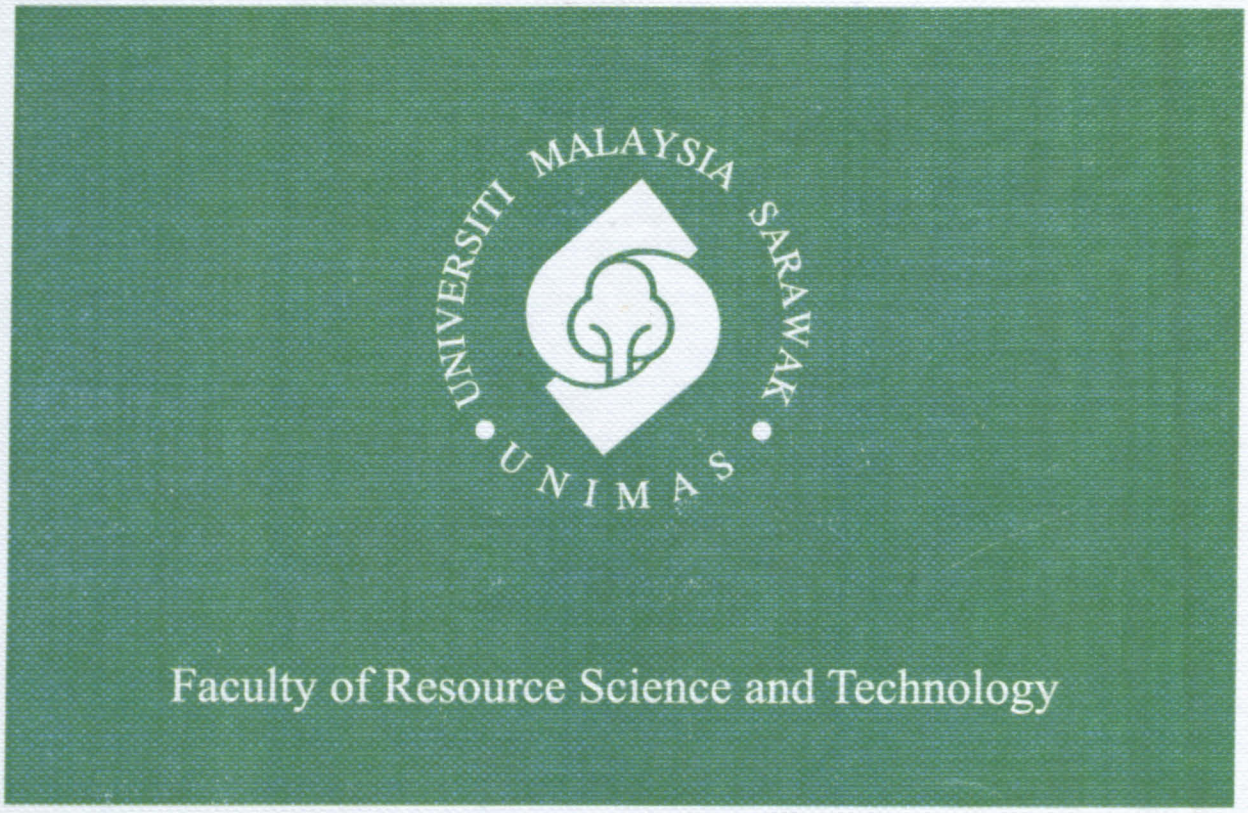

Screening of Local Herbs Against *A. flavus*

Zulhafeez Bin Hasbollah

Bachelor of Science with Honours
(Resource Biotechnology)
2017

UNIVERSITI MALAYSIA SARAWAK

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Alhamdulillah, I am able to complete my thesis and project successfully. This thesis cannot be completed without support from other people help and guidance.

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LIST OF ABBREVIATIONS

Aspergillus flavus	*A. flavus*
Antimicrobial Resistance	AMR
Potato Dextrose Agar	PDA
AmB	Amphotericin B
Species	*Spp.*
µg	microgram

Screening of Local Herbs Against *A. flavus*

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ABSTRACT

Antifungal agents are one of the efficient categories of drugs that so far well developed for improving animal and human health. Due to the increasing rate of resistance of *Aspergillus flavus* to the commonly used antifungal agent, plant extracts are of new interest as antiseptics and antimicrobial agents in the field of medicine and agriculture.The antifungal agents made from local herbs have various advantages such as they are biodegradable in nature, do not cause effect on non target species, cost less and less toxic compared to the synthetic antifungal agents. Hence, this study aims to identify potential local herbs (*Pandanus amaryllifolius, Zingiber officinale* and *Allium sativum*) that can inhibit the growth of *A. flavus* and then obtain the crude extract from selected local herbs to screen for its antifungal activity against *A. flavus*. The screening of antifungal activity of selected local herbs crude extract in methanol against *A. flavus* was performed by agar dilution method. They had been tested against *A. flavus*. Result showed methanol crude extract of *Zingiber officinale* and *Pandanus amaryllifolius* at 1.2% concentration retard the mycelia growth on *A. flavus*. The methanol crude extract of *Allium sativum* did not showed growth retardation at any concentration used.

Keywords: antifungal activity, *A. flavus*, plant crude extract, local herbs

ABSTRAK

Ejen antikulat adalah salah satu kategori berkesan ubat yang setakat ini yang maju untuk meningkatkan haiwan dan kesihatan manusia. Oleh kerana peningkatan kadar rintangan Aspergillus flavus (A. flavus) kepada ejen antikulat biasa digunakan, ekstrak tumbuhan adalah kepentingan baru sebagai antiseptik dan ejen antimikrob dalam bidang perubatan. Ejen antikulat dibuat daripada herba mempunyai pelbagai kelebihan seperti mereka boleh dilupuskan dalam alam semula jadi, tidak menyebabkan kesan ke atas spesies bukan sasaran, pembuatan dengan kos yang rendah sekali gus kurang toksik berbanding dengan ejen antifungal sintetik.Oleh itu, kajian ini bertujuan untuk mengenalpasti herba yang dapat merencatkan pertumbuhan A. flavus sekaligus mendapatkan ekstrak mentah daripada herba tempatan yang dipilih (Pandanus amaryllifolius, Allium sativum dan Zingiber officinale) dan melakukan saringan aktiviti antikulat terhadap A. flavus. Tayangan aktiviti antikulat herba tempatan yang dipilih dalam ekstrak metanol mentah terhadap A. flavus menggunakan kaedah pencairan agar. Mereka akan diuji terhadap Aspergillus flavus. Keputusan menunjukkan ekstrak metanol mentah Zingiber officinale dan Pandanus amaryllifolius pada kepekatan 1.2% membantut pertumbuhan mycelia pada A. flavus. Ekstrak metanol mentah Allium sativum tidak menunjukkan sebarang perencatan tumbesaran bagi setiap kepekatan yang digunakan.

Kata kunci: aktiviti antikulat, A. flavus, ekstrak mentah tumbuhan, tumbuhan herba tempatan

1.0 INTRODUCTION

Aspergillus flavus in general has been in the public attention mainly due to the fact it is the second leading cause of chronic aspergillosis. Previous studies by Margaret *et. al* (2013) have shown that there is an increasing rate of Aspergillosis morbidity and in recent years. Inhalation of *Aspergillus* spores will cause Aspergillosis, that is a type fungal disease (Bansod & Rai, 2008). Not only that, in agriculture, fungi cause crop losses worldwide. Fungal diseases have a significant economic impact on plant yield and quality. For example, in the United States alone, the average economic loss from mycotoxins approximately $932 million (CAST, 2003). Specifically, *A. flavus* is able to cause diseases in economically important crops, such as maize and peanuts by contaminating it with mycotoxin known as aflatoxin (Hedayati *et. al*, 2007; Yu, Cleveland, *et. al*, 2005).

Although antifungal agents are crucial in treatment of fungal infection, the available antifungal drugs are limited mainly to the slow development of new antifungal agents. Hedayati *et. al* (2007) reported that there are only two widely used antifungal drugs in medicine to treat *Aspergillus sp.* infection which are amphotericin B and itraconazole. This unresolved problem has led to the lack of a concerted effort to the discovery of new antifungal agents in clinical area, thus, the quality of patient care is indirectly being affected (Daly & Kavanagh, 2001; Hedayati *et. al*, 2007).

The usage of synthetic fungicide in agriculture to eliminate *Aspergillus flavus* in crops will cause negative environmental and health-related effects (Soković *et. al*, 2013; Ziveai *et. al*, 2013). Several human pathogenic micro-organisms developed multiple drug resistance in due to indiscriminate use of commercial antimicrobial drugs and commonly used in the treatment of diseases (Khalili *et al.*, 2012). In addition, report done by cause resistance to azole fungicides used in agriculture. Recent research by Wiederhold & Patterson (2015) revealed that *Aspergillus flavus* have developed resistance to azole which is the commonly used antifungal agent.Therefore in such situations, the discovery to use of bioorganic fungicide with biodegradable as an alternative is currently needed to replace the commercial synthetic chemical fungicide (Al-Samarrai *et. al*, 2012;

Zahari & Halimoon, 2014). It is suggested the usage of natural products such as plant extract to be less damaging in order to control the pests and fungal diseases, and fungal diseases (Amrita & Richa, 2014). These views are consistent with those of Negri *et al.* (2014), when the report claimed that in general alongside the development of synthetic drugs, it is significant to focused on natural products with antifungal properties. In fact, major drug classes such amphotericin B, the gold standard and armamentarium, and thlipopeptide caspofungin that is widely used and approved as antifungal drugs in recent years, are derived from natural products (Vengurlekar *et. al*, 2012).

In this study, local herbs were studied for their antifungal properties against *A. flavus*. The local selected herbds were *Zingiber officinale, Pandanus amaryfolius* and *Allium sativum*. López-Muñoz *et. al* (2006), stated that the uses of medicinal herbs to prevent and cure diseases are increasing every year and their preventive use for the treatment of food borne pathogens is believed to be safer than synthetic antibiotics. Maceration is the method employed followed by using methanol as solvent together with using rotary evaporator to obtain the crude extracts from herbs. Then, the crude extract was diluted into several concentration together with the media. Lastly, antifungal assay of *A. flavus* was carried out using agar dilution method.

The objectives of this study were as follow. Firstly, to identify potential local herbs that can inhibit the growth of *A. flavus*. Secondly, to compare the effect of different concentration of crude extract from local herbs against *A. flavus*.

2.0 LITERATURE REVIEW

2.1 Medicinal Plants

Medicinal values of plants are important to mankind especially in the field of herbal medicine. Several international agencies are taking an interest to encourage the use and researches on medicinal plants in the developing countries (Yulia, 2005). Most of traditional medicine comes from medicinal plant, especially herbs. The knowledge of this medicinal plant had been passed from one generation to another generation.

Nowadays, the old system of herbal medicine is being revived by day-to-day practice for its long-lasting curative effect, easy availability, natural way of healing, and less side-effects, so that today herbal medicines are gaining importance and expanding throughout the world (Firenzuoli & Gori, 2007). The importance of medicinal plants is not limited only in traditional medicine but also modern medicine. Nijar (1996) reported that 74% of the 119 drugs available in the market today are discovered from a pool of traditional herbal medicine.

2.1.1 Plant Crude Extract

Plant crude extract contain bioactive compounds. Typically bioactive compounds of plants are produced as secondary metabolites (Bernhoft, 2010). Hussain *et al.* (2011) states that plant and fungal extracts and compounds contain physiologically active biochemicals have great potential for producing new agents of great benefit to mankind. For instance, systematic screening of secondary metabolites of folk herbs and fungi may result in the discovery of novel and effective antimicrobial compounds. One of the components in plant crude extract is flavonoid, which is also called as vitamin P, is one of the largest classes of naturally-occuring polyphenolic compounds. Flavonoids are only being synthesis in plant but not in animal.

There are several types of flavonoids which capable to show antibacterial activity. Those types are flavones, flavonols, flavanones, and isoflavones (Harborna & Williams, 2000). Next, the compound tannin, which is known also as tannic acid are water-soluble polyphenols that are

present in many plants. Tannins are found to inhibit the growth of many microorganisms such as fungi, yeasts, bacteria, and viruses (Chung *et al.,* 1998).

Furthermore, alkaloids are a large and structurally diverse group of natural products of microbial, plant and animal origin. Individual alkaloids are assigned names in various ways, but almost all names end with the letters "-ine". Alkaloids have commonly served as scaffolds for important antibacterial drugs such as metronidazole and the quinolones (Cushnie *et al.,* 2014). Not to mention bioactive compound saponins are glycosides found abundantly in plants (Cheeke, 1971) which are found to be to have various pharmacological activities such as antibiotic, antifungal, antiviral, hepatoprotective anti-inflammatory and anti-ulcer (Oakenfull and Fenwick, 1981).

2.1.2 Extraction of Plant Crude Extract

Sasidharan *et al.* (2010) described that the basic operation will include steps, such as pre-washing, drying of plant materials or freeze drying, grinding to obtain a homogenous sample and often improving the kinetics of analytic extraction and also increasing the contact of sample surface with the solvent system. Proper actions will be taken to assure that potential active constituents are not lost, distorted or destroyed during the preparation of the extract from plant samples. The selection of solvent is depending on the specific nature of the bioactive compound being targeted.

Different solvent systems are available to extract the bioactive compound from natural products. The extraction of hydrophilic compounds uses polar solvents such as methanol, ethanol and distilled water. According to Tiwari *et. al,* (2011) the plant parts of each medicinal plant (100g) will be soaked in ethanol,shaked in Erlenmeyer flasks, filtered, concentrated under reduced pressure, to obtain the crude extract. Existing classical techniques is used in order to obtain bioactive compounds from plants, are Soxhlet extraction, maceration and hydrodistillation. Maceration technique is used in homemade preparation of tonic due to its inexpensive way to obtain essential oils and bioactive compounds.

2.2 Description of local herbs

2.2.1 *Pandanus amaryllifolius*

Pandanus amaryllifolius or commonly known as "daun pandan" or "pandan wangi" is from the screw pine family. In Southeast Asia, it is used in culinary as an additive to give a pleasant fresh hay like aroma (Chong *et al.*, 2012). It is an evergreen perennial aromatic plant, a cultivated plant now found worldwide due to importation and human migration (Dumaoal *et. al*, 2010). According to Fatihanim *et al.* (2007) and Kumar *et al.* (2007) reported that pandan-derived compounds and fractions have shown potential antioxidant and also anticancer activities. The previous reported by (Tan *et al.*, 2008; Ooi *et al.*, 2005; Ooi *et al.*, 2004) also mentioned pandan-derived compounds and fractions have demonstrated selective antibacterial and antiviral activity. However, the antifungal properties of *Pandanus amaryllifolius* are not yet scientifically established.

2.2.2 *Zingiber officinale*

Zingiber officinale also known as ginger, is a medicinal plant that has been widely used in Chinese and Ayurvedic as herbal medicines all over the world, for a wide array of unrelated ailments that include arthritis, rheumatism, sprains, muscular aches, pains, sore throats, cramps, constipation, indigestion, vomiting, hypertension, dementia, fever, infectious diseases and helminthiasis. Recently, scientist are interested in ginger, and several scientific investigations have been done to isolate and identify the active constituents of ginger. *Zingiber officinale* have been reported to be effective against *Candida Albicans* (Supreetha *et al.*, 2011)

2.2.3 *Allium sativum*

Allium sativum also known as garlic belongs to the family *Alliaceae*. Its close relatives include the onion, shallot, and leek. It has been used throughout recorded history for both culinary and medicinal purposes. It has a characteristic pungent, hot, flavour that mellows and sweetens considerably with cooking. Garlic has been used as medicine in various cultures for many years, dating as far back as the time that the Egyptian pyramids. Traditional medicine claimed garlic

could prevent heart diseases including atherosclerosis, high cholesterol, high blood pressure, and to improve the immune system as well as protection against cancer (Benkeblia,2004). However, there is only few studies conducted to test *Allium sativum* extracts for its antifungal properties especially against *Aspergillus sp.*

2.3 *Aspergillus flavus*

There are only few fungi have had as broad an economic impact like *Aspergillus flavus* (*A. flavus*) because it is a pathogen of plants, animals and insects, and crops (Klich, 2007). *A. flavus* grows on a wide range of agricultural crops and foods and contaminates them through secretion of Aflatoxin (Ziveai *et al.,* 2013). The major is characteristics of *A. flavus* is the presence of distinctive spore-bearing structure, known as the aspergillum (Klich, 2007). *A. flavus* produces the highly regulated mycotoxin, Aflavatoxin B1. Not only that, *A. flavus* is second only to *Aspergillus fumigatus* to cause human invasive aspergillosis. Previously, only few drugs could treat *A. flavus* infection (Hedayati *et al.*, 2007).

2.4 Antifungal agents

In medicine, according to Dixon & Walsh (1996), an antifungal agent is a drug that selectively eliminates fungal pathogens from a host with minimal toxicity to the host. Antifungal drugs are divided into 4 main classes which are the polyenes, azoles, allylamines and echinocandins. The most widely used antifungal drugs are in the azole class. The azole class is further split into two types that are the imidazoles and also triazole (Sorrel, 2007). Regarding *Aspergillus flavus* infection, known as Aspergillosis the common antifungal drugs available used for treatment are amphotericin B (AmB) and itraconazole (Hedayati *et al.*, 2007).

Recent studies have been reported by Bell (2011) described that the triazoles play an important therapeutic role in the treatment of various fungal infectious diseases in children. This is because triazoles have good antifungal activity against many common fungal pathogens, and they are

available as oral dosage forms, including oral liquids. However, described that other commonly used antifungal drugs like Liposomal Amphotericin B (AmBisome) be associated with various negative effects in patients such as pulmonary toxicity and renal impairments (Cosgrove, 2016). Moreover, antimicrobial resistance (AMR) has attracted the attention of the medical profession and the society. Thus, more research have turned to the use of medicinal plants as alternative therapy for treating infectious diseases (Bansod & Rai, 2008) and the use of medicinal plants has increased due to their minimal side effects (Daly & Kavanagh, 2001).

Not only that, National Health Service (2016) states that commonly used antifungal medicine also have found to cause severe adverse reactions such as an allergic reaction like swelling of your face, neck or tongue or difficulty breathing and severe skin reaction such as peeling or blistering skin.
Next, in agriculture the antifungal agents are commonly known as fungicide. Fungicide is a specific type of pesticide that controls fungal disease by specifically inhibiting or killing the fungus causing the disease in plants. (McGrath, 2004). The use of synthetic fungicides offer great economic and social benefits because it protect and preserve the materials, food and also prevent diseases. The usage of the synthetic fungicide is a major way to control fungal disease in the world for the past decades and nowadays it plays a significant role in crop protection (Martínez, 2012). There is no doubt that the use of pesticides has large benefits to farmers, unfortunately the current usage of pesticides in agriculture can cause negative environmental and health-related effects to society (Soković *et al.*, 2013).

The antifungal agents also play an important role in the food industry. This is mainly due to fungal spoilage frequent occurrence. Fungal spoilage is known as the most common type of microbial spoilage in food that leads to significant economical and health problems throughout the world (Pawlowska, 2013). Many naturally occurring compounds, such as nisin, plant essential oils, and natamycin, have been widely studied and are reported to be effective in their potential role as antimicrobial agents against spoilage and pathogenic microorganisms.

Various natural compound have been widely studied and reported to be effective for food preservation. For instance, nisin, plant essential oils, and natamycin are some of the natural antimicrobials that have been applied in food processing. These natural compounds are commercially available but their efficacy, consumer acceptance and regulation are not well defined (Juneja *et. al*, 2012). Therefore, it is clear that screening of crude extracts from herbs for antifungal activity would assists in the findings of novel and effective new antifungal agents that are derrived from plants extracts.

3.0 MATERIALS AND METHODS

3.1 Local herbs sampling and preparation

All of the selected local herbs were collected from Kuching, Sarawak. The fresh leaves and stems of *Pandanus amaryllifolius*, rhizomes of *Zingiber officinale* and bulb of *Allium sativum* were used in this study. Each of the local herbs sample was carefully washed with sterilized distilled water, dried with a soft cloth, and stored in a bag before used and kept at 4 °C.

3.2 Culturing of *A. flavus*

The fungus, *A. flavus* was cultured from the stock fungal collection, Molecular Biology Lab, Faculty of Resources Science and Technology, UNIMAS. The fungus was grown on Potato Dextrose Agar (PDA) media containing 50 mg/ml ampicilin. The culture was incubated at room temperature for 7 days.

3.3 Preparation of Methanol Crude Extracts via Maceration

Based on Gahlaut et. al, (2013) the method started with, plant parts was crushed mechanically using electronic blender (Sharp EM-11) and then soaked with 99.9% methanol at room temperature in a conical flask for 3 days and was shaken periodically with the speed of 80-100 rpm using rotary shaker. This was supported by the standard protocol by Azmir, *et al.* (2013), whereby initial step in maceration techniques is to grind the plant materials into small particle is used to increase the surface area for proper mixing with solvent. After maceration process, the mixture were filtered using Whatman filter paper. Then, flitrates were evaporized using rotary evaporator at temperature ranges of 40 °C. to 50 °C. with reduced pressure to obtain the crude extracts. Percentage of pure crude extract was calculated using using the formula and concept of concentration dilution, M1V1 = M2V2. (Note: M1 and V1 are the initial concentration and volume

of the concentrated stock solution, and M2 and V2 are the final concentration and volume of the diluted solution that are wanted.

3.4 Antifungal assay

Antifungal activity of plant extracts against *A. flavus* was determined by fungal growth inhibition assay as described by Fiori *et al* (2000) with some modification. This method is also known as Agar dilution method. The crude extract obtained was dissolved and mixed with agar medium, molten Potato dextrose medium (PDA) to provide desired final concentration. After the agar solidified, mycelia plug from the 7-day-old culture of pre-prepared stock *A. flavus* was placed at the centre of Petri dish. This was done so that the mycelia plug would had direct contact with the crude extracts in the PDA.

After 7 days of incubation period, fungal growth inhibition level was observed for each concentration in 5 replicates. A negative control was set up by substituting the crude extract with fixed 5 ml of distilled water. Lastly, data of growth inhibition level of *A. flavus* were recorded and determined against the area of petri dish (aprroximately 38.49cm^2 using the formula, Area of petri dish = πr^2 where r is radius and π = 3.142)

4.0 RESULTS

4.1 Methanol crude extract from local herbs

Methanol crude extract of local herbs; *Pandanus amaryllifolius*, *Zingiber officinale* and *Allium sativum* was successfully obtained using 100% absolute methanol as solvent. The methanol crude extract obtained was diluted to 0.2%, 0.6% and 1.2%.

4.2 Antimicrobial activity of methanol crude extracts local herbs

Screening of the antifungal activity of methanol crude extract from local herbs; *Pandanus amaryllifolius*, *Zingiber officinale* and *Allium sativum* was performed against *A. flavus*. The results are summarized as in Table 1. Methanol crude extract of all local herbs at 0.2% and 0.6% concentration had no effect on the growth of *A. flavus*.

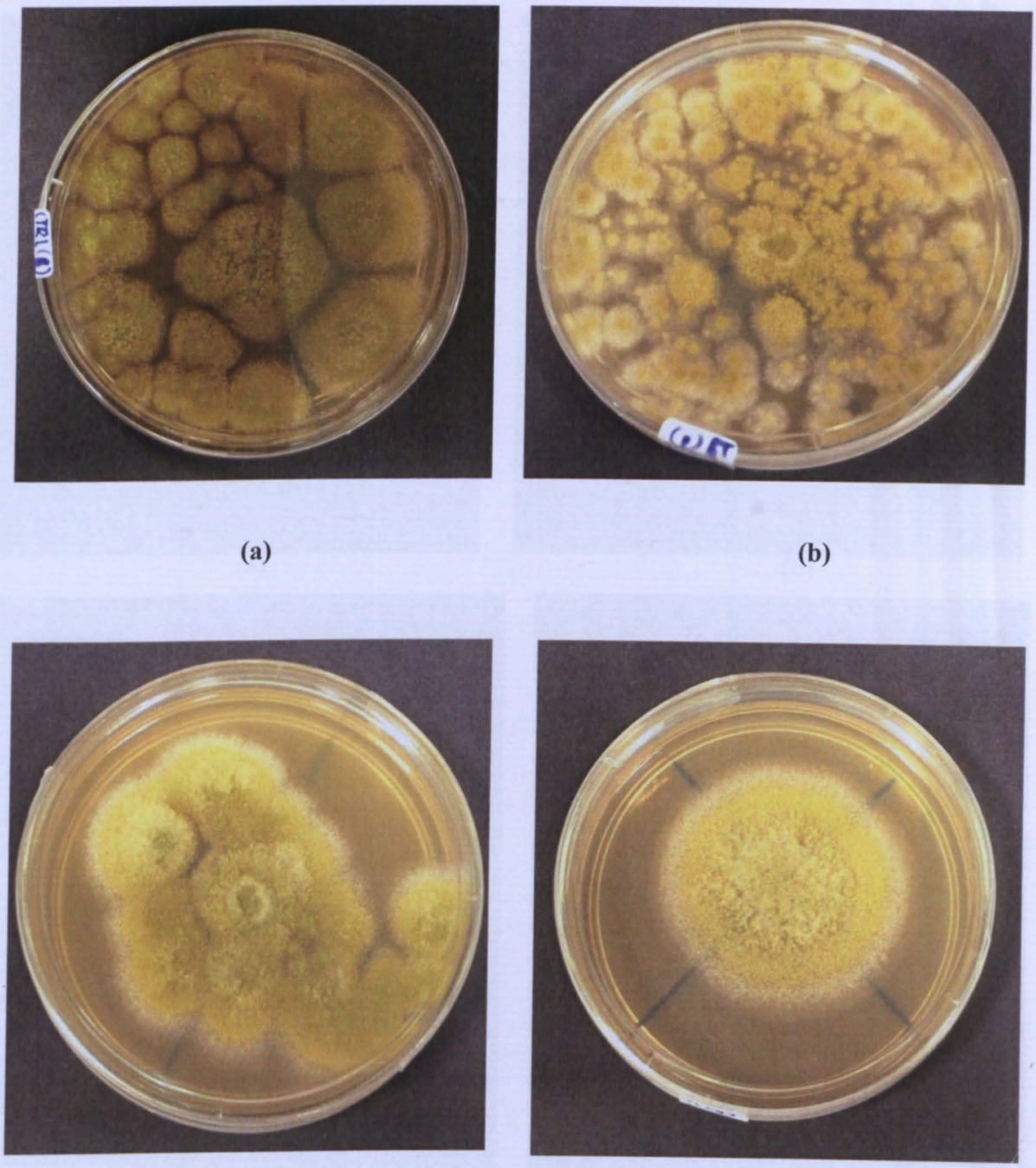
Interestingly, methanol crude extract at 1.2% concentration of *Pandanus amaryllifolius and Zingiber officinale*, showed significantly slow growth of *A. flavus* compared to their control medium.This indicates strong inhibition since the fungal growth only covers 25% area of petri dish. However, *A. flavus* was also affected by the methanol crude extract of *Allium sativum* at every concentration 0.2%, 0.6%, and 1.2% despite the presence cross contamination with bacteria. After thorough examination, this was regarded as false positive.

Table 1: Growth inhibition level of the *A. flavus* cultured on different concentrations of methanol crude extract from *local herbs; Pandanus amaryllifolius, Zingiber officinale, Allium sativum.*

Type of Local Herbs	Growth Inhibition Level			
	Control	0.2%	0.6%	1.2%
Pandanus amaryllifolius	–	–	–	+
Zingiber officinale	–	–	–	++
Allium sativum	–	–	–	–

Note: The inhibition level was reported as (–) for no inhibition: fungal growth covers approximately 70-100% of petri dish area, (+) for weak inhibition: fungal growth covers approximately 50% of petri dish area and (++) for strong inhibtion: fungal growth covers approximately 25% of petri dish area.
(number of replicates = 5, number of samples = 3)

Figure 1, Figure 2 and Figure 3 showed the antifungal assays of *Pandanus amaryllifolius* crude extract, *Zingiber officinale* and *Allium sativum* respectively. These crude extracts were the most significantly retarded in the mycelia growth of *A. flavus.*



(a) (b)

(c) (d)

Figure 1: Antifungal assays with crude extract *Zingiber officinale* at different concentration. a: control, b: 0.2 % concentration, c: 0.6% concentration, & d: 1.2% concentration

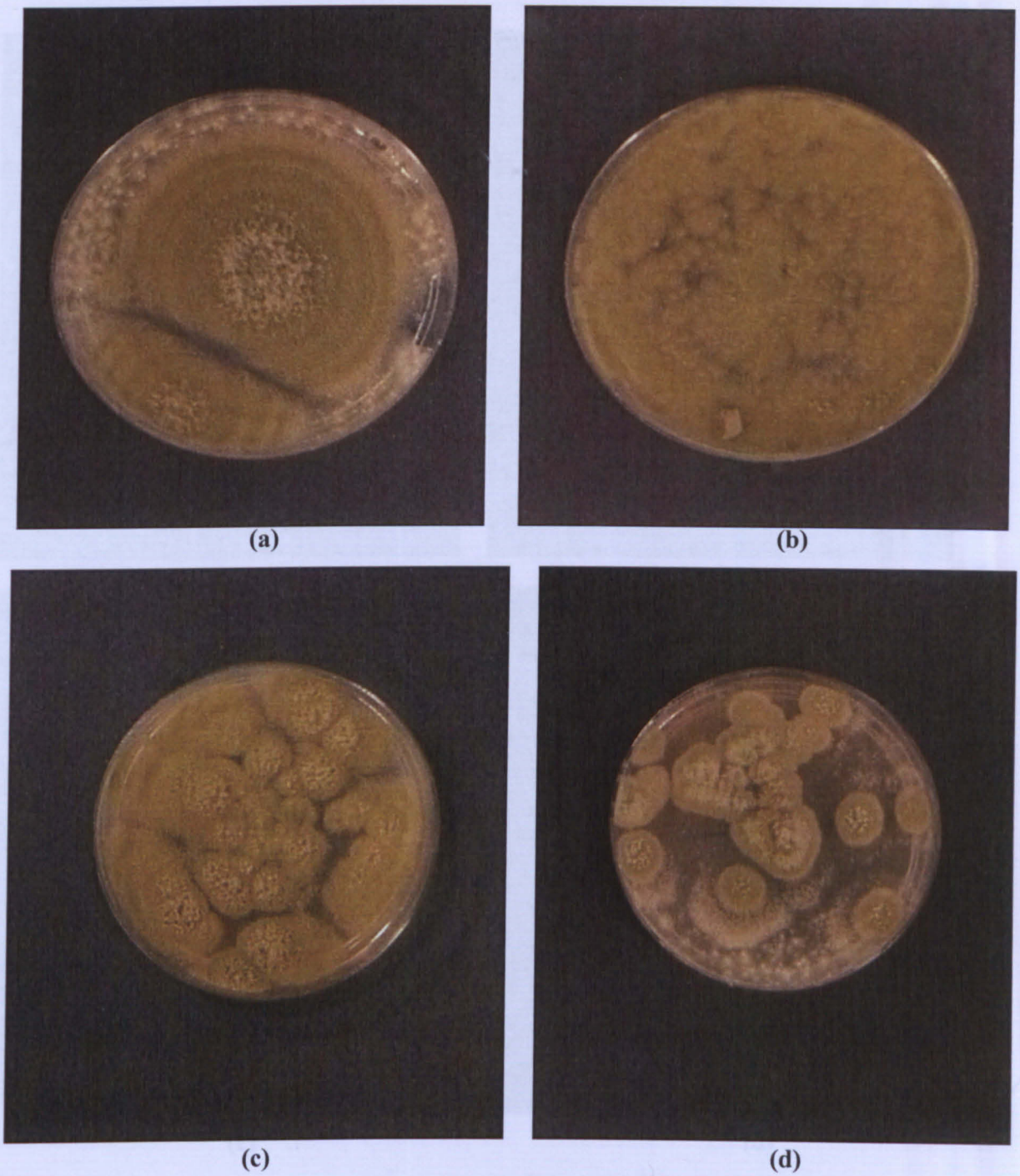


Figure 2: Antifungal assays with crude extract *Pandanus amaryllifolius* at different concentration. a: control, b: 0.2% concentration, c: 0.6% concentration, & d: 1.2% concentration