspergillus* infection. Other species that is very common are *Aspergillus flavus*, *Aspergillus terreus* and *Aspergillus niger*. However, *A. flavus* is generally present more in the air compared to *A. fumigatus* which present in the environment (Hedayati *et al.*, 2007). *A. flavus* is the most ubiquitous species of fungi and is highly aerobic in oxygen rich areas and grow as molds. They usually grow in the trees as well as plants and contaminates the starchy foods like breads and tapiocas. *A. flavus* is the main producer of Aflatoxin B_1, the most effective naturally occurring carcinogen which is associated with some pathologies and usually targets the livers (El Khoury *et al.*, 2017).

Local herbs are usually used in household for fragrance, flavouring, spices, aroma and herbal teas. Herbs are referred as medicinal plants due to the complexity and the uses of active ingredients (Singab, 2015). Herbs are also used as cancer fighting agents (Singab, 2015). The natural bioactive compounds that are found in herbs, medicinal plants and flower leaves like alkaloids, tannins, saponins, flavonoids, steroids and glycosides may act as defence and protection system against diseases (Nagarajan *et al.*, 2016). The presence of bioactive compounds in the plants affects the fungal agents which causes it to decompose easily (Balamurugan, 2014). Apart from that, local herbs have the capability to function as

antifungal and antimicrobial due to the phenolic and flavonoids compounds in the leaves (Klich, 2007).

In developing countries, many people are dependent on traditional practitioners of medicinal plants to meet their health care needs (Sanchez, 2015). Although, conventional medicines are still available in market nevertheless herbal medicines are maintained in the popularity due to their historical, personal attitude, philosophy and cultural reasons (Ohemu *et al.*, 2017). The contents of the herbs are safer compared to synthetic drugs without causing any side effects while enriched with nutrients and minerals which the body requires (Li, 2000). *A. flavus* can contaminate wide range of cereals and crops such as nuts, garlic and maize. According to Zain (2011), *A. flavus* has been reported to contaminate the product and produce aflatoxin which are mutagenic and carcinogenic. The extracts from the local herbs may offer an alternative way to protect the food or crops from contamination of the fungi (Vratnica, 2016).

Local herbs used in this study were *Carica papaya* (papaya), *Piper betle* (betel) and *Hibiscus rosasinesis* (hibiscus) leaves. This study attempted to screen the antifungal compounds against *A. flavus*. The fungal growth inhibition to different concentrations of plant extracts enabled to determine the antifungal activity of the *Carica papaya* (papaya), *Piper betle* (betel) and *Hibiscus rosasinensis* (hibiscus) leaves.

Therefore, the objectives of this study are,

i. To determine the antifungal properties of the local herbs using methanol extraction.
ii. To determine antifungal activity of the herbs against *A. flavus*.
iii. To investigate the minimal fungicidal concentration of the local herbs against *A. flavus*.

2.0 LITERATURE REVIEW

2.1 *Aspergillus flavus*

Aspergillus flavus is a saprophytic soil fungus that contaminates the crops together with Aflatoxin B_1, cancer causing agent. It is the second most intrusive aspergillosis after *A. fumigatus* (Augustina *et al.*, 2016). It grows by creating branching filaments which is known as hypae. The network of hypae which is known to be mycelium breaks down complex foods by secreting enzymes (Scheidegger, 2003). *A. flavus* is mostly reported as food-borne fungus as it has the capability to decay the seeds, crops and the vegetation like maize and rice (Fakruddin *et al.*, 2015). Its colony characteristics on potato dextrose agar (PDA) media are granular, velvety, flat, yellow at first then quickly changes to green, may have white border and at the lower surface will be reddish gold (Nyongesa *et al.*, 2015). Their conidiophores arise separately from the substratum which is 0.5 to 1.5 mm long and usually rough with heavy walls and colourless (Akare *et al.*, 2016). The rate of growth of *A. flavus* is also rapid where it would mature in three days. This fungi grows best at an optimum temperature of 37°C. In high temperature of the plant storage or in excessive moisture environment, there will be an increase in the production of aflatoxin in the *A. flavus* and causes cancer among the animals and the human beings (Villers, 2014). *A. flavus* absorb nutrients from the host plant via hydrolytic activity where the enzymes like amylase, pectinase and cellulase will be utilised. The pathogenicity of *A. flavus* is caused by these hydrolytic enzymes (Jiujiang *et al.*, 2005).

2.2 Aflatoxin

Aflatoxins are metabolites that produced by some species of molds which grow on the food (Obesity, 2015). *A. flavus, A. parasiticus, A. nomius, A. tamari and A. bombycis* produces aflatoxin and they are toxic to both humans and animals. There are several types of aflatoxin but only four major types of aflatoxins that causes disease in humans which are Blue 1 (B1), Blue 2 (B2), Green 1 (G1) and Green 2 (G2) (Thanaboripat, 2011). Continuous intake of food contaminated with aflatoxin causes the development of liver cancer due to the accumulation of aflatoxin in body. The effect of aflatoxins on health of human, the antifungal and natural plant extract provides an alternative way to prevent fungal contamination of food (Thanaboripat, 2011). Aflatoxins are normally found in peanuts, corn, rice, tree nuts, dried fruit and spices and stored under damped condition (Obesity, 2015). Extract and powders of various herbs have been reported to consists of antimicrobial activity against aflatoxin producing fungi and some of them inhibits the aflatoxin formation (Thanaboripat, 2011). The mycotoxigenic fungi will be reduced if plant products like plant extracts were consumed.

2.3 Local Herbs

Local herbs have been used by humans as food for many generation mainly to treat illnesses. There is a scientific evidence saying that accumulation of herbs contains medicinal properties which helps to prevent diseases (Lai *et al.,* 2014). The plants and the secondary metabolites constituent are usually used in traditional herbal medicines (Li, 2000). The secondary metabolites like alkaloids, flavonoids, saponins and tannins can protect the plants from the fungi (Njoki *et al.,* 2017). The plant extract activity from herbs on fungi has been studied in large number of researches in different parts of the world (Akbar, 2015). According to World Health Organization (2002), about 80% of the African and Asian

depends on traditional herbal medicine that are mainly used for primary health care. Herbal products are often cheaper and safer compared to modern medicines (Ekor, 2014).

2.4 *Carica Papaya* L.

Carica papaya is known as papaya or paw-paw that belongs to the family of *Caricaceae* and its exact source is from tropical America have been originated from Southern Mexico and Central America (Senthilkumaran, 2014). Normally, *C. papaya* would be small, has green broad leaf which are shiny, succulent, has non-woody trunk, unbranched and is an agroforestry species (Yogiraj *et al.*, 2014). The plant is very sensitive to strong winds and requires high temperature to produce a quality fruits while the humidity must be at medium to low level. They do have flowers too where it comes in a fleshy with creamy coloured and nice fragrance. The papaya fruit will be green when unripe and once riped it becomes yellow or reddish orange. The raw fruit has low calories and rich in vitamin A, B, C, minerals and calcium (Aravind *et al.*, 2013). Their young leaves can be cooked and are eaten raw in some areas in Africa. *C. papaya* has many medicinal uses and pharmacological action with crude extract of the parts of papaya plant to treat various diseases (Boshra *et al.*, 2013).

Figure 1: *Carica papaya tree*
(Source: https://www.amazon.de/RARIT%C3%84T-Papaya-Baum-verschiedene-100-120cm/dp/B075KCRYZL).

2.4.1 Application of *Carica papaya* Leaf

Carica papaya leaf have been reported to contain large amount of active compounds where the antioxidant in blood can increase and the lipid peroxidation will be reduced (Bergonio *et al.*, 2016). They are also contain antibacterial, digestive, laxatives and analgesics properties. The leaf contains tannins which help the intestines from reinfarction by the intestinal worms (Bauri *et al.*, 2015). Papaya leaves also consist of phenolic compound. According to Romasi (2011), the phenolic compound in papaya leaf has antimicrobial activity which was reported to inhibit the growth of *Rhizopus Stolonifer*, a saphrophytic fungi.

2.5 *Piper betle* L.

Piper betle are available in heart shaped, glossy and deep green in colour. For centuries, *P. betle* are used in Indian and Chinese traditional medicine and categorised in the genus of *Piper* and *Piperaceae* family (Dwivedi, 2014). They are small and climbs, fast growing, perennial and usually grows on larger trees. It is found in forest in India and in South East

Asia like China and Vietnam (Dwivedi, 2014). *P. betle* is safe to be consumed for stress, hepatotoxicity and hematotoxicity. The secondary metabolites in *P. betle* leaf like alkaloids, flavonoid and phenolic compounds were reported with several pharmacologic activities and these causes it to be used for antimicrobial, antioxidant, anti-inflammatory, anticancer antispasmodic and antioxidant activities (Zahira, 2016).

Figure 2: *Piper betle*
(Source: https://www.srilankanspices.com/sl_spices_betel.html).

2.5.1 Applications of *Piper betle* leaf.

Piper betle leaf is very important in ancient culture. The tradition of betel leaf chewing after meal by Indians was practiced before 75 and 300 AD which is described in the book of ancients like Ayurveda, Sushruta and Samhitas (Toprani, 2013). It is still practiced in India. The leaves are often used for human life on the culture, social and its significance during the marriages and ceremonies. Besides that, betel leaves play an important role in human health. However, in South East Asia, *P. betle* control the periodontal diseases. The crude extract of the *P. betle* showed antibacterial activity towards the *Streptococcus sanguis*, *Streptococcus*

mitis and *Actinomyces viscosus* which are the dental plaque early colonizer (Fathilah, 2011). According to Patra *et al* (2016), there is review saying that the leaves of *P. betle* contains healing power. The leaf extract would help in treating gastric ulcers and treat warts and make the scars not visible. Besides that, the leaf extract can be added with honey to treat the phlegm and cough. There are no side effects shown by consuming the extract of betel leaves. (Dinesh *et al.*, 2016).

2.6 *Hibiscus rosasinensis* L.

Hibiscus rosasinensis has medicinal value. It is also an ancient medicine and has beneficial effects to human body. It is originated from Asia and Tropical Africa (Daudu *et al.,* 2015). It is a Chinese rose and belongs to the family of *Malvaceae*.Their flower comes in many colours which are pink, orange, red , yellow and white and in trumpet shape. Their leaves and flower have numerous action including antibacterial, antipyretic, antioxidant, anti-inflammatory and antifungal effect (Sobhy *et al.*, 2017). Hibiscus flower comes in many types which are *H. amottianus*, *H. mutabilis*, *H. tiliaceus* and *H. hirtis*. Their flowers are used as demulcent and emollient that can be mixed with coconut water to be used for labor purposes. The roots are consumed mainly as demulcent and for coughs and the leaves are used as emollient and laxatives (Punasiya, 2014).

Figure 3: *Hibiscus Rosasinenesis*
(Source: http://blog.nurserylive.com/2016/10/26/hibiscus-a-natural-skin-care-plant-and-gardening-in-india).

2.6.1 Applications of *Hibiscus rosasinesis* Leaf

Hibiscus rosasinensis is a shrub. The leaves are glossy green, oval in shape and usually large in size. The measurement of the leaf is between 7 and 15 inches long. According to Pharmacognosy Department of ISF Pharmacy College, it is said that fresh hibiscus leaf are high in moisture. The leaves have been investigated and proven to contain several classes of secondary metabolites like flavonoids, terpenoids, steroids, alkaloids and phenols (Divya, 2013). According to Vasudeva (2008), flavonoids and phenolic are the main classes of substance in *H. rosasinensis* leaves. These compounds consist of antibacterial, antioxidative, antifungal, anti-inflammatory and antifertility activity. Leaves of *H. rosasinensis* also enhances the growth of hair and heals cancer (Mondal *et al.* 2016). Besides that, its leaves has shown an inhibitory effect on the microorganisms called *Staphylococcus aureus* which causes staph infections (Foundation, 2004).

2.7 Screening of Antifungal Activity

The leaf extract of the plants have been investigated for antifungal activities.There are some studies showed that the leaves of some plants have an amazing fungicidal activities than other parts of a plant (Dellavalle *et al.*, 2011). Various method for antifungal activity for instance poisoned food techniques and agar disc diffusion was carried out (Gakuubi, 2017).

2.7.1 Poisoned food technique method

Antifungal activity in particular plant extracts can be evaluated using the poisoned food technique. This technique is used to determine the inhibitory efficacy of mycelial growth that shows the ability of the fungi to grow on the media (Gakuubi, 2017). The diameter of colonies of the test fungi in poisoned food plates should be less than the colony diameter in control plates as this shows the antifungal potential of the plant extracts (Balamurugan, 2014). The results of antifungal screening on different plant extract will show different antifungal extent against *A. flavus* (Kumar, 2013).

2.7.2 Agar disc diffusion method

Agar disc diffusion method was first developed in 1950's by W. Kirby and A. Bauer (College, 2011). This diffusion is suitable for polar samples, the samples that easily diffuse into the agar and may penetrate into the agar even in minute amount (Scorzoni *et al.*, 2007). Agar disc diffusion is used in many clinical microbiology laboratory for daily test of antimicrobial testing. There are many advantages of this method which is simple, low cost, have the ability to test large number of microorganisms and easy to intepret the results provided (Balouiri *et al.*, 2016). In this method, the discs contain the solution of substance to be tested. The occurrence of inhibitory area surrounding the disc determines the antifungal efficacy. When there is no growth surrounding the disc, it is called as a zone of inhibition (

College, 2011). The zone of inhibition is measured using ruler and recorded in centimetre (cm). Agar disc diffusion method is not suitable to determine minimum inhibitory concentration (MIC). This is because, it is impossible to quantify the amount of antimicrobial agents diffused in the agar (Balouiri *et al.*, 2016).

2.8 Methanol Extraction

Extraction is the separation of medicinally active portions of plant tissue by using selective solvents in standard extraction procedures (Handa *et al.*, 2008). The purpose of the extraction is to separate the soluble plant metabolites while the products obtained from plants are in liquid, semisolids or powder forms. The initial crude extract obtained from extraction contains complex mixture of plant metabolites like alkaloids, phenolics, flavonoids and terpenoids. The identified compounds from plants are aromatic or saturated organic compounds and usually obtained via ethanol or methanol extraction. Furthermore, the solvents used for investigation of antimicrobial activity in plants are ethanol, methanol and water (Das *et al.*, 2010). More bioactive flavonoids are found in ethanol 70% and it is highly polar than pure ethanol. Ethanol can easily penetrate in the cellular membrane to extract the ingredients from plant materials. Methanol with boiling point of 64.7 °C is used often compared to ethanol due to the high boiling point, 78.4 °C (Version, 2011). The methanol solvent then evaporated in rotary evaporator and the product to be extract will be less damaged than the ethanol extract. Methanol is more polar than ethanol because of its cytotoxic nature and unsuitable in the kind of studies where incorrect results will be produced (Tiwari, 2011).