FRACTIONATION OF ANTIFUNGAL COMPOUNDS FROM

Mangifera pajang

By

TOH ZI XIN

A project report submitted to the Department of Chemical Science

Faculty of Science

Universiti Tunku Abdul Rahman

in partial fulfillment of the requirements for the degree of

Bachelor of Science (Hons.) Chemistry

May 2018

FRACTIONATION OF ANTIFUNGAL COMPOUNDS FROM

Mangifera pajang

ABSTRACT

Mangifera pajang is a plant species from Anacardiaceae family, which is the mango family. It can be found in the lowland rain forests of Borneo including Sabah, Sarawak, Brunei and Kalimantan. The fruits of this plant species are oval in shape and they have brown and coarse skin. The methanol extract of the kernel of *Mangifera pajang* was determined for its antifungal activities against two fungus species which are *Candida albicans* and *Candida parapsilosis.* By using normal phase column chromatography, the methanol extract was separated into 27 fractions and they were combined into 15 fractions after checking the purity of each fraction by normal phase Thin Layer Chromatgraphy (TLC). The fractions were tested for their minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) against *Candida albicans* and *Candida parapsilosis*. Fractions 8 and 9 showed the lowest MIC and MFC values with concentration at 0.0003-0.0006 mg/mL against *Candida parapsilosis.* For *Candida albicans*, fraction 7, 8 and 9 showed the lowest MIC and MFC values at 0.0013 mg/mL. Since fraction 8 and 9 showed strongest antifungal activity against both fungi, they were analyzed under HPLC by using gradient elution

method with acetonitrile and water. From the chromatogram, both fractions contain many minor and major peaks. The antifungal activity could be due to any components in these peaks.

ABSTRAK

Mangifera pajang adalah spesies tumbuhan dari keluarga *Anacardiaceae*, yang merupakan keluarga mangga. Ia boleh didapati di hutan hujan rendah Borneo termasuk Sabah, Sarawak, Brunei dan Kalimantan. Buah-buahan spesies tumbuhan ini berbentuk bujur dan mereka mempunyai kulit berwarna coklat dan kasar. Ekstrak metanol kernel *Mangifera pajang* ditentukan untuk aktiviti antikulatnya terhadap dua spesis kulat iaitu *Candida albicans* dan *Candida parapsilosis*. Dengan menggunakan kromatografi lajur fasa biasa, ekstrak metanol dipisahkan kepada 27 pecahan dan digabungkan ke dalam 15 pecahan selepas memeriksa kesucian setiap pecahan oleh fasa normal Thin Layer Chromatgraphy (TLC). Fraksi telah diuji untuk menentukan minimum inhibitory concentration (MIC) dan minimum fungicidal concentration (MFC) terhadap *Candida albicans* dan *Candida parapsilosis*. Kedua-dua pecahan 8 dan 9 menunjukkan nilai MIC dan MFC terendah dengan kepekatan 0.0003-0.0006 mg / mL terhadap *Candida parapsilosis.* Bagi *Candida albicans*, pecahan 7, 8 dan 9 menunjukkan nilai MIC dan MFC terendah pada 0.0013 mg / mL. Oleh kerana pecahan 8 dan 9 menunjukkan aktiviti antikulat terkuat terhadap kedua-dua kulat, mereka dianalisis di bawah HPLC dengan menggunakan kaedah elusi gradien dengan asetonitril dan air. Daripada kromatogram, kedua-dua fraksi mengandungi banyak puncak rendah dan tinggi. Kegiatan antikulat mungkin disebabkan oleh salah satu puncak dari puncak-puncak tersebut dan siasatan lanjut diperlukan.

ACKNOWLEDGEMENT

Firstly, I would like to express my sincere appreciation to my Final Year Project supervisor, Dr. Khoo Kong Soo, who has provided me with the information, guidance and given me the chance to carry out this project. Furthermore, I would also like to thank my co-supervisor, Dr. Sit Nam Weng, for guiding me to complete this project and provided me with any resources that I needed to proceed with my lab work for this project.

On the other hand, I would like to show my sincere gratitude to my senior, Ooi Jiaxin, who have given me many useful tips to do this project. I would also like to thank the postgraduate student of Dr. Sit Nam Weng, Kong Chi Kei who has guided me in carrying out the bioassay for this project. Moreover, I would like to thank all those lab officers of the Chemistry Department for helping me and teach me how to handle all those equipment and reagents.

Last but not least, I would also like to thank my family, friends and course mates for showing their support and patience for me to complete this project.

DECLARATION

I, Toh Zi Xin, hereby declare that this thesis prepared by me is based on my original work done for the project except for the quotations and citations mentioned in this thesis; which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

_____

(TOH ZI XIN)

APPROVAL SHEET

This project titled **"FRACTIONATION OF ANTIFUNGAL COMPOUNDS FROM *Mangifera pajang*"** was prepared by TOH ZI XIN and submitted as a partial fulfillment of the requirements for the degree of Bachelor of Science (Hons.) Chemistry at Universiti Tunku Abdul Rahman (UTAR).

Approved by:

_____

(Assoc. Prof. Dr. Khoo Kong Soo) Date: ……

Supervisor

Department of Chemical Science

Faculty of Science

Universiti Tunku Abdul Rahman

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: _____

PERMISSION SHEET

I hereby certify that **TOH ZI XIN** (ID No.: 14ADB01911) has completed this dissertation titled **"FRACTIONATION OF ANTIFUNGAL COMPOUNDS FROM *Mangifera pajang*"** under the supervision of Dr. Khoo Kong Soo (Supervisor) and Dr. Sit Nam Weng (Co-supervisor) from the Department of Chemical Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

_____

(TOH ZI XIN)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIOS

C. albicans	*Candida albicans*
C. parapsilosis	*Candida parapsilosis*
HPLC	High Performance Liquid Chromatography
MFC	Minimum Fungicidal Concentration
MIC	Minimum Inhibitory Concentration
M. pajang	*Mangifera pajang*
RPMI-1640	Roswell Park Memorial Institute- 1640
Spp.	Species
TLC	Thin-Layer Chromatography

Chapter 1

INTRODUCTION

1.1 Plant Metabolites

The human daily diet is dependent on plants because those plants constituents have nutritional value that is essential for human. These plant compounds such as carbohydrates, amino acids as well as lipids are synthesized by green plant through photosynthesis and they are known as primary metabolites which directly participate in growth, metabolism and development of plants.

Apart from primary metabolites, plants biosynthesize at least a million other compounds. Selective expression of these compounds has help scientists to group the plants into various chemotaxonomic groups. These compounds are known as secondary metabolites because they do not contribute directly to the primary functions of the plants. Secondary metabolites used to be considered as waste products from the plants but there is growing evidence that secondary metabolites can ensure the survival of a plant species that synthesize them, for example to protect the plants from harmful and pests or as a defense system. The examples of secondary metabolites are alkaloids, tannins and terpenoids (Badal and Delgoda, 2017).

1.2 Importance of Antifungal Properties of Plants

From 1950 to 1970, fungi were not considered as a serious pathogen to human because the annual death rate due to candidiasis was stable. However, from 1970 onwards, the death rate increased tremendously which raise a serious issue among our population. Invasive fungal infections has pose a severe threat to our health and they cause about 1.5 million deaths per year. One of the reason is due to the widespread use of the antifungal agents (Campoy and L. Adrio, 2017).

Amphotericin B was the only drug used to cure serious fungal diseases for nearly 30 years while imidazoles in late 1980s and triazoles in early 1990s were used to treat local and systemic fungal infection which are much safer and effective. However, the increased frequency of using these antifungal drugs has cause the fungus to develop resistance and immune to these antifungal agents. Thus, the prevalence of resistance to antifungal agents had risen significantly in the past decade (Tiwari and Mishra, 2011). Furthermore, some antifungal agents are harmful to our human body. For example, one of the side effect of amphotericin B is causing nephrotoxicity while azole antifungals can cause gastrointestinal, endocrinologic and hepatic disease (Teodoro et al., 2015).

It is very crucial to explore other new categories of antifungal drugs or agents to deal with these fungal infections. Recently, the study on natural products and their

derivatives has increased tremendously because those secondary metabolites from plants such as saponins, phenolic acids and flavonoids show significant antifungal activity. Furthermore, plants are relatively cheap biological material that consist various bioactive compounds with certain biological activities (Tiwari and Mishra, 2011). Thus, the studies and researches on antifungal properties of plants are important to discover the potential of the plant species to become an alternative for other currently known antifungal drugs.

1.3 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent that prevent the growth of a particular microorganism (Razin and Tully, 1996). MIC can only show us that the growth of the microorganism is inhibited at that particular concentration but it cannot tell whether the concentration has killed the microorganism or just stop them from growing further.

1.4 Minimum Fungicidal Concentration (MFC)

Minimum Fungicidal Concentration (MFC) is the lowest concentration of an antifungal agent that yield around 99 to 99.5 % of killing activity towards the

particular fungus species (Espinel-Ingroff et al., 2002). At this concentration, the fungus will be destroyed and they will not be able to grow anymore.

1.5 *Mangifera species*

1.5.1 Origin

The genus *Mangifera* belongs to the Anarcardiaceae family which consist of 73 genera and around 830 species. The main centre of diversity for *Mangifera* is located at tropical and subtropical Asia, which is from the equator to 27^{o}N latitude. The highest species diversity falls in Borneo, Sumatra and Malay Peninsula. The majority of the species in this genus are canopy and emergent trees of the tropical lowland rainforests. The examples of *Mangifera* species are *M.indica, M. bompardii, M. orophila, M. sylvatica, M. dongnaiensis* and etc (Litz, 2004).

1.5.2 Taxonomy

The classification of genus *Mangifera* is given below:

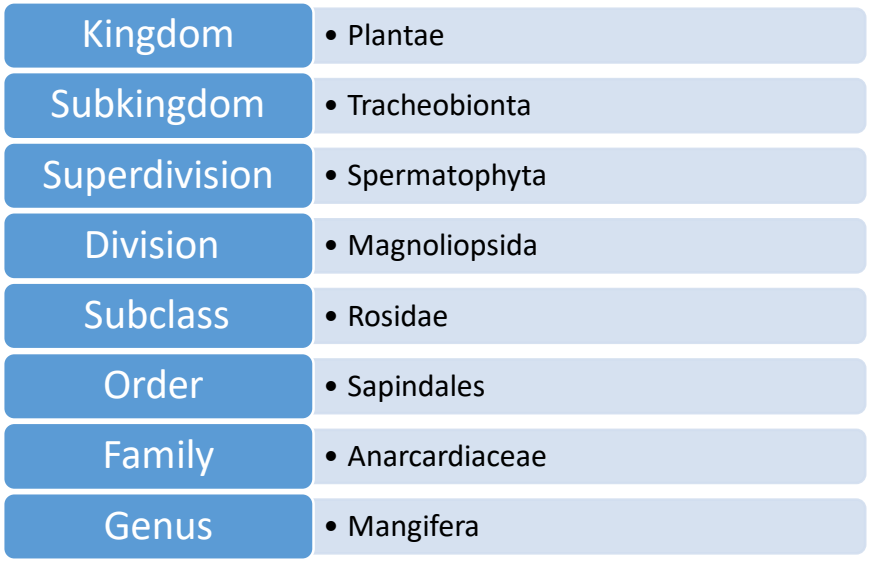


Figure 1.1: Taxonomical classification of *Mangifera* (Ediriweera, Tennekoon & Samarakoon, 2017).

1.5.3 Species of *Mangifera* and their pharmacological activities

Mangifera indica, which is commonly known as mango is grown in many countries around the world, especially tropical countries. It had been cultivated for at least 4000 years with more than 1000 varieties in cultivation. It occupied the second position as a tropical crop because this plant species is rich in micronutrients and macronutrients. *M. indica* has many medicinal properties such as anticancer, antipyretic, anti HIV, antibone resorption, antifungal, antibacterial, antithelmintic, anti-inflammatory, antidiarrheal, hypolipidemic and antidiabetic. This species is rich in provitamin A compound, alpha-carotene, lutein, beta-carotene, tannins, quercetin, kaempherol, caffeic acid, gallic acid, catechins,

mangiferin and other compounds that have huge potential to deal with those kind of diseases (Ediriweera, Tennekoon & Samarakoon, 2017).

Mangifera foetida is also known as "horse mango". It is found in Southeast Asia. The fruit peel of *Mangifera foetida* which is rich in gallic acid, protocatechuic acid and vanillic acid had showed antioxidant activity while the leaf extract of *M. foetida* demonstrated an antimicrobial activity against *S. aureus*. Another *Mangifera* species is *Mangifera sylvatica*. The pulp of *Mangifera sylvatica* contains mangiferin, quercetin, kaempherol, p-coumaric acid, gallic acid and ellagic acid. These compounds possess medicinal benefits such as anti-cancer, anti-allergic, anti-ulcer, neuroprotective, antimicrobial and antiviral (Khoo et at, 2016).

Extracts of *Mangifera zeylanica*, also known as Sri Lanka mango can initiate apoptosis in cancer cells of breast and ovary. Ediriweera, Tennekoon & Samarakoon (2017) reported that chloroform extract of bark which consists of chloromangiferamide and bromomangideric acid and chloroform extract of fruit peel and flesh of *M. zeylanica* which consists of linoleic acid and α-tocopherol showed anticancer effect.

1.6 *Mangifera pajang*

According to the Plant List of 2013, only eight out of 133 species of *Mangifera* are accepted species name while the name of the rest of species are unresolved, including that of *Mangifera pajang* (Theplantlist.org, 2013). *Mangifera pajang* is a plant species that belong to Anacardiaceae family and belongs to the genus *Mangifera*. It can be found in the lowland rain forests of Borneo including Sabah, Sarawak, Brunei and Kalimantan. Apart from knowing as Bornean mango, its local name in Sabah and Brunei is bambangan while its other local name in Sarawak and Kalimantan is mawang and embang respectively (Tangah et al., 2017)

.

1.6.1 Botanical description

The tree of *Mangifera pajang* can grow more than 30 m tall with smooth or broadly fissured grey bark. Its mature leaves are elliptic-oblong and dark green while its young leaves can show bronze to pink colour. On the other hand, the flowers of this species have a characteristic purplish-red colour on the inside and pinkish white on the outer surface (Tangah et al., 2017). Figure 1.2 shows an illustration of the tree of *Mangifera pajang* while Figure 1.3 shows the appearance of the flowers and leaves of *Mangifera pajang*.

Figure 1.2: Tree of *Mangifera pajang* (Tangah et al., 2017)

Figure 1.3: Leaves and flowers of *Mangifera pajang* (Tangah et al., 2017)

One of the most interesting fact about *Mangifera pajang* is it has the largest fruit in the genus which weight about 0.5 – 1.0 kg each. Unlike other *Mangifera* species which bear fruits with smooth, red, orange, yellow or green skin, the fruits of *M. pajang* have a brown and rough skin while the pulp is yellow in colour with sweet and sour taste (Preedy, Watson and Patel, 2011). Figure 1.4 shows the outlook of fruits of *M. pajang*.

Figure 1.4: Fruits of *Mangifera pajang* (Tangah et al., 2017)

1.6.2 Present-day cultivation and usage

The fruit pulp, which make up 60-65% of the total weight, is used to make food, but the fruit peel (around 10-15% of the total weight) and kernel (approximately15-20% of the total weight) are usually thrown away. Nowadays, this plant species is planted in the backyards of the local people's house or

cultivated commercially in larger farms. The peel of the fruits can be used to cook curry while in Sarawak, the young leaves of *M. pajang* are consumed as vegetable along with shrimp paste and chilli. In Sabah, fermented *M. pajang* fruits is also a famous snack among the Kadazan-Dusun community (Preedy, Watson and Patel, 2011).

1.7 *Candida* species

There are approximately 8.7 million eukaryotic species including around 611000 species of fungi which make up roughly 7% of the previous number. Out of this 7% of fungi, only about 600 species are human pathogens. For example, dermatophytes and *Malassezia* species will cause mild skin infections. Furthermore, *Aspergillus fumigatus, Cryptococcus neoformans, Candida albicans,* and *Histoplasma capsulatum* are the examples of fungi that cause systemic infection that threaten life. In United States, the fourth most common reason for hospital-acquired systemic infections is due to *Candida* species (Mayer, Wilson and Hube, 2013).

Candida organisms are fungi that exist mostly in unicellular form. In other words, they are yeasts which is around 4-6 µm, have thin cell wall, and form blastospores to reproduce by budding. They can form smooth, gleaming, creamy white colonies. Furthermore, they can develop well on agar plates and vented routine

blood cultures bottles without the need for specific fungal cultivation medium (Bennett, Dolin and Blaser, 2014). There are more than 200 identified *Candida* species but only a few species can raise health issues in human including *C. tropicalis, C. albicans, C. krusei, C. parapsilosis, C. stellatoidea, C. glabrata, C. pseudotropicalis, C. intermedia, C. lusitaniae,, C. dubliniensis, C. kefyr,* and *C. guilliermondii* (Long, Prober and Fischer, 2017).

1.7.1 History of *Candida* Infections

In 1861, Zenker described the first case of deep-seated Candidiasis followed by the discovery of the first case of *Candida*-induced endocarditis in 1940. Since 1970, the extensive use of antibiotics drugs had increased the cases of all forms of *Candida* infection significantly. From 2000 to 2005, the cases of candidemia-related hospitalizations per 100,000 population in United States had surged by 52% and approximately 2 billion dollars are spent on the treatment of *Candida* infection in U.S.. Despite of the increased utilization of the antibiotics drugs, the rising of the cases of human immunodeficiency virus (HIV) infection, the use of medical technology named therapeutic modalities for organ transplantation and life support have also contributed to the increasing incidence of *Candida* infections (Bennett, Dolin and Blaser, 2014).

1.7.2 *Candida albicans*

Candida albicans is a fungal pathogen that raises serious health issues in human

body. It can cause two main types of infection which are superficial infection including oral and vaginal candidiasis and systemic infection that can cause death. An estimation of 75% of the population worldwide consist of *Candida albicans* in their oral cavity. It is harmless to healthy individuals but harmful to slightly or severely immunocompromised person such as those who suffered from AIDS or cancer, which are related to 40% death rate (Potrykus et al., 2013).

1.7.3 *Candida parapsilosis*

In United States, *Candida parapsilosis* is one of the common pathogens that come after *Candida albicans*. It can be isolated from human, domestic animals, soil, marine environment as well as insects. In 1982, it was first isolated from a stool specimen and assumed to be non-pathogenic but now, it is recognized as a pathogen that cause invasive disease in individuals especially those who are immunocompromised. Some of the examples of clinical manifestation of *Candida parapsilosis* are vulvovaginitis, endocarditis, arthritis, peritonitis, keratitis, urinary tract infections, and onychomycosis. The average mortality rates due to *Candida parapsilosis* is 28.5 % (Singaravelu, Gácser and Nosanchuk, 2014).

1.7.4 Current anti-*Candida* drug

The five major classes of antifungal agents currently used against those major fungal pathogens, including *Candida* species are azoles, polyene, echinocandins, fluoropyramidines and allylamines/thiocarbamates (Maurya et al., 2013).

Polyenes such as nystatin and amphotericin-B will act on the cellular membrane of the fungus containing ergosterol by developing ionic channels and affect the proton gradient (Martins et al., 2015).

Azoles such as fluconazole and ketoconazole will affect the biosynthesis of the ergosterol while allylamines and thiocarbamates will cause ergosterol depletion and increases the levels of squalene which is toxic and it will cause cell membrane become permeable and affect the cellular organization (Martins et al., 2015). On the other hand, fluropyramidines can inhibit nucleic acids while echinocandins inhibit the glucan synthesis. Both of them are effective against *Candida* cells (Pereira Gonzales and Maisch, 2012).

1.8 Objectives of the Project

The main objective of this project is to study the antifungal activity of the methanol extract of the kernel of *Mangifera pajang* against *Candida albicans* and *Candida parapsilosis*. This project was also carried out to determine the suitable solvent system to separate the components in the methanol extract of *Mangifera pajang* using thin-layer chromatography (TLC) before separate them using column chromatography.

CHAPTER 2

LITERATURE REVIEW

2.1 Natural compounds in *Mangifera pajang*

The main compounds in the fruit peel, pulp and kernel of *M. pajang* are phenolic acids and flavonoids. Flavonoids are 15-carbon compounds made up of C6-C3-C6 skeleton including two benzene rings which are connected by an oxygen-containing pyrene ring while phenolic acids are phenol containing one carboxylic acid functionality (Tangah et al., 2017).

2.1.1 Phytochemical compounds in the fruit peels, pulp, leaves and stem bark

The peel of this plant species is rich in flavonoids and phenolic acids. The major groups of phenolic acids in the fruit peel are *p*-coumaric acid and gallic acid with a concentration of 13 mg/g and 21 mg/g respectively. Apart from peel of the fruits of *M. pajang*, phytochemical investigation has also been carried out on other plant parts such as the pulp, leaves and stem bark. The fruit pulp contains naringin (1450 μg/g) and hesperidin (930 μg/g). On the other hand, methyl gallate, quercitrin, lupenone and lupeol are found in leaves while sterols such as β-sitosterol, stigmasterol and terpenoids such as *trans*-sobrerol, mangiferonic acid, lupeol, 3β-hydroxy-cycloart-24-ene-26-oic acid and 3β,23-dihydroxy-cycloart-

24-ene-26-oic acid are discovered in the stem bark (Tangah et al., 2017). Table 2.1 demonstrates the type of natural compounds found in the fruit peel, pulp, leaves and stem bark of *M. pajang.*

Table 2.1: Classes of natural compounds found in the fruit peel, pulp, leaves and stem bark of *M. pajang* (Tangah et al., 2017).

Natural compound classes and names	Plant parts
Flavonoids	
Daidzein	Pulp, Peel
Hesperidin,	Pulp, Peel
Catechin,	Peel
Diosmin,	Peel
Epicatechin,	Peel
Morin,	Peel
Kaempferol,	Pulp, Peel
Naringin,	Pulp, Peel
Luteolin,	Pulp, Peel
Rutin	Peel
Quercetin	Pulp, Peel
Genistein	Pulp
Quercitrin	Leaf
Aromatic esters	
Ethyl gallate	Peel

Methyl gallate	Peel, Leaf
Carotenoids	
α-Carotene,	Pulp, Peel
β-Carotene	Pulp, Peel
Phenolic acids	
Vanillic acid	Peel
Chlorogenic acid	Pulp, Peel
Ferulic acid	Peel
Ellagic acid	Peel
4-hydroxybenzoic acid	Peel
Gallic acid	Peel
Pyrogallic acid	Peel
Protocatechuic acid	Peel
p-coumaric acid	Pulp, Peel
Caffeic acid	Pulp
Sterols	
β-sitosterol	Stem bark
stigmasterol	Stem bark
Terpenoids	
3β-hydroxy-cycloart-24-ene-26-oic acid	Stem bark
3β,23-dihydroxy-cycloart- 24-ene-26-oic acid	Stem bark
Lupeol	Leaf, Stem bark

Lupenone	Leaf
trans-sobrerol	Stem bark
Mangiferonic acid	Stem bark
Xanthone	
Mangiferin	Peel
Xanthophylls	
Cryptoxanthin	Pulp, Peel
Cis-Cryptoxanthin	Pulp, Peel
Vitamins	
Ascorbic acids	Pulp

2.1.2 Phytochemical compounds in the kernel of *Mangifera pajang*

The methanol extract of the kernel of *M. pajang* will be tested for its antifungal properties against *C. parapsilosis* and *C. albicans*. Abu Bakar et al. (2009) stated that the methanol extract of the kernel contained a very high amount of total phenolic content which is around 10 % of its total weight. The phenolic content is the highest in the kernel (103.30 mg GAE/g) followed by the seed (22.93 mg GAE/g) and then the flesh (5.96 mg GAE/g). Moreover, Abu Bakar et al. (2009) also reported that the kernel consists of the highest flavonoid content (10.98 mg GAE/g), followed by the peel (7.50 mg GAE/g) and then the flesh (0.07 mg GAE/g).

In the kernel, ferulic acid and diosmin are the two major chemical components with a concentration of 5.334 mg/g and 2.386 mg/g respectively (Preedy, Watson and Patel, 2011). Table 2.2 reveals the type of natural compounds found in the kernel of *M. pajang*.

Table 2.2: Classes of natural compounds found in the kernel of *M. pajang* (Tangah et al., 2017)

Natural compound class	Natural compound name
Flavonoids	diosmin, hesperidin, naringin and rutin
Phenolic acids	caffeic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid, gallic acid and sinapic acid
Aromatic ester	benzyl alcohol, benzaldehyde, methyl gallate

2.2 Antifungal activity of *Mangifera pajang*

Ahmad et al. (2015) reported the first antimicrobial study on *Mangifera pajang*. The results revealed that none of the extracts (chloroform, ethyl acetate, petroleum ether and methanol) from the kernel, leaves and stem bark showed antifungal activity against *Aspergillus chraceaus*, *Candida*

albican, and *Sacchoromyces cerevisiae*. The antifungal assays involved disc diffusion method which involve placing a sample on a 6mm diameter of paper disc and then put them onto a plate for the fungus to grow. They were incubated at 30-37^{o}C for 24-48 hours and the diameters of the areas of complete inhibition were measured (Ahmad et al., 2015).

Kong Chee Kei, the master student of my co-supervisor, had tested the antifungal activity of the methanol crude extract of the kernel of *Mangifera pajang* and found out that there was a significant antifungal properties against *Candida albicans* (C.K. Kong 2018, personal communication, 24 January). It was different from the report of Ahmad et al. (2015). The differences in the result might be due to the method used for determining the antifungal properties was different. Microbroth dilution method was used by the student to determine the MIC of the crude extract instead of agar diffusion method.

Furthermore, the difference in the extraction procedure might also contribute to the dissimilar results. From the report of Ahmad et al. (2015), they extracted 180 g ground kernel of *Mangifera pajang* successively, each three times with petroleum ether, chloroform, ethyl acetate and methanol respectively for about three days by cold maceration method. On the other hand, the master student also extracted the kernel successively, each two times with hexane, chloroform, ethyl acetate, ethanol, methanol and water for two days. The differences of solvent used

to extract the kernel would affect the compounds being extracted into the solvent which in turn affected the antifungal properties of that particular extracts.

2.3 Antifungal activity of other *Mangifera* species

Ahmed et.al. (2005) had studied the antifungal activity of the 200 mg/mL solutions of aqueous, methanol, and chloroform extracts of *Mangifera indica* seed kernels against *Candida albicans* and *Aspergillus niger*. From the results, only methanol and chloroform extracts exhibited high activity against *C.albicans* but inactive against *A. niger*. Ahmed et al. (2012) also reported that the methanol extract of the kernel of *M. indica* which consists of terpenes, flavonoids, tannins and coumarins showed antifungal activity against C. albicans with an inhibition zone of 23 mm. The results was same to those reported by Ahmed et.al. (2005).

Phytochemical screening test carried out by Talba et al. (2014) and Kaur et al. (2010) showed that the kernel of *M. indica* consists of saponins, tannins, glycosides, unsaturated sterols, carbohydrates and flavonoids. Apart from the seed kernel, Kanwal et al. (2010) also isolated five flavonoids from the leaves of M. indica and found out that all the compounds showed high antifungal activity against *Aspergillus fumigatus*, *Alternaria alternata*, *Macrophomina phaseolina*, *Aspergillus niger*, and *Penicillium citrii*.

2.4. Natural antifungal compounds against *Candida* species

Plants have the ability to produce aromatic compounds with various functional group and most of them are secondary metabolites. These metabolites act as a defense system that protect the plants from the infection of microorganism or predation by herbivores and other insects. Some secondary metabolites that plants used as their pigments, odors or even flavours are found to be possessed with useful medicinal properties such as antifungal activity (Tiwari and Mishra, 2011).

Phenolic compounds form a major categories of plant secondary metabolites because currently, there are more than 8000 structures of phenolic compounds being identified. Phenolic compounds are compounds that contain one or more aromatic ring together with one or more hydroxyl groups. They can be divided into polyphenols and simple phenols. Polyphenols with at least two phenol group are known as flavonoids while those with three or more phenol groups are classified as tannins. Currently, flavonoids, tannins and phenolic acids are the main focus of scientific researches but other classes such as coumarins, xanthones, lignins and chalcones also present in our natural environments (Dai and Mumper, 2010).

2.4.1 Phenolic acids

Phenolic acids are phenol containing one carboxylic acid functionality and they are derivatives of hydrobenzoic, phenylpropionic, phenylacetic and hydrocinnamic acids. Instead of existing in their free form, they commonly exist in the form of esters, amides or glycosides in nature. (Teodoro et al., 2015). Phenolic acids derivatives isolated from certain plant sources show antifungal activity which are shown in Table 2.3.

Table 2.3: Antifungal activity of phenolic acids against *Candida* species (Martins et al., 2015)

Phenolic acids	Source	Candida species	MIC
	Lythrum salicaria	*C. albicans*	2500 µg/mL
Gallic acid	*Paeonia rockii*	*C. albicans*	30 µg/mL
	Pelargonium reniforme	*C. albicans*	500 µg/mL
Salicylic acid	*Halimodendron halodendron*	*C. albicans*	150 µg/mL
Vanillic acid	*Stenoloma chusanum*	*C. albicans*	50 µg/mL
Caffeic acid	*Potentilla sp*	*C. albicans*	780–1560 µg/mL

Figure 2.1 shows the structures of some phenolic acids derivatives such as gallic, caffeic, vanillic and salicylic acid.

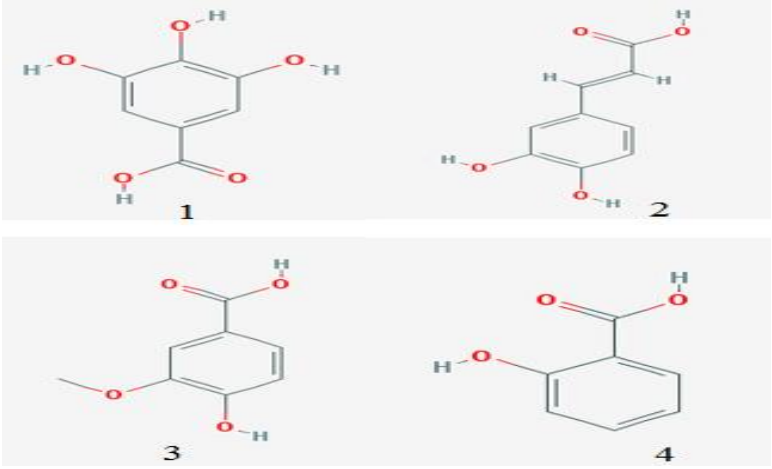


Figure 2.1: Structures of gallic (1), caffeic (2), vanillic (3) and salicylic acid (4) (Pubchem.ncbi.nlm.nih.gov, 2018).

The phenolic acids derivatives extracted from different sources may have variable MIC values against *Candida* species because of different amount of phenolic acids derivatives present in different plants, different solvents used for extraction as well as other compounds in the extracts may act synergistically with the phenolic acids to increase the overall antifungal activity (Teodoro et al., 2015).

For the mechanism of action, only some phenolic acids have been studied. Sung and Lee (2010) had suggested that caffeic acid derivative can affect the *Candida* cytoplasmic membrane, causing a change in the hydrophobic nature of the cell

surface and charge and finally causing the leakage the cytoplasmic content. The other possible mechanism was suggested by Ma et al. (2010) that caffeic acid and quinic acid can inhibit the synthesis of 1,3-β-glucan which is one of the important component of the fungal cell wall. Cheah, Lim and Sandai (2014) reported that isocitrate lyase enzyme in the cell was inhibited after treated with caffeic acid. Isocitrate lyase is an enzyme that enables *C. albicans* to continue survive in glucose-depleted situations and establish candidiasis. Ferulic acid can also inhibit the growth of *C. albicans* by inhibit the transportation in the mitochondrial membranes of fungi cells, causing uncoupling oxidative phosphorylation and inhibit NADH dehydrogenase of the inner membrane of mitochondria (Sachikonye and Mukanganyama, 2016).

The mode of actions of other phenolic compounds also give some hints to propose the mechanism of phenolic acids (Teodoro et al., 2015). For example, eugenol and methyleugenol reduce the amount of ergosterol biosynthesis in *Candida* which in turn disrupting the cell membrane (Ahmad et al., 2010). Furthermore, some phenolic compounds also trigger apoptotic mechanisms in *Candida sp.* For example, eugenol can induce apoptosis by inhibits cell cycle at G1, S, and G2-M phase in *C.albicans* (Zore et al., 2011). On the other hand, the hindering effect of carvacrol, thymol and baicalein on the drug transporter pumps in *Candida* has been confirmed using rhodamine 6G dye. This will cause the accumulation of antifungal agents inside the *Candida* cell which make the cell more vulnerable to the antifungal drugs (Teodoro et al., 2015).

2.4.2 Flavonoids

Flavanoids are 15-carbon compounds made up of C6-C3-C6 skeleton including two benzene rings which are connected by an oxygen-containing pyrene ring. It can be further divided into six major groups which are flavanones, flavonols, flavone, isoflavones, flavanols and anthocyanins (Tangah et al., 2017). They have been discovered to possess antifungal activity against C*andida* species. The following Table 2.4 shows the antifungal activity of some flavonoids against C*andida* species.

Table 2.4: Antifungal activity of flavonoids against *Candida* species. (Martins et al., 2015)

Flavonoids	**Sources**	**Candida species**	**MIC**
Kaempferol	Origanum cutidens	*C. albicans, C.glabrata*	0.5 µg/mL
	Origanum vulgare subsp. gracile	*C. albicans, C.glabrata*	0.1 µg/mL
Quercetin	Buddleja salviifolia	*C. albicans, C.glabrata*	125 µg/mL
Naringenin	Origanum acutidens	*C. albicans, C.glabrata*	10.3 µg/mL
Morin	Origanum vulgare subsp. gracile	*C. albicans, C.glabrata*	42.8 µg/mL

Figure 2.2 shows the structures of kaempferol, morin, quercetin and naringenin.

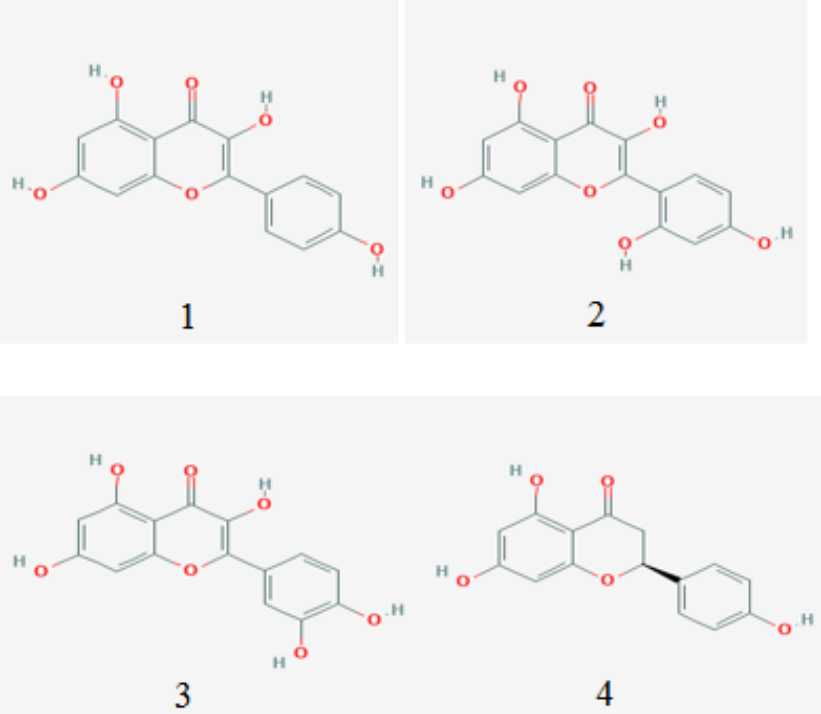


Figure 2.2: Structures of kaempferol (1), morin (2), quercetin (3) and naringenin (4) (Pubchem.ncbi.nlm.nih.gov, 2018).

The antifungal activity of flavonoids is perhaps due to their ability to form complex with extracellular and soluble proteins as well as complex with the cell walls of fungus (Sachikonye and Mukanganyama, 2016). Sachikonye and Mukanganyama (2016) had reported that the number and location of hydroxyl groups can affect the ability to complex with extracellular or other major proteins in fungal cells. Besides that, flavonoids with more lipophilic nature may also interrupt the membrane of the fungus (Tiwari and Mishra, 2011). This is

supported by a study that reported that the antifungal activity of flavonoids increases when the length of alkyl chain increases (Sachikonye and Mukanganyama, 2016).

2.4.3 Tannins

Tannins can be divided into hydrolysable tannins, condensed tannins and pseudo tannins. Hydrolysable tannins such as ellagitannins and gallotannins can be hydrolyzed by acid or enzyme while condensed tannins are derived from flavonols, flavan-3,4-diols and catechins. Pseudotannins are compounds with lower molecular weight and do not yield Goldbeater's test. The antifungal mechanism of tannins may be due to the deprivation of substratum and iron, inhibition of the extracellular enzyme of fungus as well as oxidative phosphorylation inhibition (Sanches et al., 2005). The following diagram shows the structure of gallotannins.

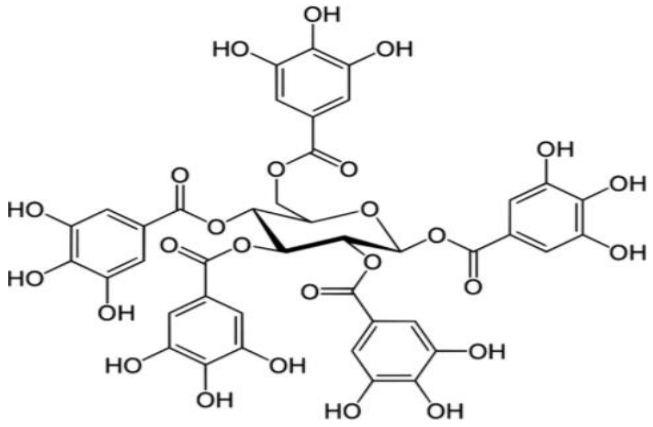


Figure 2.3: Structure of gallotannins (Ma et al., 2015)

2.4.4 Coumarins and Xanthones

Coumarins are one of the phenolic compound that contain fused benzene and α-pyrone rings. This class of compounds have been reported to activate the macrophages which could treat fungal infection (Tiwari and Mishra, 2011). Godoy et al. (2005) had reported that the antifungal activity of the monosubstituted coumarins under investigation did not rely on the pattern of substitution in coumarin nucleus and also the properties of the substituting group. It had been suggested that even though the structure-activity relationship might be discussed, the hypothesis about a plausible species-specific antifungal activity cannot be ignored.

Xanthones consists of two benzene rings links together through a carbonyl group and an oxygen. It is a planar molecule and contain different substituting group attached to it (Tiwari and Mishra, 2011). Pinto et al. (2011) had reported that the existence of hydroxyl group on the structure was essential for its antifungal activity and the nature of the substituent group will affect its antifungal activity. The table below shows the antifungal activity of coumarins and xanthones against *Candida* species.

Table 2.5: Antifungal activity of coumarins and xanthones (Marcondes et al., 2015; Martins et al., 2015; Montagner et al., 2008)

Coumarins			
Mammeisin	*Kielmeyera*	*C. albicans*	512 µg/mL
	Genre	*C. tropicalis*	512 µg/mL
		C. krusei	512 µg/mL
6-methylcoumarin	Commercial	*C. parapsilosis*	512 µg/mL
	compound	*C. albicana*	500 µg/mL
Xanthones			
Mangiferin	Commercial	*C. parapsilosis*	15.7 µg/mL
	compound	*C. albicans*	40 µg/mL

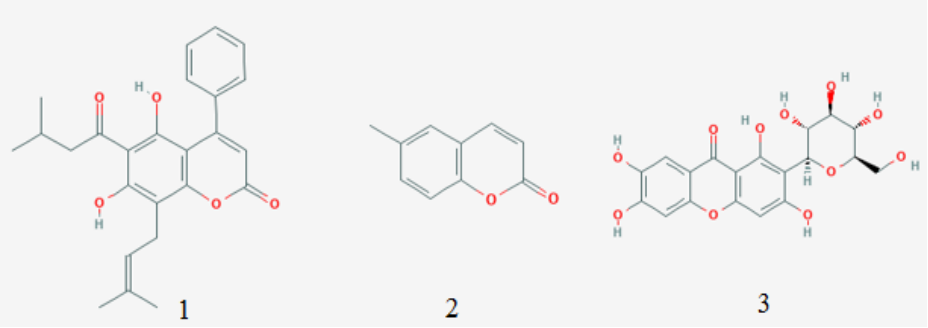


Figure 2.4: Structure of mammeisin (1), 6-methylcoumarin (2) and mangiferin (3) (Pubchem.ncbi.nlm.nih.gov, 2018)

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Thin-Layer Chromatography (TLC)

In this project, thin-layer chromatography was used to find out which solvent system is the most suitable to separate the compounds in methanol extract of the kernel of *Mangifera pajang* before the compound within the methanol extract is separated by column chromatography. The other function of TLC is to check the purity of the fractions collected after the methanol extract is separated by column chromatography.

3.1.1 Preparation of Extract for TLC

To prepare the *Mangifera pajang* methanol extract for TLC, 1 mg of the plant extract was measured into a pre-weighed sample vial. 1 mL of AR grade methanol was used to dissolve the extract in the sample vial until they are fully dissolved.

3.1.2 Preparation of Mobile Phases for TLC

Several combination of mobile phases were tried for TLC tests to separate the

compounds in *Mangifera pajang* extract. The solvents that had been used were chloroform, ethyl acetate, hexane, acetone, acetonitrile, formic acid, acetic acid, methanol and dichloromethane in different combination and ratio. The total volume of the mobile phase used was 2 mL. For example, to prepare a mobile phase that contained ethyl acetate:hexane in ratio 8:2, 1.6 mL of ethyl acetate were mixed with 0.4 mL of hexane. Only 2 mL was needed for the mobile phase because the TLC developing chamber (baby chamber) has a small volume.

3.1.3 Preparation of Developing Chamber

Normally, a TLC developing chamber has to be prepared from scratch. For example, a clean 250 mL of beaker is filled with 10 mL of mobile phase. Then, a piece of filter paper is put inside the beaker in order for it to absorb the solvent and saturated the chamber with the solvent vapour. This step is to prevent the mobile phase on the developing TLC plate from evaporated to the surrounding which will affect the separation of the compound on the plate. The beaker will be covered with an aluminum foil to prevent the evaporation of solvent.

A Camag's® developing chamber which is also known as baby chamber was used to develop the TLC. It is more efficient than the TLC developing chamber made by conventional way. The first reason is the amount of solvent used is only 2 mL which can help to reduce the organic solvent waste, reduce the amount of solvent

used and save the cost. Secondly, no aluminum foil is needed to cover the top of the chamber because the chamber come along with a lid.

To prepare a developing chamber for this project, the chamber was filled with 2 ml of mobile phase. Then, a piece of filter paper was folded and placed inside the developing chamber. The lid was covered to prevent the evaporation of the solvent which will affect the ratio of the solvents in the mobile phase. After the whole piece of filter paper was saturated with the mobile phase, a TLC developing chamber was prepared.

3.1.4 Preparation of TLC Plates

The normal phase TLC plates were cut in a length of 8 cm and width of 2 cm. A length of 1 cm was measured from the bottom of the TLC and a line was drawn with a pencil. This line is known as baseline and the extract will be spotted on this line. Then, 0.5 cm from the top of the TLC plate was measured and a line was drawn using a pencil. This line is the solvent front line.

3.1.5 TLC Test

The dissolved extract was spotted at the center of the baseline on TLC plate and let dry. Then, the TLC plate was carefully put into the developing chamber and closed with a lid. After the mobile phase had travelled to the solvent front line on the TLC plate, the TLC plate was removed from the developing chamber and left aside in the fume hood until all the solvent had been evaporated.

Two visualizing agents had been used which are TLC visualizer and iodine vapour. The TLC visualizer can reveal the spots on the TLC plates by using short UV wavelength (266nm) and long UV wavelength (354 nm). In the case of iodine vapour, an iodine chamber was needed. The chamber was prepared by placing some iodine crystals into a 200 mL wide-mouth jar and covered it with a lid. The chamber was saturated with iodine vapour which was produced from the sublimation of the solid iodine crystals. After that, the TLC plate was placed into the chamber for a few seconds with the lid covered. Any spots on the plates will be stained with brown colour. Those spots observed under these two visualizing methods were marked with pencil and the images were captured.

3.2 Glass Column Chromatography

From the TLC analysis, chloroform: ethyl acetate: methanol: formic acid is the most suitable mobile phase to separate the compounds in the extract.

3.2.1 Preparation of the Column

A 3.5 cm diameter glass column was clamped vertically onto a retort stand. Then, a layer of roughly 0.5 cm of anhydrous sodium sulphate was added into the column. Sodium sulphate was used to eliminate any moisture from the sample extract,

solvent or silica gel. A dropper was use to rinse the inner wall of the column to wash down any remaining sodium suphate that stick to the wall of the column. The layer was rinsed with 100 % chloroform by using dropper instead of pouring the solvent directly into the column to ensure that the solvent will not directly impact with the sodium sulphate layer and causing it to be slanted. After that, the valve was closed to allow the chloroform level above the sodium sulphate layer.

105 g of pre-dried silica gel was prepared by dried in an oven (65^{o}C) for 1 day. A portion of the silica gel was transferred to a 250 mL beaker and mixed with certain amount of 100% chloroform enough to produce a slurry mixture. The mixture was transferred to the glass column immediately. A 250 ml conical flask was placed beneath the glass column and the valve was opened to enable the chloroform to be eluted from the column. Meanwhile, the glass column was tapped gently and carefully with a rubber tube to remove any air bubbles between the silica gel and ensure that the silica gel become more packed. It was made sure that the solvent level did not exceed below the silica gel level so that the silica gel did not dry up and crack. This is because this will cause the column cannot be used for separation. The solvent collected in the conical flask was re-used several times by adding the solvent back into the column again to pack the column more efficiently. These steps were repeated until all 105 g of silica gel had been packed inside the glass column. The mobile phase level was ensured to maintain above the packed silica gel.

3.2.2 Preparation of Extract (Dry loading)

10 g of silica gel was prepared in a dry 250 ml beaker, covered with an aluminum foil, and dried in an oven (65^{o}C) for about 1 day. On the other hand, 2.2738 g of *Mangifera pajang*'s methanol extract was weighed into a 250 mL beaker and dissolve with a minimum amount of methanol because the extract is best soluble in methanol. The dissolved extract was transferred to the 10 g silica gel in 250 mL beaker by using a dropper and they were mixed thoroughly together by using a spatula and allowed to dry to become powder form. It was observed that the silica gel was coated with the brown colour extract.

3.2.3 Separation of Compounds in Plant Extract

By using a spatula, the silica gel coated with the plant extract was transferred to the column with care to ensure that the silica gel had a flat surface and did not disturb the level of the packed silica gel. A dropper was used to rinse the wall of the column with chloroform to wash down any silica gel together with the extract. After all silica gel with the extract were settle down, the valve was opened to collect the fractions with 250 mL conical flasks.

It was ensured that the level of the mobile phase in the column did not below the silica gel level in the column. Thus, the column was constantly refilled with the mobile phase needed before the mobile phase close to the surface of the silica gel. The separation process was aided with the purge of nitrogen gas in order to speed

up the elution proces. This is known as flash chromatography. The volume, combination and composition of the mobile phases used for the column chromatography were shown below:

Table 3.1: Mobile phases used for column chromatography

Mobile phases	Ratio	Volume used (mL)
Chloroform: Methanol: Formic acid	17:3:0.2	300
Ethyl acetate: chloroform: Methanol: Formic acid	3:14:3:0.2	300
Ethyl acetate: chloroform: Methanol: Formic acid	5:12:3:0.2	300
Ethyl acetate: chloroform: Methanol: Formic acid	7:10:3:0.2	300
Ethyl acetate: chloroform: Methanol: Formic acid	9:8:3:0.2	300
Ethyl acetate: chloroform: Methanol: Formic acid	11:6:3:0.2	300
Ethyl acetate: chloroform: Methanol: Formic acid	13:4:3:0.2	300
Ethyl acetate: chloroform: Methanol: Formic acid	15:2:3:0.2	300
Ethyl acetate: Methanol: Formic acid	17:3:0.2	300
Ethanol	1	500
Methanol	1	500

3.3 Bioassay

3.3.1 Preparation of Reagents and Fungus

3.3.1.1 Culture medium, RPMI-1640

2 packs of RPMI-1640 were added into a 1 L Schott bottle. Then, distilled water was used to rinsed the interior of the packet to wash down the remaining powder into the Schott bottle. After that, 69.09 g of MOPS and 900 mL of distilled water were added together into the same Schott bottle. The mixture was stirred using a magnetic stirred bar on top of magnetic stirring hot plate without heating. After all the powder were dissolved and become homogeneous, a dropper was use to add 5 M of NaOH drop by drop to increase the pH of the solution until 7. After that, distilled water was added until the total volume of the solution become 1 litre. Then, the solution in the bottle was filtered into two autoclaved 500 mL Schott bottle, sealed with parafilm and then stored in the fridge.

3.3.1.2 Amphotericin B (Antibiotics)

1.0 mg of Amphotericin B powder was measured and transferred to a 15 mL centrifuge tube along with 1 mL of dimethyl sulfoxide (DMSO) and mixed well. Then, 320 µL of the solution was transferred to another 15 mL centrifuge tube which contain 9680 µL of RPMI-1640. After mixed well, another eight 1.5 mL

centrifuge tube were each filled with 1 mL of the solution. After that, the surface of the tubes were covered with aluminium foil and stored in a fridge because the antibiotics is light-sensitive (Drugs.com, 2018).

3.3.1.3 70 % Ethanol

368 mL of 95 % ethanol and 132 mL of distilled water was measured and transferred to a spray bottle and mixed well.

3.3.1.4 Preparation of Agar Plates

In order to prepare 500 mL of Sabouraud Dextrose Agar 4% (SDA 4%) broth, 32.5 g of SDA 4% powder was measured and transferred to a 1 L Schott bottle. Then, 500 mL of distilled water was added into the bottle. After the solution in the Schott bottle was autoclaved, the agar solution was poured into the Petri dishes until each of the dish was half-filled. Around 20 agar plate can be prepared with 500 mL of SDA 4% agar solution. The whole pouring process was conducted in a laminar air flow. After the agar solution in the Petri dishes were cooled and solidified, they were sealed with parafilms and kept.

3.3.1.5 Cultures of Fresh Batch of *Candida albicans* and *Candida parapsilosis*

The master plates of *Candida albicans* and *Candida parapsilosis* were used to sub-culture the fungus on the agar plates prepared in 3.3.1.4. Firstly, the loop of

the metal wire of inoculating loop was burned with Bunsen burner until the tip became glowing-red in order to sterilize it. After it was cooled, it was used to scrap a little amount of fungus from the master plate and smeared gently onto the surface of the agar plate. Then, all the agar plates were sealed and placed in the fungus incubator (35 ^{o}C) for 2 days in order for the fungus to grow.

3.3.1.6 Preparation of filtered and unfiltered methanol: water solution (2:1)

In order to prepare filtered methanol: water mixture (2:1), 30 mL of methanol was mixed with 15 mL of distilled water in a 100 mL beaker. A 5 mL syringe, which was attached with a 0.45 µm of Nylon syringe filter was used to filter the mixture into a plastic tube with cap. Thus, a filtered and sterilized methanol: water solution was prepared. On the other hand, unfiltered methanol: water solution was prepared by using the same method as filtered one but without the filtration process.

3.3.1.7 Preparation of Fractions for bioassay

If the amount of the fractions was higher and significant, an appropriate amount of it was transferred to a sample vial and dissolved with a certain amount of unfiltered methanol: water solution (2:1) to produce a 10 mg/mL solution. After that, solution was filtered into 1.5 mL Eppendorf tubes by using a 5 mL syringe attached to a 0.45 µm of Nylon syringe filter. The filtered fractions in the Eppendorf tubes were labelled properly and kept in the fridge.

If the amount of the fractions was very little, it was directly dissolved with an appropriate amount of filtered methanol: water solution (2:1) to form a 10 mg/mL solution. After labelled properly, they were also kept in the fridge.

3.3.2 Process of Bioassay

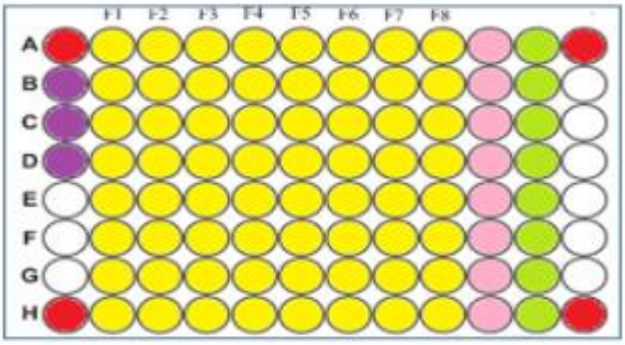
All the procedures of bioassay were carried out carefully in a laminar flow cabinet in order to prevent any contamination of the fractions, agar plates as well as the apparatus and reagent used for the assay because it will affect the result.

Before conducting the bioassay, some of the apparatus were UV-sterilized for around 15 minutes in the laminar flow. The apparatus included inoculating loop, Bunsen burner, igniter, 1 box of 10 μL micropipette tips, 2 boxes of 100 μL micropipette tips, 1 box of 1000 μL micropipette tips, 100-1000 μL micropipette, 10-100 μL micropipette, 1-10 μL micropipette, 96-well plates, multichannel micropipette, test tubes with caps and 25 mL reservoir. Some of the items cannot be sterilized under UV light such as RPMI 1640 medium, fractions, amphotericin B and fungus on agar plates.

After the items in the laminar flow cabinet were UV-sterilized, the outer surface of the bottles, tubes and agar plates that held the RPMI 1640 medium, fractions,

amphotericin B and fungus were sprayed with 70% ethanol solution for decontamination and transferred into the laminar flow cabinet. Gloves was worn when handled these works and it were also decontaminated by 70% ethanol solution.

Then, each column and wells of the 96-well plate were labeled properly according to the number of fractions, antibiotics and other aspects which was shown in Figure 3.1 below.



- 50 µL of fraction + 50 µL of medium + 50 µL of fungus

- Sterility control (100 µL medium)

- Growth control (50 µL medium + 50 µL of fungus)

- Negative control (75 µL medium + 25 µL of fraction)

- Positive control (50 µL medium + 50 µL of antibiotic + 50 µL of fungus)

Figure 3.1: Illustration of the utilization of 96- well plate for MIC assay

Table 3.2: Function of each control on 96-well plate

Type of control	Function
Positive control	- To show the expected positive result (Increased in the formation of precipitates at decreasing concentration of antibiotics) - To determine whether the fungus is drug resistance
Negative control	- To show the expected negative result and to determine whether the fraction had been contaminated by fungus
Growth control	- To determine whether the fungus can grow properly
Sterility control	- To determine whether the 96-well plate and medium is contaminated

3.3.2.1 Orders of Addition of Reagents and Fractions

The medium, RPMI 1640 was poured into a 25 mL reservoir. Then, multichannel micropipette with 8 10-100 µL micropipette tips attached was used to transfer 50

µL of the medium to all the wells in the fractions columns, the column for amphotericin B and the positive control. Then, the volume of the multichannel micropipette was adjusted to transfer 75 µL of the medium to the wells of negative control and 100 µL to the four wells of sterility control.

After that, a 10 – 100 µl micropipette was used to transfer 50 µL of each fraction to the first row of the fractions' well and another 25 µL for the well of negative control corresponding to each fraction. On the other hand, 50 µL of Amphotericin B was also added to its own well on first row. After all fractions and medium had been added to their corresponding wells in an appropriate amount, serial dilution was conducted for the fractions and Amphotericin B.

3.3.2.2 Serial dilution for fractions and Amphotericin B

A multichannel micropipette with appropriate number of 100 µL micropipette tips corresponding to the total number of fractions and Amphotericin B in the first row was pre-adjusted to 50 µL. Then, it was used to mix the mixture in the fractions' and Amphotericin B's well by taking in and out the mixture for roughly 15 to 20 times. After that, 50 µL of each mixture was transfered to the wells in second row. The process was repeated again for the wells in second row and then transferered again to the subsequent row until the final row. On that time, 50 µL of the mixture in the final row was withdrew and discarded.

3.3.2.3 Preparation of Fungus

One sub-cultured fungus agar plate cannot be used for all sets of replicates in the bioassay. For each set of replicate, only one sub-cultured fungus plate can be used. Thus, in order to avoid confusion, each capped test tube (used to contain the fungus solution) was labelled properly according to the number of replicate.

Approximately 3 mL of the medium was added into the capped test tube. Then, the metal loop of the inoculating loop was burned in the flame of Bunsen burner until it turned glowing-red. After it was cooled, it was used to transfer a little amount of fungus into the medium prepared in the test tube. The loop was heated with the flame again for decontamination so that it can be used for next preparation. After the fungus was transferred to the medium and mixed well, its optical density was measured with spectrophotometer at 530 nm after a blank (RPMI 1640) was used to calibrated the instrument. If its absorbance fell between 0.12-0.15, the fungal solution can be used for bioassay. If it was below the range, more fungus was added until the absorbance fall within the desired range. On the other hand, more medium was added to dilute the fungus solution if the absorbance was above the range.

Optical density measurement of microorganism cultures is common to identify the essential cultures parameters such as cell concentration. It is based on the amount of light scattered by the cells in the cultures rather than the absorbance. The

instrument will measure the decrease in transmittance which is caused by the scattering effect from the cell suspension. Thus, the higher the cell suspension, the greater the scattering effect and the higher the absorbance (Matlock, 2017).

For one 96-well plate, around 4995 µL of medium and 5 µL of fungus solution are required. Thus, if the replicate consists of two 96-well plates, it need to transfer 9990 µL of medium and 10 µL of fungus solution to a reservoir and mix together. The mixture need to be mix thoroughly because the fungus added was in a very small amount.

After that, multichannel micropipette was used to transfer 50 µL of the diluted fungus solution to each of the well of the plates that consists of column fractions, Amphotericin B, negative control and positive control except the wells of sterility control. Lastly, the plates were closed with the caps, sealed with parafilm and incubated in the fungus incubator oven (35 ^{o}C) for 2 days.

3.3.2.4 Addition of p-iodonitrophenyltetrazolium violet (INT) indicator

During the next day of incubation, the plates were took out from the incubator oven. Firstly, INT indicator was poured on the reservoir. Then, multichannel micropipette was used to transfer 50 µL of the indicator to each of the well that consists of column fractions, Amphotericin B, negative control, positive control

as well as the wells of sterility control. After that, the plates were sealed with parafilm and put back into the incubator oven to incubate one more day.

3.3.3 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

For the minimum inhibitory concentration (MIC) assay, the concentration of the first well (counted from last row) that showed precipitates less than 50 % of the precipitates formed in growth control well will be considered as the MIC for the particular fraction.

For those wells which have less than 50 % precipitates, 20 µL of solution from the well was transferred to a pre-segmented agar plate. On each plate, there were eight segments and they were labelled with the corresponding mixture added on it. The solutions on the agar plates was swabbed across the surface of agar plate within their own segments by using a cotton swabs. The process was repeated until all the solution from the wells which have less than 50 % precipitates were transferred. The agar plates were sealed with parafilm and incubated in the fungus incubator oven (35 ^{o}C) for 2 days. After 2 days, the plates were observed for any growth of fungus. If there was colonies shown on a particular segments, it means that particular fractions only inhibit the growth of the fungus instead of killing the fungus. The lowest concentration of the fraction at which no visible growth of

fungus was observed will be identified as minimum fungicidal concentration (MFC).

3.4 High Performance Liquid Chromatography (HPLC)

The fractions with the lowest MIC have the highest antifungal activity. Thus, those fractions with lowest MIC were analyzed under HPLC to determine how many compounds are there in the fractions. Shimadzu HPLC instrument was used to carry out the analysis. The column used was 4.6 x 150 mm, 5 µm Purospher® STAR RP-18 endcapped and the detector used was Photodiode Array detector (PDA) which can detect the absorption of the compounds over a wide range of UV to visible light wavelength region.

0.1 mg/mL solution was prepared from each fraction selected for analysis. The best separation was tried to obtain with different mobile phase combination, instrument settings such as flow rate, temperature, injection volume, type of elution such as isocratic or gradient elution and type of column.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Thin-Layer Chromatography

In order to prepare the sample for TLC, about 1 mg of the methanol crude extract of the kernel of *Mangifera pajang* was dissolved in 1 ml of methanol. This is because the crude product is more soluble in methanol and the solvent is very volatile. After the sample was spotted on the TLC, the solvent can be evaporated faster and leave behind the sample so that the TLC separation process can be carried out. Before development of the TLC, the TLC plate together with the sample spotted on it was view under the UV light in order to figure out whether the sample can be view under long (366 nm) or short (254 nm) UV wavelength. For this *Mangifera pajang* methanol extract, it only fluoresce under short UV wavelength.

The most suitable mobile phase to separate the compounds in the crude extract was ethyl acetate: chloroform: methanol: formic acid. Figure 4.1 (a) and (b) below show the spots on the TLC plates after developed with the mobile phases and view under short UV wavelength.

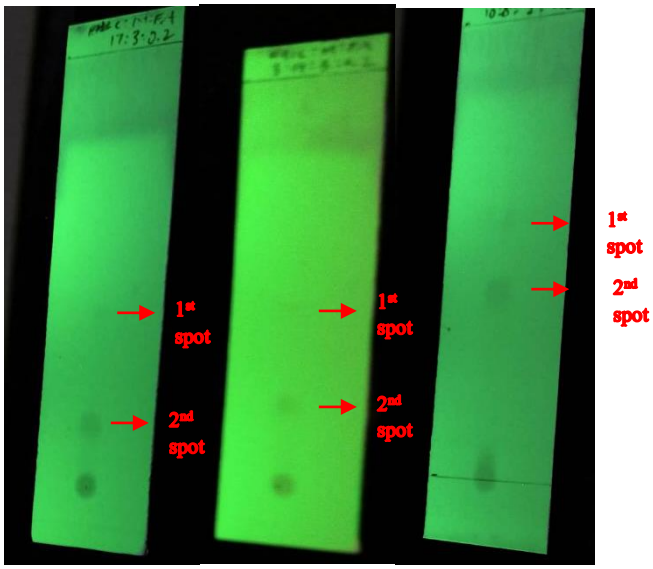


Figure 4.1 (a) Figure 4.1 (b) Figure 4.1 (c)

Figure 4.1 (a): TLC plates developed with chloroform: methanol: formic acid in ratios of 17: 3: 0.2

Figure 4.1 (b): TLC plates developed with ethyl acetate: chloroform: methanol: formic acid in ratios of 3: 14: 3: 0.2

Figure 4.1 (c): TLC plates developed with ethyl acetate: chloroform: methanol: formic acid in ratios of 10: 7: 3: 0.2

Formic acid is an essential component in this mobile phase because without it, the second spot cannot be separated well and gives a very bad tailing effect as shown in Figure 4.2 below:

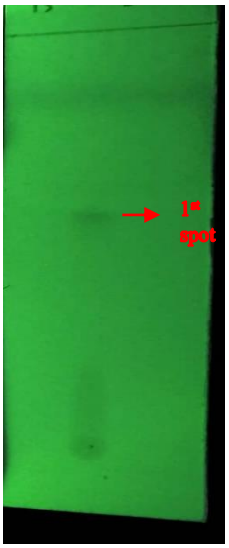


Figure 4.2: TLC plate developed with ethyl acetate: Chloroform: methanol in ratios of 10: 7: 3

The tailing is due to the presence of acids, bases or highly polar compounds in the crude extract which adhere to the absorbent strongly. This will cause the compounds hard to move upwards which disturbs the migration of the solutes across the stationary phase. Addition of a trace amount of acids or bases can minimize the strong interaction between the compound and adsorbent as well as increase the polarity of the mobile phase. This reduces the tailing effect and give a nicer spot shape (Dolan, n.d.). After tested, formic acid reduced the tailing effect of the spots much better than acetic acid and ammonia.

When increasing the polarity of the mobile phase by increased the percentage of ethyl acetate, the spots travelled a longer distance on the TLC plate which mean

that the compounds in the spot have a higher polarity because it is more soluble in higher polarity mobile phase and migrate further. Table 4.1 below shows the retardation factors of the spots on the three TLC plates shown in Figure 4.1 (a), (b) and (c).

Table 4.1: Retardation factor for the spots on each TLC plate

Mobile phase	**Distance travelled by mobile phase, D (cm)**	**Distance travelled by spots, L (cm)**	**Retardation factor, R_f = L / D**
Chloroform: Methanol: Formic acid (17: 3: 0.2)	6.5	First spot = 1.0	First spot = 0.154
		Second spot = 2.5	Second spot = 0.385
Ethyl acetate: Chloroform: Methanol: Formic acid (3: 14: 3: 0.2)	6.5	First spot = 1.4	First spot = 0.215
		Second spot = 3.0	Second spot = 0.462
Ethyl acetate: Chloroform: Methanol: Formic acid (10: 7: 3: 0.2)	6.5	First spot = 2.5	First spot = 0.385
		Second spot = 3.8	Second spot = 0.585

4.2 Glass Column Chromatography

Column chromatography was performed in order to separate all the compounds in

the crude extract into several fractions according to their polarities. This will cause the fractions collected from the column to have lesser compounds, which make the next purification step much easier. After carried out column chromatography, 27 fractions were collected. These fractions were further combined into 15 fractions by performing TLC on each fraction to check their purity. Those which had almost similar or completely identical TLC separation pattern were mixed together and become a single fraction. This is to reduce the amounts of fractions required for bioassay which reduces the workload. The figures below show the TLC result for each fraction.

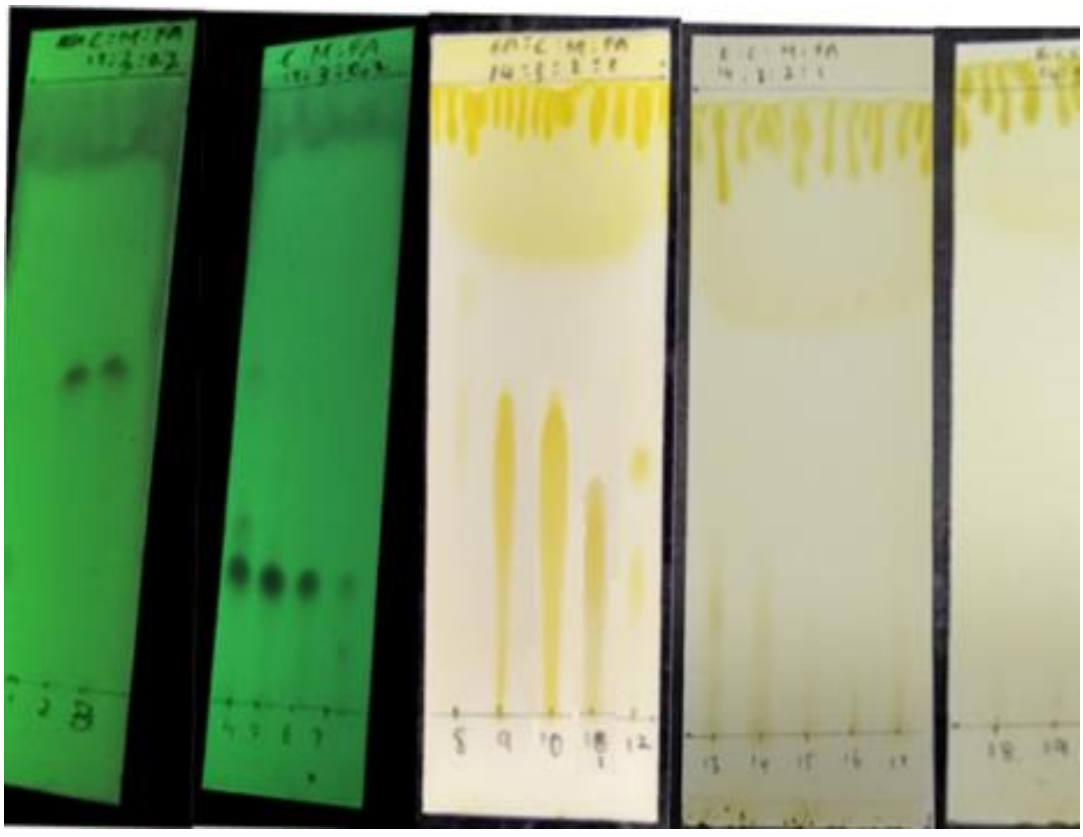


Figure 4.3 (a): TLC result for fraction 1 to 19 (Start from left to right)

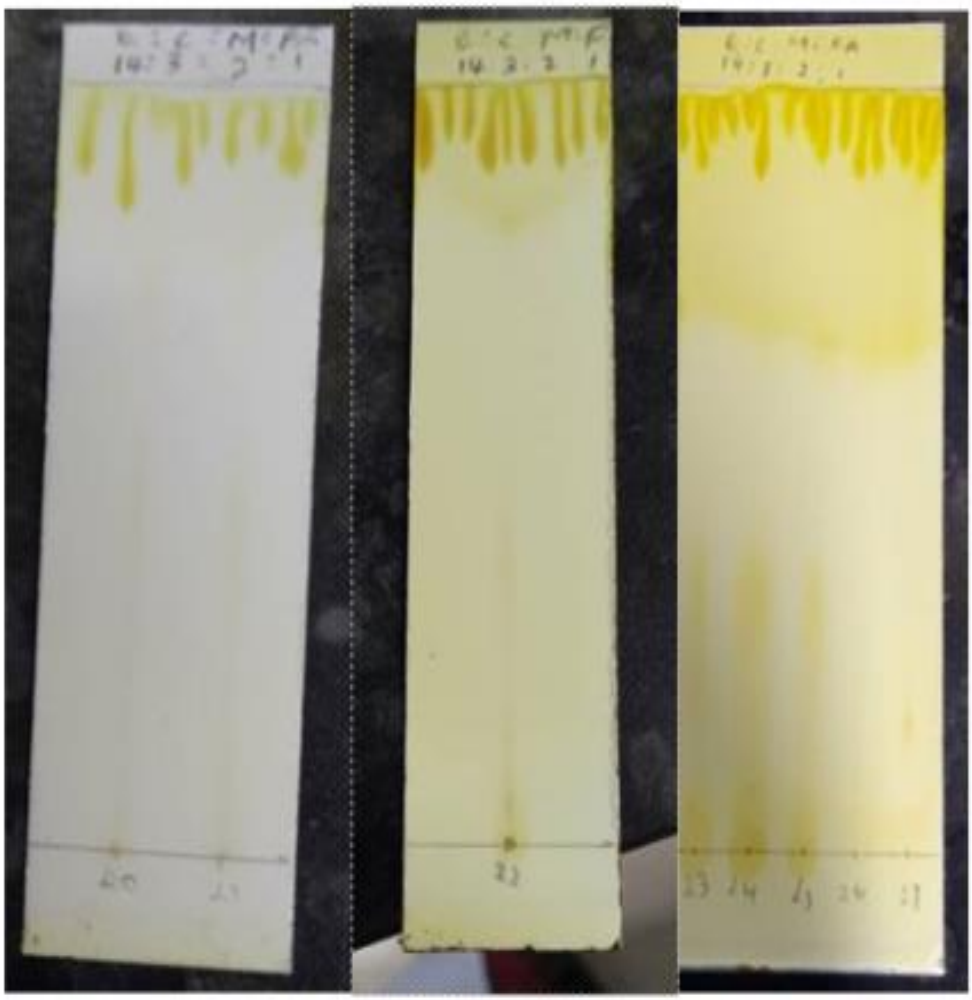


Figure 4.3 (b): TLC result for fraction 20 to 25 (Start from left to right)

For fractions 1 to 7, the mobile phase used was chloroform: methanol: formic acid in ratios of 17: 3: 0.2. From the result, fraction 1 did not show any spot under short UV wavelength but it still considered as an individual fraction because it was collected as a narrow green band as shown in Figure 4.3 below that differentiate itself from others band.

Figure 4.4: First band separated in column chromatography

Fraction 2 and 3 showed similar spot at approximately same distance from the baseline. Thus, these 2 fractions were combined to become a single fraction. For fraction 4, 5, 6 and 7, each of them was considers as a single fraction because the TLC result did not show significant similarities. From fraction 8 onwards, the polarity of the compounds increased and the previous mobile phase cannot elute the compounds from the baseline. Thus, ethyl acetate: chloroform: methanol: formic acid in ratios of 14: 3: 2: 1 was used. From fraction 8 to 12, only 9 and 10 were combined together since their TLC result were roughly similar to each other. From fraction 13 onwards, the tailing effect was greater and significant. Fraction 13 and 14 were pull together as a single fraction while fraction 15 until 19 were mixed together and considered as an individual fraction. Fractions 20 and 21 were combined while fraction 23 until 27 were mixed together as a single fraction. Thus, a total of 15 fractions were obtained. The following figure shows the summary for the combination of those fractions.

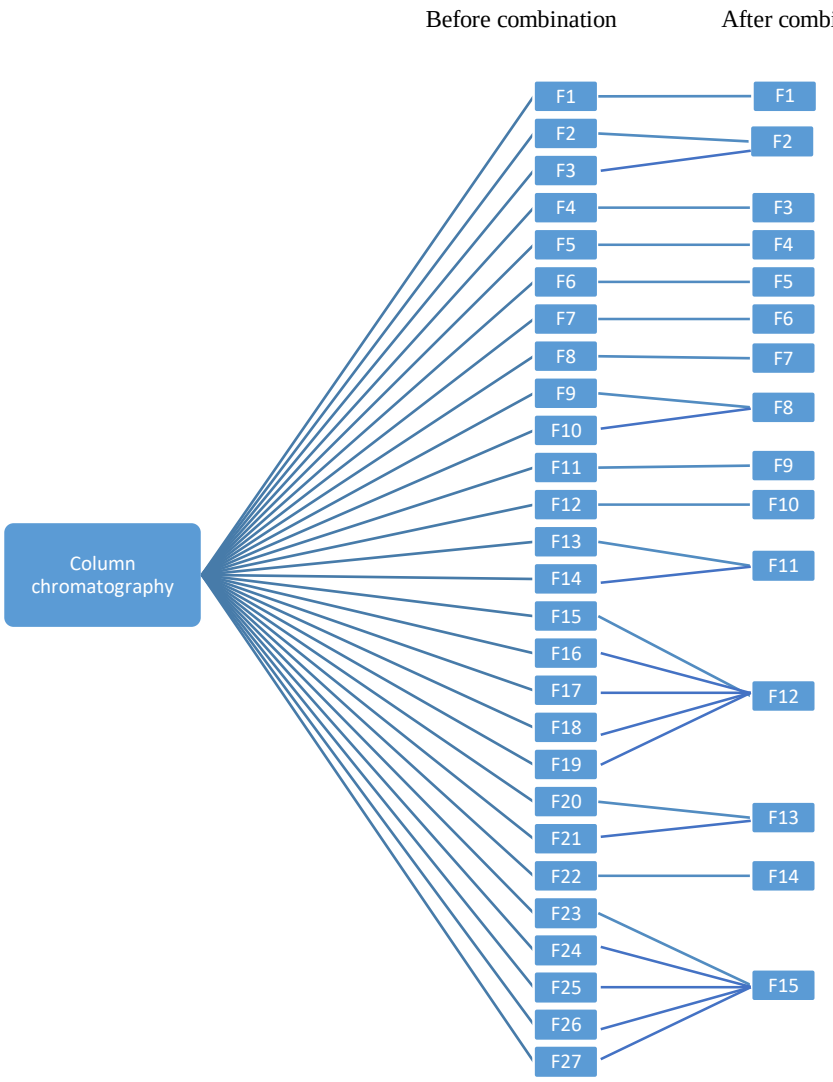


Figure 4.5: Flowchart for the combination of the fractions

Although the tailing effect on fractions 13 until 27 make it unable to clearly differentiate one fraction with another, it is not a significant problem because the main objective of this project is to determine the antifungal activity of the *Mangifera pajang* extract. Only the fraction with the highest antifungal activity will become the main target which will be proceed to more work such as further purification and be tested again for their antifungal activity respectively. Thus, the

purities of the fractions are not the main concern at this stage. Table 4.2 below show the mass of each fraction after drying.

Table 4.2: Mass of each fraction collected and total mass

Fractions	Mass (g)
1	0.0020
2	0.1056
3	0.0962
4	0.0722
5	0.0632
6	0.0026
7	0.0167
8	0.7484
9	0.1131
10	0.0858
11	0.3299
12	0.0287
13	0.0277
14	0.115
15	0.0658
Total mass collected	1.8729

$$\text{Recovery yield of total fractions} = \frac{\text{Total mass of fraction collected}}{\text{Mass of sample used}} \times 100\ \%$$

$$= \frac{1.8729\ \text{g}}{2.2738\ \text{g}} \times 100\ \%$$

$$= 82.37\ \%$$

From Table 4.2, fraction 8 has the highest mass while fraction 1 has the lowest mass. The recovery yield of the fractions was not 100 % because some of the compounds adhered strongly to the silica gel of the column which was unable to be removed even though the silica gel was eluted with 100 % ethanol and 100% methanol which has high polarity.

4.3 MIC (Minimum Inhibitory Concentration) Test for Bioassay

The minimum inhibitory concentration of the 15 fractions collected were determined against *Candida albicans* and *Candida parapsilosis*. The MIC values were determined by observed the first well, counted from the bottom in each column that contain purplish-red precipitates that was less than 50 % of the precipitates observed in growth control well.

4.3.1 MIC against *Candida parapsilosis*

Firstly, 10 mg/mL solution was prepared from each fraction and used for the MIC test. However, only fraction 1, 2 and 14 showed the MIC values while the rest did not formed any precipitates. This mean that the fractions were too concentrated enough to inhibit or kill the whole fungus population in all diluted concentration. If the concentration of the fraction was 10 mg/mL, then the concentration of the first well will be 2.5 mg/mL due to dilution with medium and fungus solution. The subsequent concentration of fraction was 1.25, 0.63, 0.31, 0.16, 0.08, 0.04 and 0.02 mg/mL in each column. The figure below shows the MIC result on 96-well plate for both replicate.

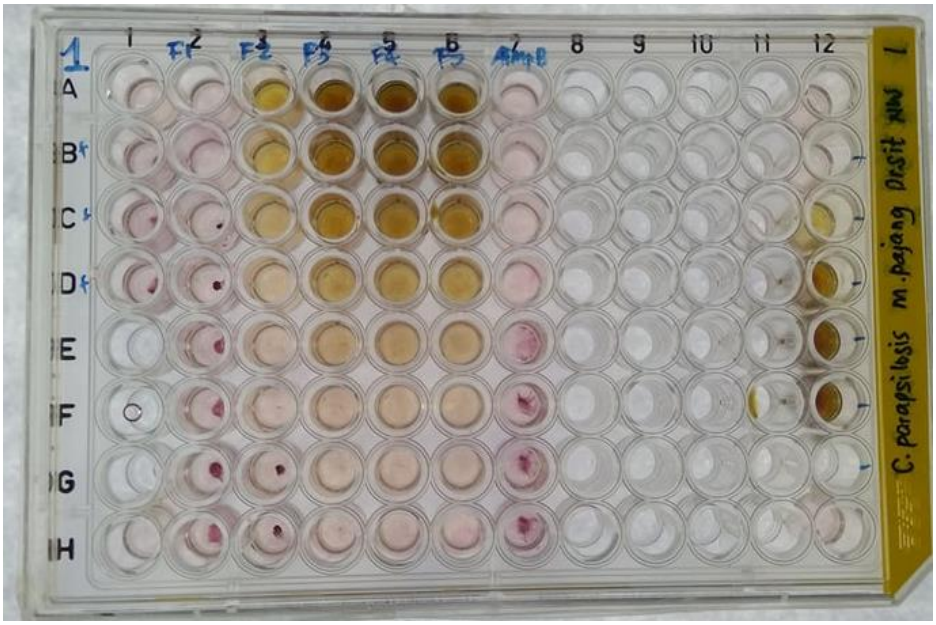


Figure 4.6 (a): First replicate for MIC test of fraction 1-5 against *Candida parapsilosis*

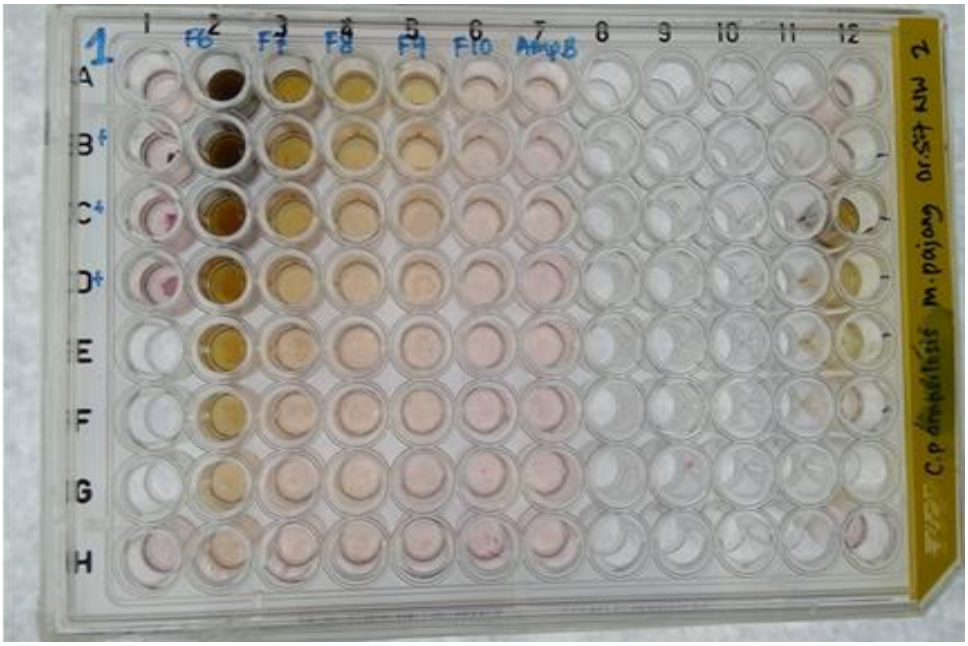


Figure 4.6 (b): First replicate for MIC test of fraction 6-10 against *Candida parapsilosis*

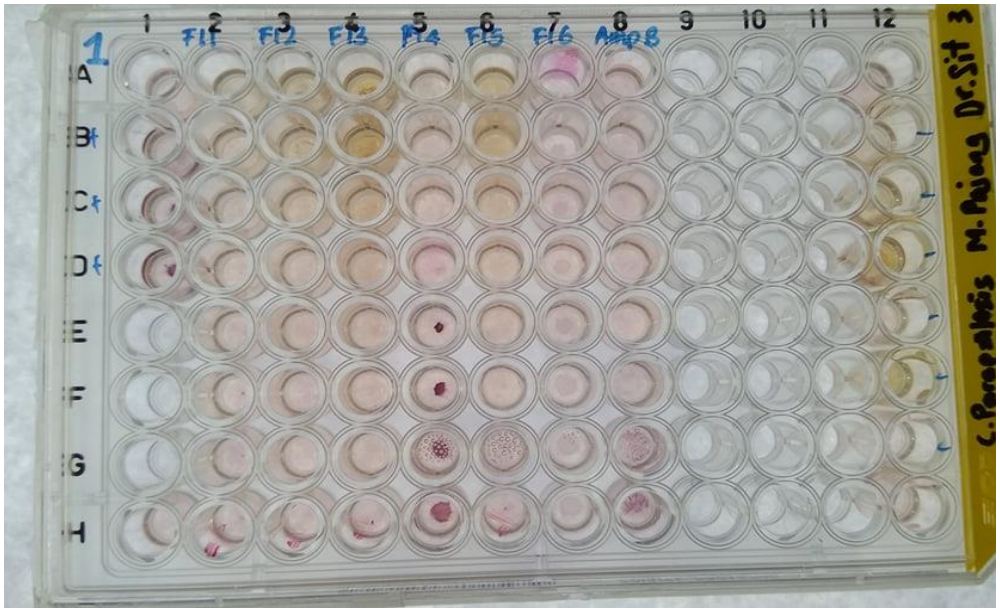


Figure 4.6 (c): First replicate for MIC test of fraction 11-15 against *Candida parapsilosis*

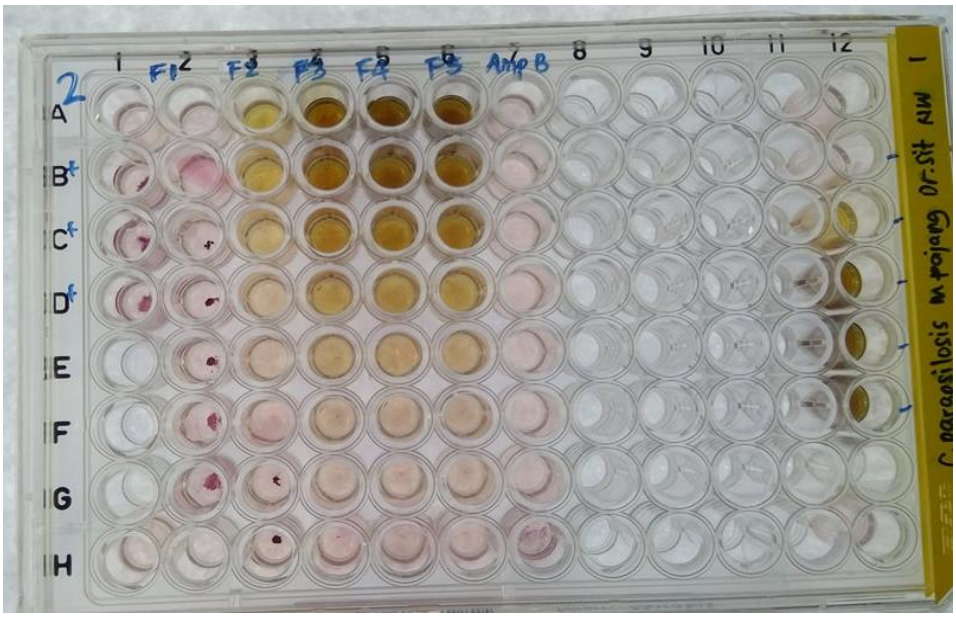


Figure 4.7 (a): Second replicate for MIC test of fraction 1-5 against *Candida parapsilosis*

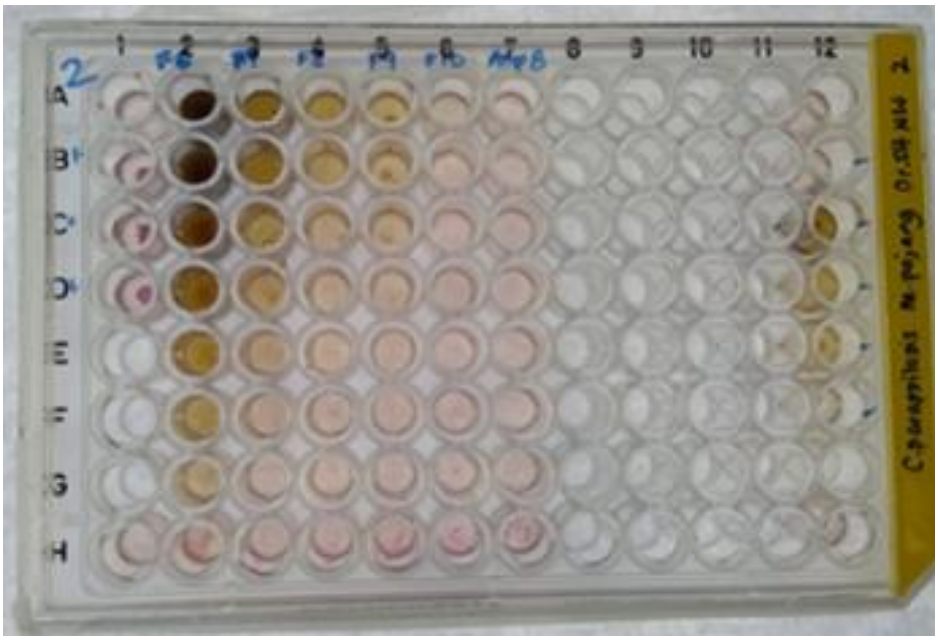


Figure 4.7 (b): Second replicate for MIC test of fraction 6-10 against *Candida parapsilosis*

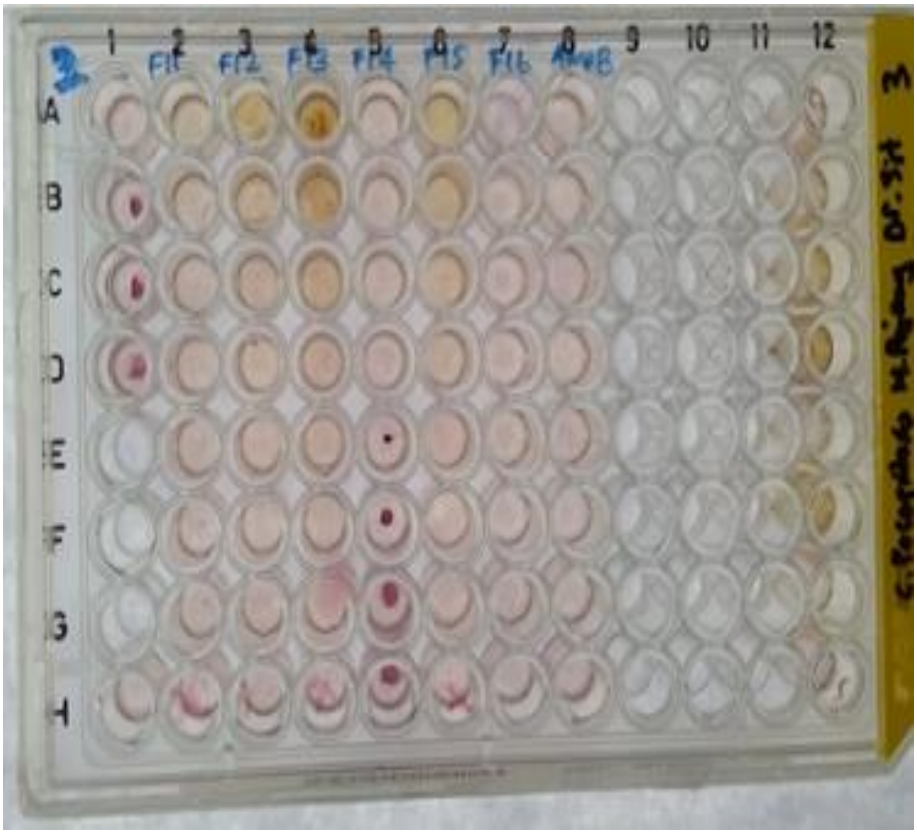


Figure 4.7 (c): Second replicate for MIC test of fraction 11-15 against *Candida parapsilosis*

Since the concentration was too high, each fraction except fraction 1, 2 and 14 was further diluted to 0.16 mg/mL. The concentration of the fraction in the first well is 0.04 mg/mL followed by 0.02, 0.01, 0.005, 0.0025, 0.00125, 0.0006 and 0.0003 mg/mL. The figure below shows the MIC result on 96-well plate for both replicate.

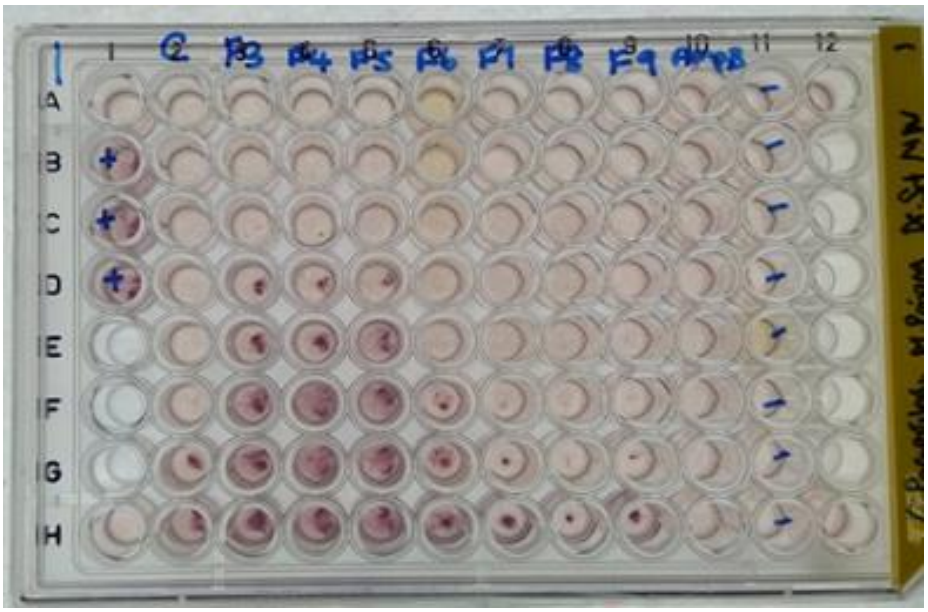


Figure 4.8 (a): First replicate for MIC test of crude extract and fraction 3-9 against *Candida parapsilosis*

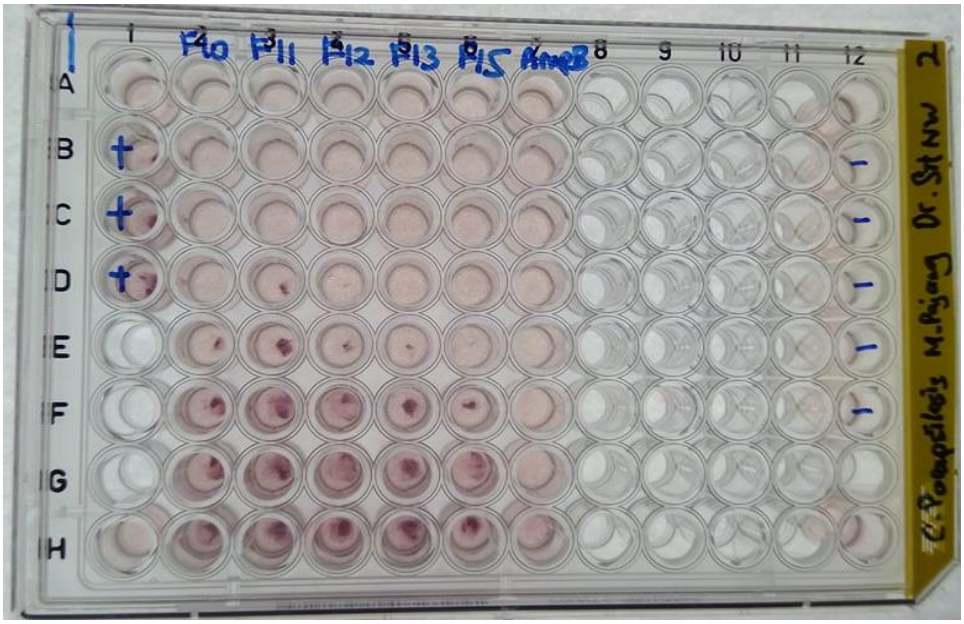


Figure 4.8 (b): First replicate for MIC test of fraction 10-15 against *Candida parapsilosis*

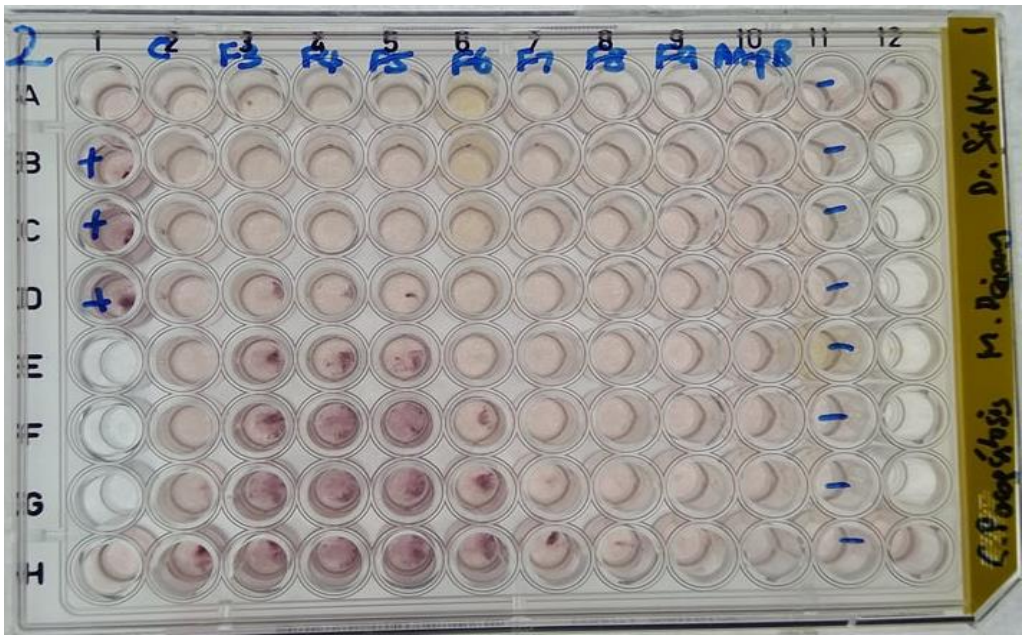


Figure 4.9 (a): Second replicate for MIC test of crude extract and fraction 3-9 against *Candida parapsilosis*

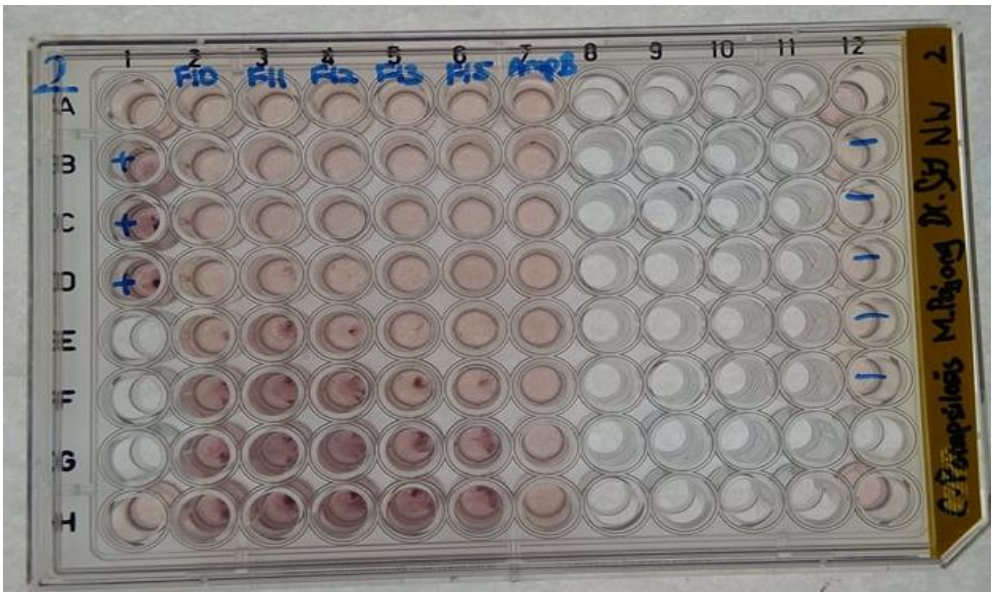


Figure 4.9 (b): Second replicate for MIC test of fraction 10-15 against *Candida parapsilosis*

The 'C' symbol labelled on column no. 2 was the crude extract being tested for its MIC in order to prove that the crude extract possessed with the antifungal activity. The precipitates formed in fraction 9 for both replicate against *Candida parapsilosis* had a quite large difference as shown in Figure 4.8 (a) and 4.9 (a). Thus, it was repeated again and was shown on column no. 9 of the second plate for each replicate in Figure 4.10 (b) and 4.11 (b) below. It showed that fraction 9 had a MIC of 0.0003 mg/mL for both replicate which can combine with the first replicate in Figure 4.8 (a) to give a MIC range from 0.0003-0.0006 mg/mL. The positive control in Figure 4.6 (b), (c), 4.7 (a), (b), (c), 4.8 (a), (b) and 4.9 (a), (b) did not show any precipitates which means that the fungus were killed or inhibited by Amphotericin B at all concentration. This might be due to the excess volume of Amphotericin B transferred to the positive control or the concentration of Amphotericin B used was too high due to wrong volume transferred during preparation. The table below shows the MIC of each fraction against *Candida parapsilosis*.

Table 4.3: MIC of each fraction against *Candida parapsilosis*

Fractions	Minimum Inhibitory Concentration (mg/mL)
1	1.2500
2	0.0800
3	0.0100
4	0.0100

5	0.0100
6	0.0025
7	0.0006-0.0013
8	0.0003-0.0006
9	0.0003-0.0006
10	0.0050
11	0.0100
12	0.0050
13	0.0025
14	0.3100
15	0.0025

From the table, fraction 1 had the highest MIC value which was 1.25 mg/mL while fraction 8 and 9 had the lowest MIC value which was around 0.0003-0.0006 mg/mL.

4.3.2 MIC against *Candida albicans*

As almost all the fractions had quite high antifungal activity against *Candida parapsilosis*, another concentration of fractions was prepared at 0.63 mg/mL except fraction 1 because the amount of it was very little and whole fraction 1 had been used up for the determination of MIC against *Candida parapsilosis*. The concentration of the fraction in the first well was 0.16 mg/mL followed by 0.08,

0.04, 0.02, 0.01, 0.005, 0.0025 and 0.00125 mg/mL. The figure below shows the MIC result on 96-well plate for both replicate.

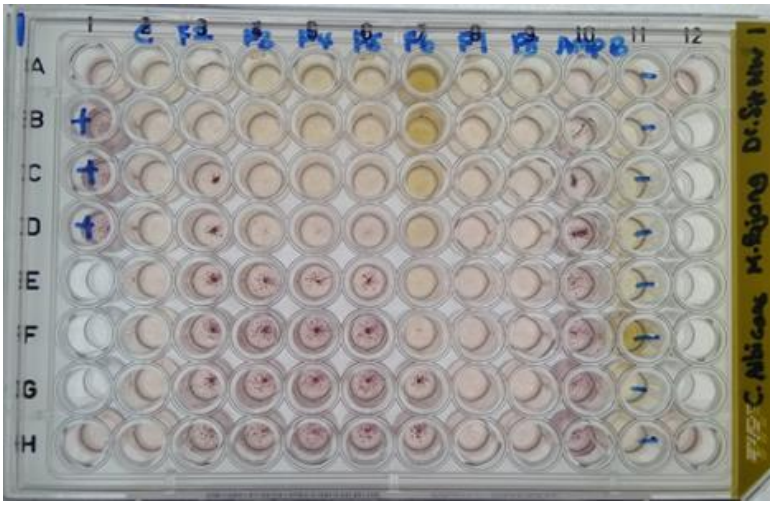


Figure 4.10 (a): First replicate for MIC test of crude extract and fraction 2-8 against *Candida albicans*

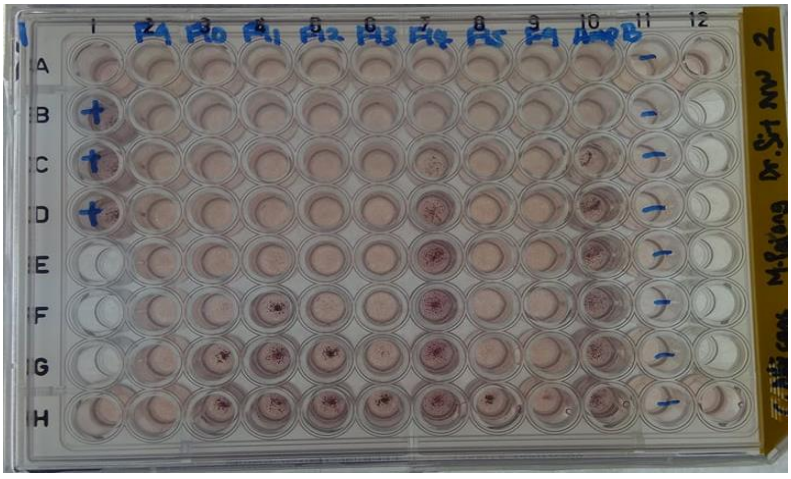


Figure 4.10 (b): First replicate for MIC test of fraction 9-15 against *Candida albicans* and fraction 9 against *Candida parapsilosis* at column No. 9.

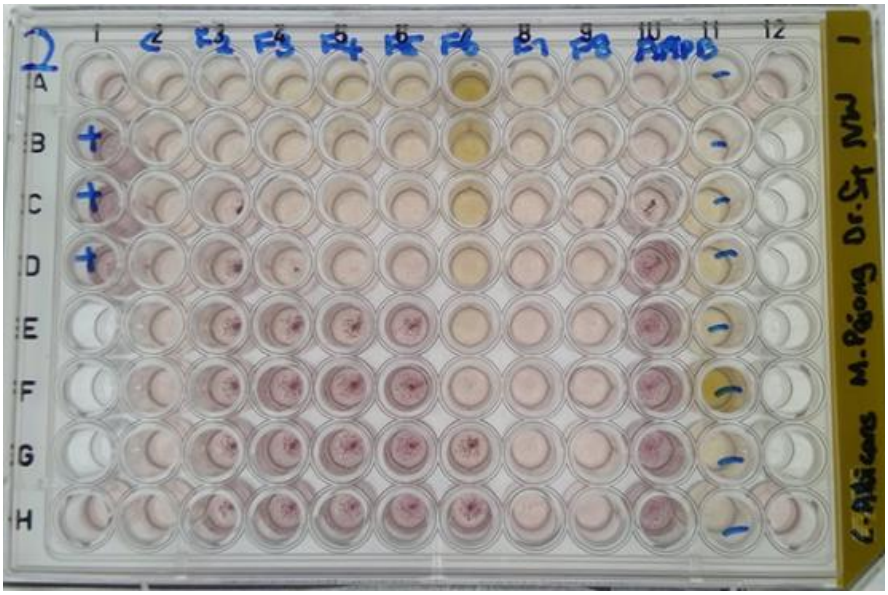


Figure 4.11 (a): Second replicate for MIC test of crude extract and fraction 2-8 against *Candida albicans*

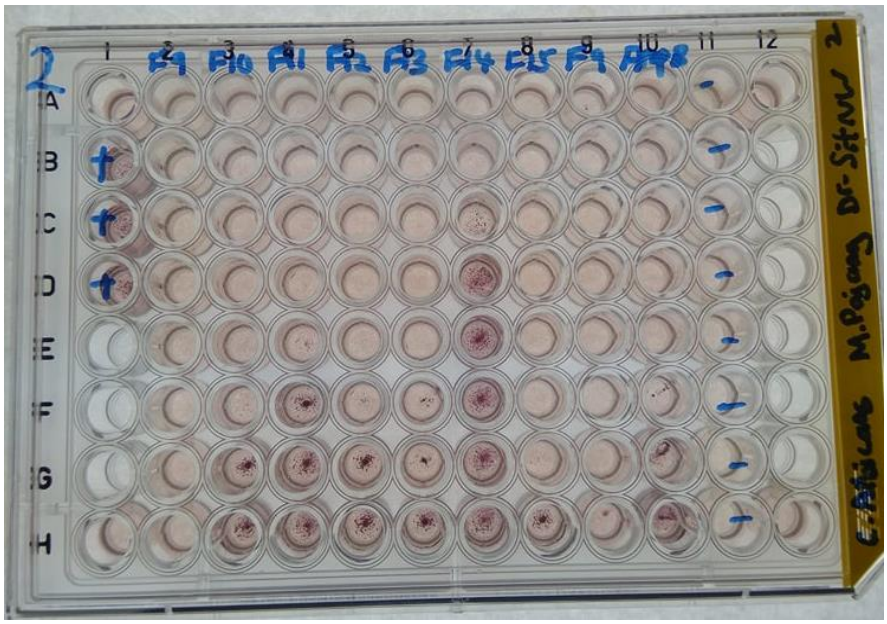


Figure 4.11 (b): Second replicate for MIC test of fraction 9-15 against *Candida albicans* and fraction 9 against *Candida parapsilosis* at column No. 9.

The 'C' symbol labelled on column no. 2 was the crude extract being tested for its MIC in order to prove that the crude extract possessed with the antifungal activity. Fraction 7, 8 and 9 showed a very trace amount of precipitates in the last wells and their MIC values cannot be determined because the first concentration at which the precipitates formed is less than 50 % of the precipitates observed in growth control well was unknown. However, the last well was considered as their MIC because they had the strongest antifungal activity (lowest MIC) among those fractions while fraction 8 and 9 also had the lowest MIC values against *C. parapsilosis* while fraction 7 was second lowest. It will be meaningless to further dilute the fractions to determine the exact MIC since it already proved that these three fractions had greater antifungal activity against *C. albicans* and *C. parapsilosis*. The table below shows the MIC values against *Candida albicans.*

Table 4.4: MIC of each fraction against *Candida albicans*

Fractions	Minimum Inhibitory Concentration (mg/mL)
1	-
2	0.0800
3	0.0400
4	0.0400
5	0.0400
6	0.0050
7	0.0013

8	0.0013
9	0.0013
10	0.0050
11	0.0100
12	0.0050
13	0.0050
14	0.0800
15	0.0050

From the table, fraction 2 and 14 had the highest MIC value which was 0.0800 mg/mL while fraction 7, 8 and 9 had the lowest MIC value which was around 0.0013 mg/mL.

4.4 MFC (Minimum Fungicidal Concentration) Test for Bioassay

This test was used to determine the antifungal action of the compounds within each fraction , whether they just inhibited the fungus growth or they killed the fungus. The table below shows the minimum fungicidal concentration of each fraction against *Candida albicans* and *Candida parapsilosis.*

Table 4.5: MFC of each fraction against *Candida albicans* and *Candida parapsilosis*

Fraction	**Minimum Fungicidal Concentration (mg/mL)**	
	Candida parapsilosis	***Candida albicans*.**
1	1.2500	-
2	0.6250	0.0800
3	0.0100	0.0400
4	0.0100	0.0400
5	0.0100	0.0400
6	0.0025	0.0050
7	0.0006 - 0.0013	0.0013
8	0.0003 - 0.0006	0.0013
9	0.0003 - 0.0006	0.0013
10	0.0050	0.0050
11	0.0100	0.0100
12	0.0050	0.0050
13	0.0025	0.0050
14	0.3100	0.0800
15	0.0025	0.0400

From the table, it showed that fraction 8 and 9 had the lowest MFC values against *Candida parapsilosis* while fraction 7, 8 and 9 exhibited lowest MFC values against *Candida albicans.* Almost each of the fractions have the similar MIC and MFC values which mean that the compounds within the fractions were strong enough to kill the fungus population instead of just inhibited their growth.

From the MIC and MFC values of all the fractions, fraction 8 and 9 had the lowest MIC and MFC against *Candida parapsilosis*, while fraction 7, 8 and 9 had the lowest MIC and MFC values *against Candida albicans.* However, only fraction 8 and 9 became the fraction of interest because both of them exhibited significantly strong antifungal properties against both of the fungus and their amount was large enough to proceed to further work. The amount of fraction 7 was only 0.0167 g while fraction 8 and 9 had a mass of 0.7484 g and 0.1131 g respectively. Thus, fraction 8 and 9 were analyzed using HPLC to determine the number of compounds present inside each fraction.

4.5 High Performance Liquid Chromatography (HPLC)

After several trials, it was found out that acetonitrile and water was the best mobile phase to separate the compounds within fraction 8 and 9 by using gradient elution method. The column used was 4.6 x 150 mm, 5 µm Purospher® STAR RP-18 endcapped and the injection volume was 20 µL. The concentration of the

fractions tested was 0.1 mg/mL. The following table shows the parameter of the gradient elution.

Table 4.6: Parameter for gradient elution

Time (min)	**Flowrate (ml/min)**	**Composition (%)**	
		Acetonitrile	**Water**
0	0.8	0	100
10	0.8	20	80
15	0.8	30	70
20	0.8	50	50
25	0.8	75	25
32	0.8	100	0
35	0.8	100	0

At 0 min, the composition of the water was 100 % which slowly decrease to 0 % when the time reached 32 min. The following figures shows the chromatogram of fraction 8, 9 and the blank under 210, 254, 280 and 320 nm.

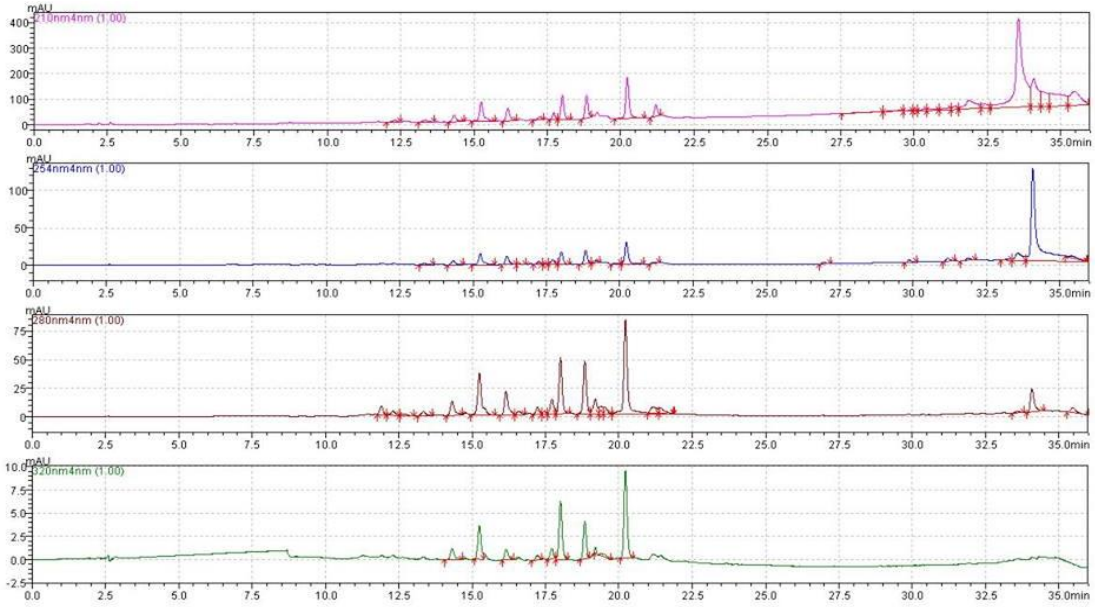


Figure 4.12: Chromatogram of fraction 8 under 210, 254, 280 and 320 nm (from top to bottom)

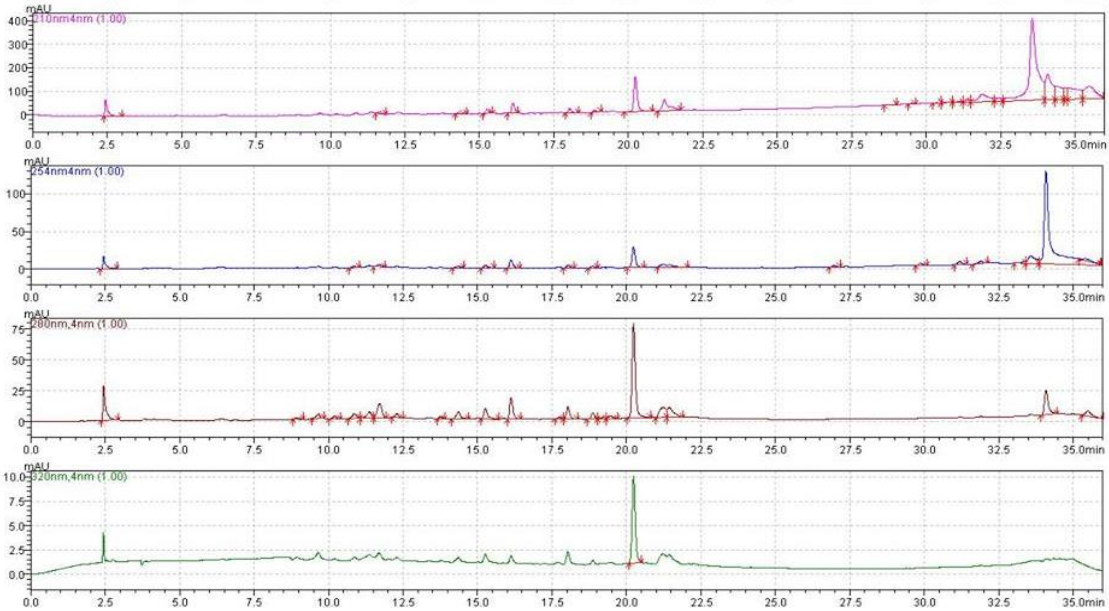


Figure 4.13: Chromatogram of fraction 9 under 210, 254, 280 and 320 nm (from top to bottom)

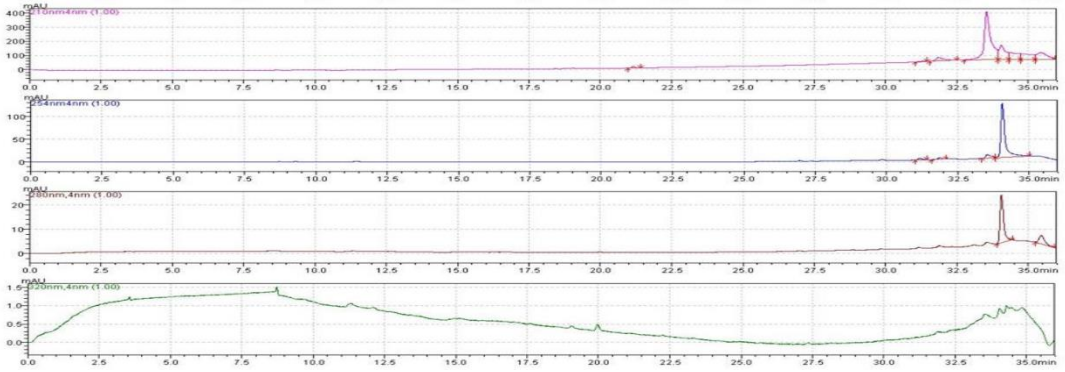


Figure 4.14: Chromatogram of blank under 210, 254, 280 and 320 nm (from top to bottom)

The chromatogram of the blank shows that the peak beyond 30 mins are belong to the solvent. The peaks in the chromatogram of fraction 8 and 9 that beyond 30 mins are belong to the solvent instead of the compounds. For fraction 8, there are many peaks shown in between 12.5 min and 22.5 min. There might be at least six compounds present inside the fraction due to the presence of six sharp, distinguishable peaks. The absorbance of those peaks are the strongest at 210 nm, followed by 280 nm, 254 nm and lastly, 320 nm.

Fraction 9 also showed many peaks with retention time between 10.5 min and 22.5 min in which some peaks were similar to those present in fraction 8. Both of these fractions might contain some similar compounds but the amount of them might lesser in fraction 9 due to lower intensity. The most distinguishable peaks are at 2.5 min and around 20 min. The peak at around 20 mins was present in both fraction with almost similar intensity. The compound in this peak might be the

major component in both fraction due to its highest intensity in both fractions Similar to fraction 8, the absorbance of those peaks are also the strongest at 210 nm, followed by 280 nm, 254 nm and 320 nm.

This analysis shows that both of the fractions contain many compounds. The high antifungal activity might be due to any one of them or those compounds act synergistically to give a higher antifungal properties.

CHAPTER 5

CONCLUSION

The mobile phases used to separate the compounds in the crude extract was mixture of ethyl acetate: chloroform: methanol: formic acid. From the bioassay, the methanol extract of the kernel of *Mangifera pajang* contains strong antifungal properties against *Candida albicans* and *Candida parapsilosis*. Almost all the fractions collected from column chromatography inhibit the fungus population by killing action. This was proved by the data in which almost each of the fractions have the similar MIC and MFC values which mean that the compounds within the fractions kill the fungus population instead of just inhibited their growth. Fraction 7, 8 and 9 had the lowest MIC values against *Candida albicans* at 0.0013 mg/ml and while fraction 8 and 9 had the lowest MIC values against against *Candida parapsilosis* at 0.0003-0.0006 mg/ml. Their MFC values were similar to their respective MIC values. Fraction 8 was eluted by ethyl acetate: chloroform: methanol: formic acid in ratio of 7: 10: 3: 0.2 while fraction 9 was eluted by ethyl acetate: chloroform: methanol: formic acid in ratio of 9: 8: 3: 0.2.

SUGGESTIONS FOR FURTHER STUDIES

Firstly, it will be the best if those compounds present in fraction 8 and 9 can be separated and isolated by using other chromatographic method. Then, each of the compound obtained can be identified and tested for their antifungal properties against *Candida albicans* and *Candida parapsilosis* in order to figure out which compounds are responsible for that strong antifungal activity or they act synergestically to enhance the overall antifungal properties. The compounds within those fractions are worth to investigate because they can kill the fungus at very low concentration which can be utilized for the medical application to cure fungal infection.

Furthermore, the mechanisms of the antifungal action of the compounds in fractions 8 and 9 against *Candida albicans* and *Candida parapsilosis* could be investigated to explore the potential of other classes of compounds to become an antifungal agent.

REFERENCES

Abu Bakar, M., Mohamed, M., Rahmat, A. and Fry, J. (2009). Phytochemicals and antioxidant activity of different parts of bambangan (*Mangifera pajang*) and tarap (*Artocarpus odoratissimus*). *Food Chemistry*, 113(2), pp.479-483.

Ahmad, A., Khan, A., Manzoor, N. and Khan, L. (2010). Evolution of ergosterol biosynthesis inhibitors as fungicidal against Candida. *Microbial Pathogenesis*, 48(1), pp.38-39.

Ahmad, S., Sukari, M., Ismail, N., Ismail, I., Abdul, A., Abu Bakar, M., Kifli, N. and Ee, G. (2015). Phytochemicals from *Mangifera pajang* Kosterm and their biological activities. *BMC Complementary and Alternative Medicine*, 15(1).

Ahmed, I., Tohami, M., Almagboul, A. and Verpoorte, R. (2012). Characterization of Anti-microbial Compounds Isolated from *Mangifera indica L* seed kernel. *University of Africa Journal of Science*, 2(1), pp.77-97.

Ahmed, S.O.M., Omer, M.A.A., Almagboul, A.Z., Abdrabo, A.N. (2005). In vitro antimicrobial activity of *Mangifera Indica L. Gezira Journal of Health Sciences*, 2(2), pp.82-91.

Badal, S. and Delgoda, R. (2017). Pharmacognosy: Fundamentals, Applications and Strategies. Academic press, pp.93-100.

Bennett, J., Dolin, R. and Blaser, M. (2014). *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases*. 8th ed. London: Elsevier Health Sciences, p.2879.

Campoy, S. and L. Adrio, J. (2017). *Antifungals*. Elsevier, pp.86-87.

Cheah, H., Lim, V. and Sandai, D. (2014). Inhibitors of the Glyoxylate Cycle Enzyme ICL1 in Candida albicans for Potential Use as Antifungal Agents. *PLoS ONE*, 9(4), p.e95951.

Dai, J. and Mumper, R. (2010). Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties. *Molecules*, 15(10), p.7314.

Dolan, J. (n.d.). *LC Troubleshooting: Why Do Peaks Tail?*. [online] Alfresco.ubm-us.net. Available at: http://alfresco.ubm-us.net/alfresco_images/pharma/2014/08/22/db291f1a-f9e7-48d1-9b96-8801d4514acf/article-69793.pdf [Accessed 13 Mar. 2018].

Drugs.com. (2018). Amphotericin B - FDA prescribing information, side effects and uses. [online] Available at: https://www.drugs.com/pro/amphotericin-b.html [Accessed 3 Mar. 2018].

Ediriweera, M., Tennekoon, K., & Samarakoon, S. (2017). A Review on Ethnopharmacological Applications, Pharmacological Activities, and Bioactive Compounds of Mangifera indica (Mango). *Evidence-Based Complementary And Alternative Medicine*, 2017, 1-24.

Espinel-Ingroff, A., Fothergill, A., Peter, J., Rinaldi, M. and Walsh, T. (2002). Testing Conditions for Determination of Minimum Fungicidal Concentrations of New and Established Antifungal Agents for *Aspergillus* spp.: NCCLS Collaborative Study. *Journal of Clinical Microbiology*, 40(9), p.3204.

Godoy, M., Victor, S., Bellini, A., Guerreiro, G., Rocha, W., Bueno, O., Hebling, M., Bacci Jr, M., Silva, M., Vieira, P., Fernandes, J. and Pagnocca, F. (2005). Inhibition of the symbiotic fungus of leaf-cutting ants by coumarins. *Journal of the Brazilian Chemical Society*, 16(3b), pp.670-671.

Kanwal, Q., Hussain, I., Latif Siddiqui, H. and Javaid, A. (2010). Antifungal activity of flavonoids isolated from mango (*Mangifera indica L.*) leaves. *Natural Product Research*, 24(20), pp.1907-1914.

Khoo, H., Azlan, A., Kong, K., & Ismail, A. (2016). Phytochemicals and Medicinal Properties of Indigenous Tropical Fruits with Potential for Commercial Development. *Evidence-Based Complementary And Alternative Medicine*, 2016, 3-10.

Litz, R. (2004). Biotechnology of Fruit and Nut Crops. Biotechnology in Agriculture Series (p. 40). Wallingford: CAB International.

Long, S., Prober, C. and Fischer, M. (2017). *Principles and Practice of Pediatric Infectious Diseases, 5th Edition*. 5th ed. Amsterdam: Elsevier, p.1231.

Ma, C., Abe, T., Komiyama, T., Wang, W., Hattori, M. and Daneshtalab, M. (2010). Synthesis, anti-fungal and 1,3-β-d-glucan synthase inhibitory activities of caffeic and quinic acid derivatives. *Bioorganic & Medicinal Chemistry*, 18(19), pp.7009-7011.

Ma, H., Liu, W., Frost, L., Wang, L., Kong, L., Dain, J. and Seeram, N. (2015). The hydrolyzable gallotannin, penta-O-galloyl-β-d-glucopyranoside, inhibits the formation of advanced glycation endproducts by protecting protein structure. *Molecular BioSystems*, 11(5), p.1339.

Marcondes, H., de Oliveira, T., Taylor, J., Hamoy, M., do Leonel Neto, A., de Mello, V. and Nagem, T. (2015). Antifungal Activity of Coumarin Mammeisin Isolated from Species of the Kielmeyera Genre (Family: Clusiaceae or Guttiferae). *Journal of Chemistry*, 2015, p.3.

Martins, N., Barros, L., Henriques, M., Silva, S. and Ferreira, I. (2015). Activity of phenolic compounds from plant origin against Candida species. *Industrial Crops and Products*, 74, pp.648-670.

Matlock, B. (2017). *Differences in bacterial optical density measurements between UV-Visible spectrophotometers*. [online] Thermofisher.com. Available at: https://assets.thermofisher.com/TFS-Assets/MSD/Technical-Notes/TN52236-E-Bacterial-Optical-Density-EN.pdf [Accessed 3 Mar. 2018].

Maurya, I., Thota, C., Sharma, J., Tupe, S., Chaudhary, P., Singh, M., Thakur, I., Deshpande, M., Prasad, R. and Chauhan, V. (2013). Mechanism of action of

novel synthetic dodecapeptides against *Candida albicans*. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1830(11), p.5193.

Mayer, F., Wilson, D. and Hube, B. (2013). *Candida albicans* pathogenicity mechanisms. *Virulence*, 4(2), pp.119-128.

Montagner, C., de Souza, S., Groposo, C., Delle Monache, F., Smânia, E. and Smânia Jr, A. (2008). Antifungal Activity of Coumarins. *Zeitschrift für Naturforschung C*, 63(1-2), p.22.

Pereira Gonzales, F. and Maisch, T. (2012). Photodynamic inactivation for controlling *Candida albicans* infections. *Fungal Biology*, 116(1), pp.1-10.

Pinto, E., Afonso, C., Duarte, S., Vale-Silva, L., Costa, E., Sousa, E. and Pinto, M. (2011). Antifungal Activity of Xanthones: Evaluation of their Effect on Ergosterol Biosynthesis by High-performance Liquid Chromatography. *Chemical Biology & Drug Design*, 77(3), p.219.

Potrykus, J., Stead, D., MacCallum, D., Urgast, D., Raab, A., van Rooijen, N., Feldmann, J. and Brown, A. (2013). Fungal Iron Availability during Deep Seated

Candidiasis Is Defined by a Complex Interplay Involving Systemic and Local Events. *PLoS Pathogens*, 9(10), p.e1003676.

Preedy, V., Watson, R. and Patel, V. (2011). *Nuts & seeds in health and disease prevention*. 1st ed. United States: Academic press, pp.184-187.

Pubchem.ncbi.nlm.nih.gov. (2018). *The PubChem Project*. [online] Available at: https://pubchem.ncbi.nlm.nih.gov/ [Accessed 17 Jan. 2018].

Razin, S. and Tully, J. (1996). *Molecular and Diagnostic Procedures in Mycoplasmology (Second Edition)*. 2nd ed. Academic Press, p.189.

Sachikonye, M. and Mukanganyama, S. (2016). Antifungal and Drug Efflux Inhibitory Activity of Selected Flavonoids Against *Candida albicans* and *Candida krusei*. *Journal of Biologically Active Products from Nature*, 6(3), pp.223-236.

Sanches, A., Lopes, G., Nakamura, C., Dias Filho, B. and Mello, J. (2005). Antioxidant and antifungal activities of extracts and condensed tannins from Stryphnodendron obovatum Benth. *Revista Brasileira de Ciências Farmacêuticas*, 41(1), p.104.

Singaravelu, K., Gácser, A. and Nosanchuk, J. (2014). Genetic determinants of virulence – *Candida parapsilosis*. *Revista Iberoamericana de Micología*, 31(1), p.17.

Sung, W. and Lee, D. (2010). Antifungal action of chlorogenic acid against pathogenic fungi, mediated by membrane disruption. *Pure and Applied Chemistry*, 82(1), pp.223-225.

Talba, A., Suleiman, M., Raji, M. and Oniye, S. (2014). Phytochemical Screening and In-vitro Antibacterial Activity of *Mangifera indica* (Mango) Kernel on Aeromonas caviae. *IOSR Journal of Pharmacy (IOSRPHR)*, 4(10), pp.47-48.

Tangah, J., Bajau, F., Jilimin, W., Chan, H., Wong, S. and Chan, E. (2017). Phytochemistry and Pharmacology of *Mangifera pajang*: An Iconic Fruit of Sabah, Malaysia. *Systematic Reviews in Pharmacy*, 8(1), pp.86-91.

Teodoro, G., Ellepola, K., Seneviratne, C. and Koga-Ito, C. (2015). Potential Use of Phenolic Acids as Anti-Candida Agents: A Review. *Frontiers in Microbiology*, 6.

Theplantlist.org. (2013). *The Plant List-Mangifera*. [online] Available at: http://www.theplantlist.org/browse/A/Anacardiaceae/Mangifera/ [Accessed 10 Jan. 2018].

Tiwari, V. and Mishra, B. (2011). Opportunity, challenge and scope of natural products in medicinal chemistry. Trivandrum: Research Signpost, pp.283-311.

Zore, G., Thakre, A., Jadhav, S. and Karuppayil, S. (2011). Terpenoids inhibit *Candida albicans* growth by affecting membrane integrity and arrest of cell cycle. *Phytomedicine*, 18(13), p.1189.