ISOLATION AND CHARACTERIZATION OF ANTIBIOTIC FROM ENDOPHYTE OF *Melastoma malabathricum* L. IN KUCHING AREA

JANE SEBESTIAN TAKA (18590)

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<tr>
<td>ºC</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>%</td>
<td>Percent</td>
</tr>
<tr>
<td>uL</td>
<td>Micro liter</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
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<td>mg</td>
<td>Milligram</td>
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<td>ml</td>
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<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>PDA</td>
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</tr>
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</tr>
<tr>
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<td>NB</td>
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Isolation and Characterization of Antibiotic from Endophyte of Melastoma malabathricum L. in Kuching Area

Jane Sebastian Taka
Resource Biotechnology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

A study was done to determine the presence of antimicrobial substances in endophytic fungal isolates from Melastoma malabathricum L. plants. Twelve isolates of endophytes, designated as SB1, SB2, SB3, ST1.1, ST1.2, ST1.3, ST1.4, SP1.1, SP1.2, SP1.3, SP1.4 and SM1.1, were obtained from plant samples collected from four different locations in Kuching area, which were BDC area, Tabuan area, Pending area and Matang area. Preliminary screening for antimicrobial activities using fungal colonies revealed that only three, SB1.1, ST1.3 and SP1.1 were active against all test bacteria consisted of Gram positive bacteria, namely Staphylococcus aureus, and Gram negative bacteria, namely Escherichia coli, Salmonella typhi and Enterobacter aerogenes. Subsequently, for further screening using overlay agar technique, endophytes which showed positive antimicrobial activities in preliminary screening was used. In the test, only isolates ST1.3 was active against all test bacteria, while isolates SP1.1 active only against three test bacteria, except Staphylococcus aureus. Further study using methanol extract of isolates ST1.3 and SP1.1 was carried out using agar disc diffusion method. Methanol extract of isolates ST1.3 showed positive antimicrobial activities against Staphylococcus aureus and Enterobacter aerogenes, and no positive antimicrobial activities showed for methanol extract of isolates SP1.1. The minimal inhibitory concentration (MIC) of methanol extract of isolates ST1.3 that inhibited both test bacteria was 50mg/ml. Macroscopic observations for determination of the morphological characteristics of the endophytes showed that, isolates ST1.3 colonies have dark, asexual spores and having the septate mycelium. While isolates SP1.1 colonies have white colonies, not forming spore and having the septate mycelium. Hence, isolates ST1.3 and SP1.1 were tentatively identified as Aspergillus sp and Geotrichum sp respectively.

Key words: antimicrobial activity, methanol extracts, MIC, morphological characteristics.

ABSTRAK

Kajian terhadap kehadiran aktiviti antimikrob telah dilakukan ke atas pencilan kulat endofit daripada senduduk (Melastoma malabathricum L.). Dua belas pencilan kulat endofit iaitu SB1, SB2, SB3, ST1.1, ST1.2, ST1.3, ST1.4, SP1.1, SP1.2, SP1.3, SP1.4 dan SM1.1 dipencilkan dari empat lokasi di Kuching iaitu BDC, Tabuan, Pending dan Matang. Ujian antimikrob awalan pencilan endofit menunjukkan tiga daripada dua belas pencilan, iaitu SB1.1, ST1.3 dan SP1.1 mempunyai aktiviti antimikrob ke atas ujian bakteria dari Gram positif, Staphylococcus aureus dan Gram negatif, Escherichia coli, Salmonella typhi dan Enterobacter aerogenes. Ujian kedua aktiviti antimikrob dilakukan semula ke atas pencilan kulat endofit yang menunjukkan aktiviti yang positif dari ujian awalan. Hanya pencilan ST1.3 sahaja yang merencat semua ujian bakteria, manakala pencilan SP1.1 merencat semua ujian bakteria kecuali Staphylococcus aureus. Pengekstrakan menggunakan larutan metanol telah dilakukan bagi pencilan ST1.3 dan SP1.1. Dengan menggunakan kaedah resapan agar, ujian lanjutan antimikrobial bagi ekstrak metanol menunjukkan ekstrak metanol pencilan ST1.3 mempunyai aktiviti antimikrobial yang positif ke atas Staphylococcus aureus dan Enterobacter aerogenes, dan tiada sebarang aktiviti antimikrobial ke atas bakteria ujian bagi ekstrak metanol pencilan SP1.1. Kepekatuan minimum perencatan (MIC) bagi ekstrak metanol pencilan ST1.3 terhadap ujian bakteria ialah 50mg/ml. Dalam kajian pencirian morfologi endofit bagi kulat ST1.3, morfologi koloni menghasilkan spora berwarna coklat gelap dan mempunyai miselium yang bersel, manakala kulat SP1.1 menghasilkan morfologi koloni berwarna putih, miselium bersel tetapi tidak menghasilkan spora. Oleh itu, diremehkan pencilan kulat ST1.3 merupakan kulat dari spesies Aspergillus dan pencilan kulat SP1.1 merupakan kulat dari spesies Geotrichum.

Kata kunci: aktiviti antimikrobial, ekstrak metanol, pencirian morfologi.
1.0 Introduction

The emergence of multi-resistant bacterial strains has rendered many of the currently available antibiotics to become ineffective (Strobel and Daisy, 2003). This situation thus happened widely when the bacteria have the ability to resist certain antibiotics, thus make diseases which will be difficult to be treated. Because of the emergence of new diseases and acute shortage of effective treatment drugs have become a major public health concern. Therefore there is a need to search for novel antibiotics to prevent bacterial agents from becoming resistant and cause diseases.

One of the best sources to search for antibiotics can be found in plants. Plants are recognized as rich sources of medicines because they can produce compounds that give biological activity. These compounds are produce to act as a defense system against some diseases. Naturally compounds are preferred in producing new drugs because they pose less risk to the safety and environmental problems compared to the synthetic chemical compounds. Thus, natural products from plant offer great opportunities in finding new drugs (Strobel et al., 2004).

A variety of microorganisms such as bacteria and fungi inhabit the inside of plants. These are known as endophytic microorganisms. Endophytic microorganisms that colonize the host plant do not cause any negative effects towards the host plant. This phenomenon has made endophytes to become potential sources for the search on novel natural products for medical exploitation (Ratti et al., 2007). For example, a research has been done on endophytic fungi from coffee plant where the coffee plant endophytes have showed potential production
of antimicrobial compounds against pathogenic bacteria (Sette et al., 2006). Coffee plant is one of the popular natural resources because it have high antioxidant properties in it besides having good taste, thus has been processed commercially as foods and drinks. The properties may be produced from the microorganisms in the plant such as endophytes. Therefore it has been studied for their bioactive compounds. The secondary metabolite components produced by the microorganisms are very precious source of bioactive compound.

Hence, a study on endophytes that are isolated from the *Melastoma malabathricum* plants are used as a microorganisms sample in order to discover the potential of substance that it produced to form an inhibition to the pathogenic bacteria (Ratti et al., 2007). *M. malabathricum* plant is recognized as a plant that has rich source of medicinal compound because they can produce molecules that give biological activity for medicinal agents. Because of these properties, they are suitable to be use in the study to find novel antibiotic for pharmaceutical industries.

**Objective**

1. To isolate potential antibiotic producing microorganisms from endophyte of *Melastoma malabathricum* plant.
2. To screen for antimicrobial activities of isolated endophyte against indicator strains.
3. To screen for antibacterial activity at methanol extract of endophytes.
4. To identify the isolated endophyte up to genus level.
2.0 Literature review

2.1 The emergence of bacterial antibiotic resistant

Ever since the discovery of penicillin in 1950’s, antibiotics have played an important role in the medicine for control of infectious diseases. Antibiotics acts by antimicrobial mechanism in killing or inhibiting the growth of bacteria trough interference of cell membrane, interference of cell wall synthesis, interference of protein synthesis, protein inhibition and also by inhibition of nucleic acid (McClanahan, n.d). Unfortunately, the microorganisms have developed numerous ways to combat the action of many antibiotics, thus developing antibiotic resistance. For example, pathogenic microorganisms such as methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus faecium* (VREF) have developed resistance towards the current antibiotics such as penicillin, ampicillin and metilsillin (Zhang *et al.*, 2009).

Many factors have contributed to the problem of antibiotic resistance, of which mutation is of primary importance. When bacteria change and mutate, they can become resistant towards current antibiotic by reducing the antibiotic effects on them (Florini *et al.*, 2005). Mutation may occur because of uncontrolled used of antibiotics (Guillemot *et al.*, 1998) and inappropriate use of antibiotics among humans and agricultural animals (Hughes & Anderson, 2001). In agricultural animals, antibiotics are use to treat sick animals and prevent diseases in animals and also as a growth promoters in some animals to increase their weight gain more quickly.
With the problem of bacterial resistance and the emerging of infectious diseases, therefore there is a need to find new antibiotics, chemotherapeutic agents and agrochemical compounds. To alleviate the problem posed by the development of antibiotic resistance, a research have been done on many new resources to generate a new generation of antibiotics which is more specific and thus help in overcoming or decreasing the rate of antibiotic resistance (Strobel and Daisy, 2003).

2.2 Endophyte

Endophyte refers to the fungi which live almost entirely within the leaves and stems of apparently healthy host plants causing no visible signs of infections (Isaac, 1992). Within the host tissues, endophytes do not sporulate on the host plant but passed to the new plants vegetatively by growth into ovules and penetrate into plant tissue mainly by root, stomata, flowers and cotyledons. Both fungi and bacteria are the most common microorganisms that exist as endophytes. Because endophyte colonizes living internal tissues of plant without causing any negative effects, therefore there is a mutualistic relationship between the endophyte and the host plant (Sette et al., 2006).

The endophyte-host plant relationship give antagonists effect to the host plant where the endophytic fungi can restrict the growth of the plant pathogen by some mechanisms such as antibiosis, competition and parasitism mechanisms (Karlovsky, 2000). Antibiosis mechanisms are considered important because it produce secondary metabolites such as antibiotics and toxin which will show an antagonistic activity of fungal biocontrol agents against the plant pathogen (Karlovsky, 2000). Many reports also have shown that in a
microorganism-plant relationship, endophytes can contribute to possess various types of bioactivity substances such as antimicrobial and pesticidal effect (Onifade, 2007). Besides that, endophytic associations do not lead to the development of disease symptoms but do results in some morphological and physiological changes in host tissues which increase the survival and vigour of the plants, make plants more tolerant of water stress, recover more quickly than uninfected plants and may produce growth regulators (Isaac, 1992). However endophytes may also become pathogenic when they are under certain environmental conditions which give them stress. Potentially pathogenic endophytes may inhibit their hosts by stimulating the fungi themselves (Shamount, 2000).

In the earth ecosystem, the plants that have the greatest biodiversity will seem to be having the most diverse endophytes. Therefore, ecosystem plays a main role in influencing the general metabolism of endophytic microorganisms. In addition, tropical and temperate rainforest are the most biologically diverse terrestrial ecosystem on earth such as in Malaysia. Some reviews have stated that tropical endophytes produce large numbers of active secondary metabolites compare to other fungi from other substrata (Strobel et al., 2004).

Therefore, endopytic fungal products can be use as an antibiotic. Natural products from endophytic fungi have been observed to inhibit and kill variety of harmful microorganisms such as bacteria, fungi, viruses and protozoans (Strobel et al., 2004). For example Cryptosporiopsis cf. quercina, which was an endophyte from a medicinal plant, Tripterigeum wilfordii, was an excellent antifungal activity against Candida albicans and Trichophyton spp (Strobel et al., 2004).
2.3 Secondary metabolites production from endophytes

Endophytic fungi have been recognized as a potential source to produce bioactive secondary metabolites (Muhammad, 2009). A recent study has indicated that 51% of bioactive substances isolated from endophytic fungi were previously unknown (Haiyan Li et al., 2005). Hence, the endophytic fungi are expected to be a potential source for new natural bioactive products. Secondary metabolites are structurally diverse where they show biological effects at low concentrations and also act as carriers of chemical communication in their host plant (Karlovsky, 2000).

Plants infected with endophytes are often healthier compared with endophyte free plants because the plants that have been infected by endophytes can gain protection from herbivores and pathogens because of the presence of secondary metabolites that generated in the plant tissues. A review by Schulz et al. (2002) has described the diversity of metabolites isolated from endophytic fungi. These have functions in ecological role and some metabolites are synthesized based to its particular ecological niche. The interactions may enhance the synthesis of more secondary metabolites for chemical defense mechanism.

Tropical endophytes provide more active natural products and produced larger number of active secondary metabolites as compared with temperate endophyte (Srobel and Daisy, 2003). Plant secondary metabolites may play an ecological role such as becoming the host selective toxins of phytopathogenic fungi, producing antifungal compounds from the mycoparasitic fungi, producing mycotoxins that protect fungal reproductive structure from herbivores and induce the competitive and antagonistic interaction with the hosts (Schulz et
Metabolites of endophytes can also prevent the host from fungi, pests and mammals attack, inhibiting numbers of microorganisms and showing the identity with anticancer compounds produced by host plants (Sahar et al., 2006).

Besides that, endophytic fungi can contribute the mutualistic interactions with its host by increasing the concentration of defense metabolites against pathogen, by excreting phytohormones and by increasing the general metabolites activity of the plant host (Schulz et al., 1999). Various kinds of bioactive metabolites alkaloids, such as terpenoids, steroids and aromatic compounds are found in the internal part of the plant tissues (Sahar et al., 2006). Plant infected with endophyte are often healthier because endophyte produce phytohormones, cytokines and other plant growth promoting substances and also can enhance the host’s absorption of nutritional elements such as nitrogen and phosphorus and regulate carbon-nitrogen ratio (Zhang et al., 2006).

The example of the presence of endophytes that produced secondary metabolites in infected tissues can be seen in correlations between the presence of the endophyte *Phomopsis oblongata* and the demise of the bark beetles (Isaac, 1992). It is shown that feeding of the bark beetles on the endophyte infected wood led to a reduction in the reproductive capacity and a decline in the beetle populations. This may give rise to toxic effects on the insects causing poor larval growth and development and reduction in reproductive capacity of insects. Other review on endophytes that produce secondary metabolites for antimicrobial activities can be seen in Table 2.1.
Table 2.1: Review on endophytes producing antimicrobial activities

<table>
<thead>
<tr>
<th>Plants</th>
<th>Fungus</th>
<th>Metabolites substances and biological activities towards:</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scots Pine Needles</td>
<td>Lophodermium conigenum</td>
<td>Antimicrobial activities against <em>L. seditiosum</em></td>
<td>Carrol 1988</td>
</tr>
</tbody>
</table>

2.4  *Melastoma malabathricum* as endophytes source

The genus *Melastoma* species is included in the Family Melastomaceae. This Family is divided into three subfamilies which are Astonioideae, Memecyloideae and Melastomatoideae (Huan Keng, 1986). The main features of this plant are, the height of the plant is average 1m tall, and the stems are reddish in color and covered with small rough scales. The leaves are simple and narrow with three prominent longitudinal veins. The flowers have five petals, dark purple to pinkish in color and on rare occasions they can be found in white color. And the fruits are oval in shape with purple pulp which contains tiny seeds (Huan Keng, 1986). *Melastoma* species can be spread to long distance by humans in the horticulture trade and from the garden plants can be further spread by fruit eating birds and other mammals (Starr *et al*., 2003).

This plant has been used in many traditional medicinal purposes. Table 2.2 summarized the biological activities of *M. malabathricum* for traditional medical treatments in treating illness.
Table 2.2: Scientific research on *Melastoma malabathricum*

<table>
<thead>
<tr>
<th>Plant Parts</th>
<th>Biological Activities/Traditional Use</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf</td>
<td>Reduce high blood pressure, anti ulcer, anti inflammatory, antinociceptive</td>
<td>Kim <em>et al.</em> 2000</td>
</tr>
<tr>
<td></td>
<td>Treat wounds, scars from smallpox, astringent for dysentery</td>
<td>Burkill <em>et al.</em>, 1935</td>
</tr>
<tr>
<td>Flower</td>
<td>Drink to treat stomachache, remove black spots on skin</td>
<td>Kamarudin &amp; Latiff, 2002</td>
</tr>
<tr>
<td>Roots and leaf</td>
<td>Boiled water of roots and leaf can be drink to treat leucorrhoea</td>
<td>Burkill 1966</td>
</tr>
</tbody>
</table>

2.5 The test bacteria

*S. aureus* is a widespread bacterium carried by humans that can cause a number of problems from mild skin infections to serious diseases including food poisoning, wound infections, pneumonia and toxic shock syndrome. The World Health Organization (WHO) recently reported that more than 95% of *S. aureus* worldwide is resistant to penicillin, and 60% to it is derivative of methicillin (Kardar, 2005). Appropriate antibiotic for *S. aureus* infections is a problem because they are often drug resistant.

*S. typhi* also generally exhibit multiple antimicrobial resistances, which causes serious problems in the treatment of extra intestinal invasive infections. It was reported that antibiotics given to animals to treat and prevent disease was the primary cause of drug resistance in *Salmonella* species (Otkun *et al.*, 2001). Because diseased animals are frequently treated with penicillin, ampicillin, amoxicillin, cephalexin, cefapirin, cephacetrile and cefoperazone, this may be caused of why *S. typhi* also have problems in drug resistant (Otkun *et al.*, 2001).

Same with *E. aerogenes*, they are important nosocomial pathogens that responsible for various infections including bacteremia, lower respiratory tract infections, skin and soft-tissue
infections, urinary tract infections (UTIs), endocarditis, intra-abdominal infections, septic arthritis, osteomyelitis and ophthalmic infections (Fraser, 2010). Because *Enterobacter* species possess inducible beta-lactamases which are responsible for resistance during treatment, therefore physicians treating patients with *Enterobacter* infections are advised to avoid certain antibiotics particularly third-generation cephalosporins because resistant mutants can quickly appear.

*E. coli* are Gram-negative, anaerobic and non-sporulating bacteria of clinical importance. It is commonly found in the lower intestine of warm blooded animals such as human. Most *E. coli* strains are harmless, but some are recognized as important potential pathogens in humans such as serious food poisoning in humans. Studies with *E. coli* are importance mostly in the applications of medical because this species occupy multiple niches, including human and animal host (Obire et al., 2009). *E. coli* are resistant to many antibiotics that is effective to gram-positive bacteria and may also pass on the genes responsible for antibiotic resistance to other species of bacteria such as *S. aureus*.

In this study, the choice of examples for antibiotic targets is focused on clinically important pathogens such as *S. aureus* for Gram positive bacteria and *E. coli, S. typhi* and *E. aerogenes* for Gram negative bacteria.
3.0 Materials and methods

3.1 Collections and preparations of endophytes samples

Stem and branches of *M. malabathricum* were collected from three areas around Kuching which were BDC area, Tabuan area, Pending area and Matang area. Branches were cut by using scissors and wrapped in a wet tissue to avoid moisture loss. Then, the materials were brought to the laboratory and further processed for the isolation of endophytes.

<table>
<thead>
<tr>
<th>Samples Locations</th>
<th>Endophyte Isolates</th>
</tr>
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<tbody>
<tr>
<td>BDC</td>
<td>SB1, SB2, SB3</td>
</tr>
<tr>
<td>Tabuan</td>
<td>ST1.1, ST1.2, ST1.3, ST1.4</td>
</tr>
<tr>
<td>Pending</td>
<td>SP1.1, SP1.2, SP1.3, SP1.4</td>
</tr>
<tr>
<td>Matang</td>
<td>SM1.1</td>
</tr>
</tbody>
</table>

3.2 Cultivations of endophytes on agar

Upon collecting, the samples were cut into smaller pieces of about 3 cm length by using sterilized surgical blades. Then, the samples were surface-sterilized by immersing them sequentially in 5% sodium hypochlorite for 5 minutes, 70% ethanol for 5 minutes and rinse thoroughly with sterile distilled water and blotted dry with sterile filter paper. The stems were peeled off after surface sterilization using sterile surgical blade in the sterilized cabinet. Then
they were incubated onto PDA media in room temperature until fungal growth appeared. The samples were prepared in triplicates

3.3 Subculturing of endophytes

The endophytes that showed different growth characteristics were further subcultured onto new PDA plates. Using the inoculation needle, the endophytic fungi were cultivated under aseptic conditions by using Bunsen burner and 70% ethanol as sterilization utensils. Then, they were incubated for 2 days at room temperature. The subculturing processes were done three times to obtain more pure culture.

3.4 Preparations of test microorganisms

The test microorganisms used were two Gram positive bacteria, which were S. aureus and three Gram negative bacteria which were E. coli, S. typhii and E. aerogenes. To prepare the bacteria inoculums, test bacteria were streaked onto the NA obtained from the stock bacteria and then incubated for 24 hour at 37°C. After an overnight incubation, one single colony of each test bacteria was inoculated into 3ml of NB and incubated for 24 hour at 37°C again. Before the antimicrobial screening test, the optical density (OD) of each test bacteria was measured by using spectrophotometer, and was adjusted to 0.6 OD at 520nm.
3.5 Preliminary screening of antimicrobial activities of endophytes

Preliminary screening for antimicrobial activities was done using agar overlay technique. Firstly, 0.1ml of each indicator test bacteria was pipette into 3ml of NA soft agar. The test bacteria and melted agar were mixed by vortexing the mixture. Immediately the mixtures were poured out onto V8 agar plate that contained the growing endophytes isolates. V8 agar media was formulated from the V8 juice that contained 100% of variety vegetables juice. The plates were gently shaken and evenly distribute the mixtures to avoid forming of bubbles and prevent the agitation of agar. After fully gelled, the plates were inverted and incubated for 24 hour at room temperature. Then the zones of inhibitions were observed.

3.6 Secondary screening of antimicrobial activities of endophytes

Further screening of antimicrobial activities was done on the endophytic fungi that showed positive inhibitions from the preliminary screening. The endophytes then were subcultured and grown each on the V8 agar and PDA. The same procedures of agar overlay techniques were done for secondary screening of antimicrobial activities of endophytes on same test bacteria. Then the zones of inhibitions were observed.

3.7 Screening of antifungal activities of endophytes

To test for antifungal activities, *Fusarium sp.* was used as a test fungal. The endophytic fungi that showed positive inhibition from secondary screening were subcultured onto V8 agar
media together with *Fusarium sp.* After few days, the formations of zones inhibition activities of endophytes culture towards test fungus were observed.

### 3.8 Endophytes cultivation and antibiotics extractions of metabolites

The endophytic fungi that showed the presence of zone of inhibition in secondary screening were further subcultured for antibiotics extractions of metabolites. Each endophytes were cultivated and cultured onto the V8 agar media or PDA for several weeks. After the endophytes had grown to the edge of the plate, the samples were let to dry at room temperature. The endophytes samples were dried by placing the petri plate upside down without its cover for two to three weeks. The surfaces were cleaned with 70% ethanol every day to prevent contaminations. After drying process, the agar mycelium were crushed by using mortal and pestle and subjected to methanol extractions for 4 days in sterile beaker. Then, methanol extract were filtered by using sterile filter paper. The filtrates were let to be dried at room temperature.

### 3.9 Determination of Minimal Inhibitory Concentrations (MIC)

This test was conducted to get the minimum concentrations of inhibitions from the extracted endophytes. The antimicrobial test was conducted by using Agar Disc Diffusion method. First, 0.1g of dried methanol extracts were weighed and then dissolved again with 100ul of 100% methanol and 900ul PBS to get the final concentrations of 100mg/ml of stock solutions. To prepare for the minimal inhibitory concentrations (MIC), the extracts were diluted to 50mg/ml, 25mg/ml, 12.5mg/ml and 6.25mg/ml. Then, seven sterile filter paper discs were arranged on
the MHA media that have been swabbed with the same test bacteria. Volume of 30ul of extract with 100 mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml and 6.25mg/ml concentrations were pipette onto each discs. Another one empty discs were pipette with 30ul of PBS that contain 100% methanol as a negative control and 30ul of 5x dilutions penicillin-streptomycin antibiotic solution on the other empty disc as positive control. Petri plate then incubated for 24 hour at 37°C. After overnight incubation, the diameter of zone of inhibition around each disc was measured. The lowest concentrations that formed the inhibition zone were considered the MIC value.

4.0  Morphological characterization of endophytic fungi

The characterizations and identifications of endophytes were done by microscopic and macroscopic techniques towards the endophytic fungi that showed zone of inhibiton against test bacteria. For macroscopic observations, the colony characteristic of endophytic fungi that had been observed were the color of colonies, the parameter of the colonies, the texture of mycelia, the mycelia mat and their reverse color. For microscopic examinations, the mycelial ends, the spores shape, the color of hyphae and the septate hyphae were observed.
4.0 Results

4.1 Preliminary screening of antimicrobial activities of endophytes

From the preliminary screening, antimicrobial activities were observed in three from twelve isolates of endophytic fungi. This was shown by the presence of zones of inhibitions against the test bacteria, by the isolates SB1, ST1.3 and SP1.1 (Figure 4.1; Table 4.1).

When compared to other endophytes isolates, the isolates SP1.1 clearly showed bigger zone of inhibition against all test bacteria, with diameter of zone of inhibition approximately around 3mm-4mm. In comparison, both isolates SB1 and ST1.3 showed smaller diameter of zone of inhibition against all test bacteria which range approximately around 1mm-3mm in diameter.

Figure 4.1: Preliminary screening of isolates SP1.1 showing antimicrobial activities against A: *E. coli*; B: *E. aerogenes*; C: *S. typhii*; D: *S. aureus* by producing zone of inhibitions.
Table 4.1: Results in preliminary screening of antimicrobial activities of endophytic isolates against all test bacteria on V8 agar

<table>
<thead>
<tr>
<th>Test bacteria</th>
<th>Endophytic fungi and presence of zone of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SB1</td>
</tr>
<tr>
<td><em>S. typhii</em></td>
<td>+</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>+</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>+</td>
</tr>
<tr>
<td><em>E. aerogenes</em></td>
<td>+</td>
</tr>
</tbody>
</table>

* Relative size of inhibition zone: - (no zone of inhibition); + (approximately 1mm in diameter); ++ (approximately 2mm in diameter); +++ (approximately 3mm in diameter); ++++ (approximately 4mm in diameter)