SCREENING FOR ANTIMICROBIAL ACTIVITIES IN ENDOPHYTES ISOLATED FROM *Melastoma malabathricum* IN KOTA SAMARAHAN, SARAWAK

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Screening for Antimicrobial Activities in Endophytes Isolated From
Melastoma malabathricum in Kota Samarahan, Sarawak

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This thesis was submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honours in Resource Biotechnology

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DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree qualification of this any other university or institution of higher learning.

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# Table of Content

Declaration......................................................................................................................ii

Acknowledgement.........................................................................................................iii

Table of Content.............................................................................................................iv

List of Abbreviation.........................................................................................................viii

List of Tables...................................................................................................................ix

List of Figures..................................................................................................................x

Abstract..........................................................................................................................xi

1.0 Introduction.................................................................................................................1

1.2 Antibiotics..................................................................................................................3

1.3 *Melastoma malabathricum* as Endophytes Sources..................................................4

1.4 Biology of Endophytes................................................................................................6

1.5 Production of Secondary Metabolites by Endophytes..............................................7

1.6 Test Bacteria and Test Fungal....................................................................................8

2.0 Methods and Materials..............................................................................................10

2.1 Preparation of Samples.............................................................................................10

2.2 Microorganisms Test................................................................................................10
2.3  Culturing of Test Microorganisms  

2.4  Optical Density Measurement  

2.5  Antimicrobial Test  

2.5.1  Test Bacteria  

2.5.2  Test Fungal  

2.6  Extraction of Secondary Metabolites from Endophytes  

2.7  Antimicrobial test towards extracts  

2.7.1  Standardization method  

2.7.2  Minimum Inhibitory Concentration method  

2.8  Identification and Characterization of Isolated Endophytes  

3.0  Results  

3.1  Preliminary Screening  

3.1.1  Antibacteria test  

3.1.2  Antifungal Test  

3.2  Antimicrobial Test on Methanol Extracts  

3.2.1  Antimicrobial Test for Methanol Extract  

3.2.2  Minimum Inhibitory Concentration
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>Cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celcius</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>Kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>mL</td>
<td>Mililitre</td>
</tr>
<tr>
<td>%</td>
<td>Percent</td>
</tr>
<tr>
<td>PDA</td>
<td>Potato Dextrose Agar</td>
</tr>
</tbody>
</table>
List of Tables

Table 3.1: The formation of zone of inhibition by endophytes towards test bacteria in Preliminary test. 16
Table 3.2: The formation of zone of inhibition by endophytes towards test bacteria in Secondary test 16
Table 3.3: Antimicrobial activity of methanol extracts of two endophytic fungi in M. malabathricum. 18
Table 3.4: Morphology endophytic in v8 juice media 22
Table 3.5: Microscopic observation for isolated endophytes 1c and 2d at 40x, 100x 22
List of Figures

Figure 2.1: The dilution process for extract endophytes isolates 1c and 2d. 14

Figure 3.1: The observation for samples 1c and 2d against antifungal test *F. oxysporum*. 17

Figure 3.2: Antimicrobial test for methanol extract using Minimum Inhibitory Concentration method 19

Figure 3.3: Microscopic observation of morphology of isolates 1c and 2d. 21
Screening for Antimicrobial Activities in Endophytes Isolated From *Melastoma malabathricum* in Kota Samarahan, Sarawak

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**ABSTRACT**

A study was carried out to determine the presence of antimicrobial activities in endophytes isolated from stem of *Melastoma malabathricum* in Kota Samarahan, Sarawak. A total of 20 fungal endophytes were successfully isolated from the stems and after initial observation only six isolates which were 1b, 1c, 2a, 2b, 2c and 2d were tentatively recognize as different species. All the six were screened on three different media which were Potato Dextrose Agar (PDA), V8 juice (V8) and Czapek Dox Agar (CDA). In the preliminary screening, fungal colonies 1b, 1c, 2c and 2d were active against the test bacteria consisting of *Enterobacter aeruginosa*, *Escherichia coli*, *Salmonella typhii* and *Staphylococcus aureus*. Two endophytes namely isolates 1c and 2d with higher potential of antimicrobial product were chosen for further analysis. However, in the antifungal test, zone of inhibition was not observed between isolates 1c and 2d against *Fusarium sp*. Extraction of secondary metabolites from endophyte isolates 1c and 2d was done using methanol. Methanol extract 1c and 2d were tested against test bacteria which were *Enterobacter aeruginosa*, *Escherichia coli*, *Salmonella typhii* and *Staphylococcus aureus*. There were two methodologies involve during antimicrobial screening for methanol extracts namely Standardization Volume method and Minimum Inhibitory Concentration (MIC). Only methanol extract 1c formed zone of inhibition against *Escherichia coli*, *Salmonella typhii* and *Staphylococcus aureus* in Standardization Volume method. Meanwhile, zone of inhibition was not observed with both extract methanol of isolates 1c and 2d in MIC test. In morphological studies, endophytic fungi isolated 1c showed morphology of *Zygomycetes spp*., the fungus displayed as mycelium sparse, slender, branches with no septate. Isolate 2d displayed characteristic of *Choanephora* in which showed white mycelium, conidiophores and branches at the apex. The study support that endophytes isolated from *M. malabathricum* could be useful sources of novel antibiotics for treatment in future.

Keywords: endophytes; antimicrobial activities; preliminary screening; minimal inhibitory concentration; standardization volume
ABSTRAK

Kajian terhadap kehadiran aktiviti antimikrob telah dilakukan ke atas pencilan kulat endofit daripada Melastoma malabathricum di kawasan Kota Samarahan, Sarawak. Sebanyak 20 pencilan kulat endofit berjaya dipencilkkan. Selepas pemerhatian awal hanya enam jenis kulat endofit masing-masing dilabel sebagai 1b, 1c, 2a, 2b, 2c dan 2d yang diperhatikan berbeza spesis telah dipilih. Kesemua enam kulat endofit dikulturkan ke atas tiga jenis media yang berbeza iaitu potato dextrose agar (PDA), V8 juice (V8) dan Czapek Dox Agar (CDA). Dalam ujian antimikrob awalan pencilan-pencilan endofit iaitu 1b, 1c, 2c dan 2d menunjukkan aktiviti antimikrob terhadap bakteria Enterobacter aeruginosa, Escherichia coli, Salmonella typhii dan Staphylococcus aureus. Endofit 1c dan 2d yang menunjukkan potensi yang tinggi sewaktu ujian antimikrob awalan telah dipilih untuk ujian antimikrob lanjutan. Walaubagaimanapun, bagi ujian antikulat, tiada aktiviti di antara 1c dan 2d terhadap Fusarium sp dapat diperhatikan. Hasil ekstrak methanol di uji ke atas bakteria ujian Enterobacter aeruginosa, Escherichia coli, Salmonella typhii dan Staphylococcus aureus. Terdapat dua kaedah yang digunakan dalam ujian ini iaitu kaedah penyeragaman kepekatan dan ‘Minimum Inhibitory Concentration (MIC)’. Hasil ekstrak 1c bertindak ke atas bakteria ujian Escherichia coli, Salmonella typhii dan Staphylococcus aureus dalam kaedah ‘standardization volume’. Sementara itu, tiada zon perencatan terbentuk dalam ujian MIC dapat diperhatikan. Dalam pencirian endofit pula, pencilan kulat 1c menunjukkan sifat Zygomycetes spp di mana kulat ini mempunyai struktur miselium putih yang jarang dan bercabang tetapi tidak mempunyai septa. Manakala, endofit 2d menunjukkan sifat Choanephora, iaitu miselium putih, menunjukkan konidia dan bercabang. Kajian ini menyokong bahawa pencilan dari M. malabathricum mungkin menyumbang kepada penemuan antibiotik yang berguna.

Kata kunci: endofit; aktiviti antimikrob; ujian antimikrob awalan; minimal inhibitory concentration; penyeragaman kepekatan
1.0 Introduction

The emergence of many new antibiotic-resistant bacteria in recent years has motivated researchers to discover novel antibiotics. Misuse of antibiotics has been cited as the major cause in the emergence of these antibiotic-resistant bacteria of which *Staphylococcus*, *Mycobacterium* and *Streptococcus* are most common (Mckenna, 1996). Moreover, the increasing occurrence of diseases such as acquired immune deficiency syndrome (AIDS), severe acute respiratory syndrome (SARS), influenza viruses such as H1N1 has impact on severity of opportunistic bacterial infection. Hence, there is an urgent need to discover new novel antibiotics or drugs to combat them (Strobel & Daisy, 2003; Weber *et al.*, 2007).

There are a various approaches for discovering antibiotics. The approaches include the utilization of natural sources, genomic, non-culturable bacteria, bacteriophages and synthetic route. Among these approaches, syntheses of antimicrobial substances are gaining popularity. However, isolation of antibiotics from natural sources especially microorganisms, remains popular. This is because techniques like synthetic approach may involve carcinogenic materials that may be harmful to health and the environments (Strobel & Daisy, 2003).

There are more than 120 types of medicines that have been extracted from microorganisms (Ahmed, 2008). According to Kurylowicz (1993), 50 % of the whole antibiotics isolated from microorganisms come from *Streptomycetes*. This is followed by plants (14%), fungi (10-11%) and other bacteria than the *Streptomycetes* (10%). Some of the most important antibiotics, such as penicillin are obtained from fungi. Hence, fungi still provide opportunities for discovery of new antibiotics. Although, there are several thousands of antimicrobial substance had been isolated, only 4000-5000 has been used commercially.
(Kurylowicz, 1993). This situation has meant that more work has to be done to discover effective antimicrobial substantial (Baquero & Blázquez., 1997).

In recent studies, many new bioactive compounds have been discovered from endophytic fungi. Examples of these new discovery include anti-\textit{Candida} metabolites from endophytes, antiviral and anticancer agents from endophytes (Inácio \textit{et al.}, 2006; Strobel & Daisy., 2003; Weber \textit{et al.}, 2007). Recently, endophyte with antimicrobial activity has been isolated from \textit{Melastoma malabathricum} found in the state of Selangor, Malaysia (Norefrina Shafinaz Md Nor, 2004). Because this plant is also found in Sarawak, it could be appropriate to conduct research on similar plants growing in this centre of biodiversity. Hence, the objectives of this research are:

a). To search for bioactive compounds from endophytic fungi living in \textit{M. malabathricum}.

b). To identify the endophytes by using macroscopic and microscopic technique.

c). To extract and test secondary metabolites from each endophyte against selected bacterial and fungal species.
1.2 Antibiotics

Microorganisms that live in extreme environment posses a special characteristic that enables them to survive which is they required an adaptation to fight the extreme environment by producing antimicrobial activities or antibiotics (Tortora et al., 2007). Antibiotics can be defined as drugs that can kill bacteria (Robert & Martin, 2005). After the first discovery of penicillin by Alexander Fleming in 1928 (Sabuncu et al., 2009), antibiotics has became vital in modern medicine (Coates & Hu, 2004).

The spread of bacterial resistance towards antibiotics has limited the future progress of medicine (Baquero & Blázquez, 1997). The evolution of bacteria from susceptible strains to bacterial resistance towards antibiotics has stimulates researcher to discover new antibiotics (Strobel & Daisy, 2003). Moreover, diseases such as acquired immune deficiency syndrome (AIDS), severe acute respiratory syndrome and patients with cancer and organ transplant require the discovery of new novel antibiotics and drugs (Weber et al., 2007).

Generally, the response of antibiotics on microorganisms can be divided into 2 groups which were agent that inhibit (static agent) or kill the microorganisms (cidal agent) (Lisa, 1999). Antibiotics kill microorganisms by disturbing the synthesis of cell wall by the microbes, cell membrane function, synthesis proteins, acid nucleic metabolisms and enzymatic functions (Joklik et al., 1992)

Coates et al., (2002) have proposed that there are a few ways to developing antibiotics, which are, penicillin has been discovered by scientific observation (Fleming, 1929). A natural compound contains a basic structure which is 6-aminopenicillanic acid that is used to produce analogues; amoxicillin (Rolinson & Geddes, 2007). Novel compounds have also been
developed from the non-natural chemical route include prontosil and oxazolidinones (Fernandez, 2006). Screening of compound collections with enzymes also has been used (Wang et al., 2006).

Although much research has been conducted, no new novel antibiotic has been discovered and the production of marketable antibiotics has declined. This could be explained by the decrease in research activities on natural sources of antimicrobials (Coates & Hu, 2004). A natural source has advantage for production of antibiotics due to low from toxicity, side effect but effective action of antimicrobial agents (Monagahan & Tkacz, 1990).

The current situation of antibiotic resistance may signal the end of the antibiotic era. To overcome this problem it is a must to continue research on finding novel antibiotics to overcome the world widespread emergence diseases and the spreading of antibiotic resistance

1.3 Melastoma malabathricum as Endophytes Sources

The species of *M. malabathricum* or commonly known Singapore rhododendron is included in family of Melastomaceae. It consists of 240 genera and 3000 species which are mainly distributed in tropical and subtropical areas (Dinda & Saha, 1988). The plant is bushy and can grow to 12 to 13 ft and as high as 20 ft (Deny et al., 2005). In Malaysia there are 180 species from 25 genera and known as senduduk (Henderson, 1959).

According to Burkill (1966), these plants usually grow at the edge of the stream, on landslips or old clearings and they are evergreen with flowers whole year round. There are three categories of *M. malabathricum* based on flower size and colour. Flower may be large,
medium or small with purple-magenta, pink-magenta or the rare white petals colours (Deny et al., 2005). The leaves are 0.25–2 inch wide, with stalks 0.25–0.5 inch long, flowers 1–3 inch wide, calyx closely set with short chaffy and silky or silvery scale (Deny et al., 2005). The reddish stems and leaves are covered with fine bristles, each flower has five petals and the fruits are identified as berries (Wong, 2008).

Choosing *M. malabathricum* as the source for endophytes isolation is considered reasonable since the plant has been known to show healing properties (Deny et al., 2005). From the record, *M. malabathricum* used as traditional Malay medicine to treat diarrhea, post-partum and haemorrhoids (Burkill, 1966). Furthermore, this plant has the ability to live in challenging environment condition where nutrient and water are lacking (Wong, 2008). In addition, tannin that is isolated from pink-magenta petals *M. malabathricum* is found to have antiviral activity against immunodeficiency virus (Yoshida et al., 1992). These examples have proven that natural products or antimicrobial from endophytes have the potential to inhibit or eliminates harmful disease agents not only phytopathogens, protozoans (Strobel & Daisy, 2003) but also human pathogens.

In addition, nature including plants has become an important source to discover medicinal compounds (Ahmed, 2008). Plants especially nominated as traditional medicine for examples *Erythrina crista-galli* (Weber et al., 2004), *Rhodomyrtus tormentosa*, and *Melastoma malabathricum*. Because of these, many scientists concentrate on extraction of biological active compounds from plants that are mainly used in traditional medicines (Sarac & Ugur, 2007). These enriching have stimulate researcher to conduct research on screening for antimicrobial substances produced from endophytes isolated from *M. malabathricum*. 
1.4 Biology of Endophytes

The decline in production of antibiotics from natural sources can be due to the difficulty to deal with the traditional sources of bioactive compounds including plants and a huge development in technologies of robotics, chemistry and molecular in producing antibiotics. But, when synthetic antibiotics have been proven to contain carcinogenic materials, people start to carefully study and select the biological sources (Ikram, 1995).

It has been reported in some studies that endophytic fungi causes latent fungal infection in grasses, shrubs and evergreen trees (Timothy, 1993). However, recent studies has shown that endophytes are important as source of antibiotics because natural products or antimicrobial from endophytes are effective in inhibiting or eliminating harmful disease agents such as phytopathogens, fungi, bacteria, viruses and protozoans (Strobel & Daisy, 2003).

According to Strobel and Daisy (2003), endophytes are considered as novel sources that show the potential for medical, agriculture and industry because it inhabit such unique characteristic in higher plants. Furthermore, the widespread population of endophytes that can withstand under stress environment and production of novelty biological activity isolated from endophytes complete the selection factors of novel organism.

Endophytes can be divided into the balansiaceous group that specifically colonize grasses and usually belong to ascomyceous and non-balansiaceous group that occur in almost all plant species (Schulz & Boyle, 2005). Concentration and diversity of endophytes in plant are influenced by type of tissue, age of plants and distant from inoculum source. Endophytes may live in intercellular spaces of stems, petioles, roots and leaves (Lu et al., 2006), particularly in vascular plant parts (Carol, 1988; Eshagi, 1998). Younger trees have lower
number of endophyte strains than older trees due to the high concentration of foliage in the older trees (Timothy, 1993). Trees that are further away from inoculums source will contain fewer fungal strains compared to younger trees that grow near to diverse inoculum sources (Timothy, 1993). In addition, climate is very important in the distribution of endophytes in a certain place. As shown by Taylor et al., (1999) different endophytes live in tropical and temperate area. Humidity influences the number of endophytes in a certain area, whereby, an area with high humidity have large number of endophytes (Fisher et al., 1994).

Bacon and White (2000) stated that endophytes occupy the internal part of plant tissues without causing negative infection. Aldrich-Markham et al., (2007) have stated that endophytes will be transmitted only through the seed and its entire life cycle takes place inside the plant tissue. Therefore, the non infected variety remains as non infected and the new plants will only be infected if the habitat is surrounded by infected variety. The relationship is maintained through symbiotics and mutualistic between endophytes and their host (George, 1988).

1.5 Production of Secondary Metabolites by Endophytes

Plants need a mechanism to defend themselves from any infection from microbial, herbivores thread and environmental stress. The protection system includes the production of toxic and nontoxic compounds (Zikmundova et al., 2002). The presence of endophytes inside plants helps the plant to produce new substances known as secondary metabolites or antimicrobial substances.
Strobel and Daisy (2003) reported that endophytes involve directly in the production of antibiotics, antiviral compounds, volatile antibiotics, anticancer agents, antioxidants, insecticidal activities, antidiabetic agents and immunosuppressive compounds. Moreover, the productions of secondary metabolites by endophytes are very crucial in plant protection mechanisms and valuable potential metabolites in therapeutic technology (Bills et al., 1994).

There are cases where endophyte produces mycotoxin that does not kill the host plant, but kills animal herbivores and plant pathogenic bacteria (George, 1988). For examples, *Rhabdocline parkeri*, an endophyte of Douglas fir possesses antagonistic reaction towards gall midges particularly in Douglas fir needles (Timothy, 1993). Besides, endophytes are able to stimulate the plant growth, increase disease resistance, improved plant’s ability to stand in stress environment (Sturz & Nowak, 2000). Thus, endophytes can be a source of antimicrobial diversity that can contribute to the discovering of novel antibiotics.

1.6 Test Bacteria and Test Fungal

Antibacteria test is usually carried out using Gram positive and Gram negative bacteria. This is to make sure that the screening process covers the reaction of metabolites towards the two major divisions of bacteria. It is very appropriate to choose test bacteria and test fungal that involve in screening test. Pathogenic microorganisms usually contribute to emergence of many diseases (Theuretzbacher, 2009).

Bacterial species that are usually used include *Staphylococcus aureus, Bacillus* substitute for Gram negative bacteria and *Escherichia coli, Enterobacter aeruginosa* and *Salmonella thyphii* for Gram positive bacteria. Test plant pathogenic or animal pathogenic
could be used. Antifungal activity test for plant pathogenic fungi may include the species \textit{Fusarium sp.}

Sometimes, bioactive compounds that have been isolated are tested against pathogens of human. These include \textit{Aspergillus spp} and \textit{Curvularia sp} which are opportunistic pathogens that infect person who are immunocompromised such as cancer and organ transplant (Tortora \textit{et al.}, 2005). Bacterial species include \textit{Staphylococcus aureus}, \textit{Escherichia coli}, \textit{Enterobacter aeruginosa} and \textit{Salmonella thyphii} are those whoch usually infect human and known as bacterial resistance to antibiotics (Bonhoeffer \textit{et al.}, 1997). The test organisms are selected according to the important role they have in human life and as an effort to discover novel antibiotics.

Bacterial cell have variety of shapes and sizes that responsible for cells maintaining and stress bearing elements (Huang \textit{et al.}, 2008). Both Gram negative and Gram positive bacteria are builds from polymer peptidoglycan which are glycans crosslinked by stretchable peptides (Datta \textit{et al.}, 2007). The difference between the bacteria is the thickness of cell wall; gram positive with thick peptidoglycans and gram negative with thin layer peptidoglycans (Datta \textit{et al.}, 2007).
2.0 Methods and Materials

2.1 Preparation of Samples

Stem of *M. malabathricum* was collected from Kota Samarahan, Sarawak around September to October 2009. Then, the collected samples were studied and the locations were recorded. The branches of the plant were cut into 10 cm pieces. The samples were wrapped by wet tissue and brought to the laboratory for further analysis. The stems were surface sterilized by immersing into Clorox 5 % for 5 minutes, followed by 70 % ethanol for another 5 minutes. The samples were rinsed thoroughly with sterile water for 5 minutes, and then the samples were blotted dry on sterile filter paper before culture. After that, the samples were cut by using sterile scalpel into 3 cm pieces. Then, samples were transferred onto Potato Dextrose Agar (PDA) plates. The plates were incubated for two to four days in the dark at room temperature (25±2° C). After incubation, the cultures were observed for growth of hyphal at the cut ends. The hyphal tips growth are known as endophytes, once the hyphal appears, it were transferred onto new PDA media. The endophytes were subcultured for three times to get the pure culture. After a pure culture was obtained, a culture of endophytes were transferred into PDA slant agar for stock culture and stored at 4 °C.

2.2 Microorganisms Test

There were two microorganism test conducted. Antibacterial test was carried out between Gram positive, *S. aureus*, and Gram negative bacteria, *E. coli*, *S. typhi* and *E. aeruginosa*. Antifungal test was carried out by using *Fusarium oxysporum* as test organisms. The antibacterial test was conducted on all isolates endophytes which were 1b, 1c, 2a, 2b, 2c and 2d in preliminary testing and on isolates 1b, 1c, 2c, 2d in secondary testing. Antifungal test
was carried out on 1c and 2d. Tests were conducted to ensure the isolated endophytes have antibacterial and antifungal activities. This is because further analysis test will be conducted for positive result in preliminary test.

2.3 Culturing of Test Microorganisms

Bacteria test were prepared from the stock culture. The bacteria were streak on nutrient agar (NA) and were kept inside 37 °C incubator for overnight. Then, the single colony of bacteria were inoculated into nutrient broth (NB) and incubated for 24 to 48 hours. Fungal test was cultured on PDA media.

2.4 Optical Density Measurement for Test Bacteria

After an overnight incubation, NB with test bacteria were measured for optical density (OD) by using spectrophotometer. The Optical Density (OD) was 0.6 at wavelength 520 nm. NB with free bacterium was used as control.

2.5 Antimicrobial Test

2.5.1 Test bacterial

For antibacterial test, agar overlay technique was applied. The endophytes isolates were cultured on PDA media and incubated 2 days in the dark at room temperature. Aliquots of 2-3 mL of NA soft agar were prepared in sterile bijou bottles. These melted top agar was placed in waterbath at 45-50 °C to avoid solidifying. Then, 0.1 mL of test bacteria was pippeted into NA soft agar. The mixture was homogenized in vortex mixer and poured evenly onto agar plate containing the endophyte cultures. After the NA soft agar was solidified, the agar plates were inverted upside down and incubated overnight for 37 °C. The plates were observed and
zone of inhibition were recorded. The isolates endophytes with positive results that show zones of inhibition were selected for further analysis.

2.5.2 Test fungal

Two types of endophytes which were 1c and 2d were selected for antifungal testing. Firstly, PDA plates were prepared; the endophytes were scooped out (0.5 cm) from the stock culture and transferred onto PDA plates. The test fungal (*F. oxysporum*) was scooped and placed at the center of the plates. After four days, observation was carried out.

2.6 Extraction of Secondary Metabolites from Endophytes

Each positive result of isolated endophytes was cultured on PDA media. After two weeks of growth, the cultures were dried inside laminar flow hood at room temperature and surface sterilises surrounding with 70 % alcohol were done everyday. After that, the dried agar was aseptically transferred into sterile beaker and was immersed into methanol for four days. After four days, the methanol extracts were filtered using sterile filter paper; the extracts were dried and were kept inside – 20 °C after drying process.

2.7 Antimicrobial Test Towards Extracts

2.7.1 Standardization method

The volume of the liquid extracts was measured. Aliquots of 400 µL of the extracts, from isolates 1c and 2d were measured and mix with 100 µL of sterile distilled water. The mixtures of extract and distilled water were vortex. Mueller Hinton Agar (MHA) containing test bacteria, which were *S. aureus* and *E. coli, S. typhi* or *E. aeruginosa*, were spread by sterile swab, evenly over the surface of a sterile agar plate. The Optical density (OD) measurement