SCREENING FOR ANTIMICROBIAL ACTIVITIES IN MYCOBIONTS ISOLATED FROM LICHENS COLLECTED FROM PALM TREES IN UNIMAS CAMPUS

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Bachelor of Science with Honours (Resource Biotechnology) 2010
SCREENING FOR ANTIMICROBIAL ACTIVITIES IN MYCOBIONTS ISOLATED FROM LICHENS COLLECTED FROM PALM TREES IN UNIMAS CAMPUS

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This project is submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honours (Biotechnology)

Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2010
Declaration

I hereby declare that this thesis entitled “Screening for Antimicrobial Activities in Mycobionts Isolated from Lichens Collected from Palm Trees in UNIMAS Campus” is the result of my own research work and effort. It has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged.

Signature:

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Date: 26\textsuperscript{th} May 2010
Acknowledgement

First and foremost, I owe my deepest gratitude to my supervisor, Prof. Dr. Ismail bin Ahmad for the valuable guidance and advices. This study would not be possible without the encouragement, guidance and support from Prof that helped me to develop understanding on this study. The personal guidance from Prof from the beginning until the final parts of this study has provided a good basic for me to complete this study. His wide knowledge and vast experiences have been of great value for me that provided me with detailed and constructive comments.

Besides, I am heartily thankful to my co-supervisor, Puan Fazia binti Mohamad Sinang. Puan Fazia has provided me with extra information as a guidance regarding this study. I am grateful that she is always there to inspire me and give me untiring help to work in this study. Her willingness to guide and advise has helped me to complete my Final Year Project.

Furthermore, I would like to show my gratitude to University Malaysia Sarawak (UNIMAS) that supply me with good environment and laboratory facilities to complete my study. An honourable mention to post-graduate students, lab assistants and my colleagues in Virology Laboratory who are assisted me in carrying out this study.

Lastly, I would like to thank my family and friends as well as all those who supported me during the completion of the Final Year Project.
Table of Contents

Acknowledgement........................................................................................................I

Table of Contents........................................................................................................II

List of Abbreviations.....................................................................................................V

List of Tables................................................................................................................VI

List of Figures...............................................................................................................VII

Abstract......................................................................................................................1

1.0 Introduction.............................................................................................................3

2.0 Literature Review....................................................................................................5
   2.1 Antimicrobial Agents.........................................................................................5
   2.2 Antibiotics-Resistant Bacteria..........................................................................6
   2.3 Lichens...............................................................................................................7
      2.3.1 Bioactive metabolites from mycobionts of lichens.................................9
   2.4 Palm Trees........................................................................................................9
   2.5 Agar Overlay Technique..................................................................................10
   2.6 Antimicrobial Susceptibility Testing.................................................................11

3.0 Materials and Methods..........................................................................................12
   3.1 Preparation of Media.......................................................................................12
      3.1.1 Preparation of PDA media.......................................................................12
      3.1.2 Preparation of V8 juice agar media.........................................................12
      3.1.3 Preparation of MHA media.....................................................................13
      3.1.4 Preparation of PDA slant.......................................................................13
3.2 Sampling Sites and Collections...............................................14
3.3 Surface Sterilisation..........................................................14
3.4 Fungal Cultivation.............................................................14
3.5 Isolation, Subculture and Storage of Fungal Isolates.................15
3.6 Preparation of Bacterial Culture Suspension............................15
  3.6.1 Inoculation of test bacteria ...........................................15
  3.6.2 Standardisation of bacterial inoculums ..............................15
3.7 Preliminary Antimicrobial Activity Screening..........................15
  3.7.1 Preliminary antimicrobial activity screening on PDA plate.......16
3.8 Secondary Antimicrobial Activity Screening on V8 Juice Agar Plate......16
3.9 Isolation of Antimicrobial Agent from Fungal Isolates..............17
3.10 Antimicrobial Activity Testing.............................................17
3.11 Characterisation and Identification of Fungal Isolates..............18
4.0 Results..................................................................................20
  4.1 Isolation of Lichenic Fungi..................................................20
  4.2 Preliminary Antimicrobial Activity Screening on PDA Plate........20
  4.3 Secondary Antimicrobial Activity Screening on V8 Juice Agar Plate....23
  4.4 Antimicrobial Activity Testing.............................................24
  4.5 Characterisation and Identification of Fungal Isolates..............26
5.0 Discussion..............................................................................30
  5.1 Sampling Lichens and Mycobiont Isolates..............................30
  5.2 Preliminary Antimicrobial Activity Screening on PDA Plate..........30
  5.3 Secondary Antimicrobial Activity Screening on V8 Juice Agar Plate....31
  5.4 Kirby-Bauer Disc Diffusion Susceptibility Test........................32
  5.5 Antimicrobial Activity Testing.............................................32
5.6 Characterisation and Identification of Fungal Isolates

6.0 Conclusion

References

Appendix A
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDA</td>
<td>Potato Dextrose Agar</td>
</tr>
<tr>
<td>NA</td>
<td>Nutrient Agar</td>
</tr>
<tr>
<td>NB</td>
<td>Nutrient Broth</td>
</tr>
<tr>
<td>MHA</td>
<td>Muller-Hinton Agar</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>Calcium Carbonate</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>S. pneumonia</td>
<td><em>Streptococcus pneumonia</em></td>
</tr>
<tr>
<td>C. candelaris</td>
<td><em>Chrysothrix candelaris</em></td>
</tr>
<tr>
<td>S. aureus</td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>E. aerogenes</td>
<td><em>Enterobacter aerogenes</em></td>
</tr>
<tr>
<td>S. typhi</td>
<td><em>Salmonella typhi</em></td>
</tr>
</tbody>
</table>
List of Tables

Table 4.1 Brief description of lichen samples ......................................................... 20
Table 4.2 Inhibition of test bacterial growth by eight fungal isolates grown on PDA plates ........................................................................................................ 21
Table 4.3 Inhibition of test bacterial growth by six fungal isolates grown on V8 juice agar plates ........................................................................................................ 23
Table 4.4 Inhibition of test bacterial growth by six methanol extracts ...................... 25
Table 4.5 Comparison of macroscopic characteristics of fungal isolates on PDA and V8 juice agar plates ........................................................................................................ 27
Table 4.6 Microscopic characterisation of fungal isolates ....................................... 28
List of Figures

Figure 3.1 Positions of paper discs for methanol extract and control on MHA plate.....18

Figure 4.1 Fungal isolates that formed zones of inhibition when tested with \textit{E. aerogenes}
and graphical representation.................................................................22

Figure 4.2 Methanol extracts L2(2), L2(3), L2(5) and L10(2) that exhibited zones of
inhibition towards \textit{E. aerogenes} with 5X penicillin-streptomycin as positive
control at center......................................................................................25

Figure 4.3 Methanol extracts L2(2), L2(3), L2(5) and L10(2) that formed zones of
inhibition towards \textit{E. aerogenes} with autoclaved distilled water as negative
control at center......................................................................................26

Figure 4.4 Macroscopic characteristics of fungal isolates grown on PDA plate........29

Figure 4.5 Reddish color of V8 juice agar plate changed to transparent......................29

Figure A Lichen sample L2 (A) and L10 (B)......................................................40
Screening for Antimicrobial Activities in Mycobionts Isolated from Lichens Collected from Palm Trees in UNIMAS Campus

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ABSTRACT

The emergence and spread of multi-drug resistant bacteria have become a global threat to the public health. This is due to the ineffectiveness of life-saving antibiotics. Lichens are receiving great attention from researchers as significant new sources of bioactive substances since their secondary metabolites are reported to have antimicrobial activities. This study was done to screen the antimicrobial activities of mycobionts isolated from lichens collected from palm trees in UNIMAS campus. A total of 28 fungal isolates were subjected to preliminary screening by agar overlay technique against three Gram negative bacteria, *Enterobacter aerogenes*, *Escherichia coli*, *Salmonella typhi* and one Gram positive bacteria, *Staphylococcus aureus*. Eight fungal isolates showed inhibitory effect towards Gram negative bacteria, but none inhibited Gram positive bacteria. All the eight fungal isolates were subjected to secondary screening on V8 juice agar plates for further testing. Only six of the fungal isolates formed zones of inhibition against the test bacteria. Extraction using methanol solvent was performed on the six fungal isolates to extract the active secondary metabolites. From the result of antimicrobial activity screening by Kirby-Bauer disc diffusion method, methanol extracts of L2(2), L2(3), L2(5) and L10(2) isolates formed zones of inhibition against *E. aerogenes*. The largest diameter of the zone of inhibition was observed for L10(2) which was 15.5mm, while the smallest was 9.5mm for L2(2). Macroscopical and microscopical observations using slide culture technique revealed that the fungal isolates L2(2), L2(3) and L2(5) possessed similar morphological characteristic and implied to belong to the same genus while L10(2) was a different fungi.

Key words: antibiotic-resistant bacteria, antimicrobial activity, lichen, mycobionts, zone of inhibition
Screening for Antimicrobial Activities in Mycobionts Isolated from Lichens Collected from Palm Trees in UNIMAS Campus

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ABSTRACT

The emergence and spread of bacteria that have multiple antibiotic resistances threatens public health. This is due to the ineffectiveness of antibiotics. Lichens have received attention from researchers as a source of new active compounds because secondary metabolites from lichens have been reported to have antimicrobial activity. A study has been conducted on the presence of antimicrobial activity in mycobionts from lichens collected from palm trees in the UNIMAS campus. An initial antimicrobial test was carried out on 28 pencil stubs against three Gram-negative bacteria, Enterobacter aerogenes, Escherichia coli, and Salmonella typhi, and one Gram-positive bacteria, Staphylococcus aureus. Only eight pencil stubs showed positive results against Gram-negative bacteria, but none of the pencil stubs showed antimicrobial activity against Gram-positive bacteria. Further tests were carried out on the eight remaining pencil stubs using V8 juice agar. Results showed that only six pencil stubs had antimicrobial action. Extraction with methanol was performed on these six pencil stubs to obtain active substances. The agar Kirby-Bauer method was used to test antimicrobial activity. The results showed that methanol extracts L2(2), L2(3), L2(5) and L10(2) could form a diffusion zone, but only E. aerogenes. Pencil stub L10(2) formed a large diffusion zone 15.5mm in diameter, while the smallest 9.5mm was formed by L2(2). Macroscopic and microscopic observation of slaid cultures showed that pencil stubs L2(2), L2(3) and L2(5) had the same characteristics, but L10(2) was a different pencil stub. Key words: bacteria resistant to antibiotics, antimicrobial activity, lichen, mycobiont, diffusion zone.

Kata kunci: bakteria yang rintang terhadap antibiotik, aktiviti antimikrob, liken, mykobiont, zon perencatan
1.0 Introduction

The overuse and misuse of antibiotics in the treatment of bacterial infections are triggering the increase of microorganisms that have acquired resistance, and this has led to the emergence of multi-drug resistant bacterial strains (Melgarejo et al., 2008). Drug-resistant bacteria have dramatically been contributing to the emergence of new diseases and the re-emergence of old diseases which have become one of the currently most urgent health issues (Morse, 1995). Many bacteria that were susceptible to antibiotics in the past have transformed to become resistant, while the development of antimicrobial agents is insufficient to counter with the emergence of antibiotic-resistant bacteria which multiply rapidly (Serrano, 2005). More importantly these antibiotic-resistant pathogenic microorganisms are evolving at an alarming rate.

Spontaneous mutation triggers genetic changes in bacteria and the bacteria develop resistance to antibiotics (Anderson, 2005). Genetic exchange among bacteria of both related and unrelated species causes the antibiotic-resistant genes to be incorporated into other bacteria. According to Melgarejo et al. (2008), 60% of *Streptococcus pneumoniae* strains are resistant to β-lactams and 60% of *S. aureus* strains are resistant to methicillin. Vacomycin was an alternative in treating *S. aureus* infections. However, resistance is beginning to develop towards that antibiotic as well. Consequently, it is crucial that concerted efforts be deployed in order to develop new antimicrobial agents with novel structures and activities in combating these threats.

In response to the shortage of antimicrobial agents available for treatment of diseases, medicinal chemists are still intensely developing new and improved antimicrobial agents to counteract the ability of bacteria to acquire resistance to currently used drugs in treatments of infectious disease.
Lichens have been used for medicinal purposes throughout the ages and represent an opportunity to be used as an alternative source for the development of new antimicrobial agents (Karthikaidevi et al., 2009). This opportunity arises since lichens produce secondary metabolites that function as chemical defenses against environmental stresses, thus providing support for their continued dominance (Nayanakantha et al., 2003). Various bioactive secondary metabolites have been isolated from lichens and numerous of them are used in pharmaceutical industries (Huneck & Yoshimura, 1996). These biologically active metabolites have clear significant biological roles as antibiotic, antitumoural, antimutagenic, antiviral, anti-inflammatory, plant growth inhibitor and enzyme inhibitor.

The presence of these secondary metabolites with antimicrobial activity have made lichens to become targets among researchers for new sources of bioactive substances. The discovery of biological active metabolites from lichens can augment the effort to develop novel antimicrobial agents. Hence, this study was performed to determine the biological active compounds produced in mycobionts isolated from lichens which can be used as new antimicrobial agents to overcome the limitations of drugs that still in use.

Objectives of this study are to:

1. isolate mycobionts from lichens collected from palm trees
2. extract antibiotics produced by mycobionts
3. screen the antimicrobial activity in mycobionts isolated from lichens by Kirby-Bauer disc diffusion method
4. characterise and identify the mycobionts isolated from lichens
2.0 Literature Review

2.1 Antimicrobial Agents

Serrano (2005) had defined antimicrobial agents as drugs of either natural products or synthetic chemicals that are able to kill or inhibit the growth and reproduction of microorganisms. Antimicrobial agents are one of the global priorities that help to control many infectious diseases and saved millions of lives (WHO, 2002). However, it has become apparent that the overuse and misuse of these drugs caused the evolution of bacteria that are resistant to the drugs used in common treatment and resulted in the emergence of multi-drug resistant bacteria (Lauterwein et al., 1995).

Aschenbrenner et al. (2008) have summarised that the mechanism of actions of antimicrobial agents include the prevention of the synthesis of bacterial cell wall, protein or nucleic acid, inhibition of metabolic pathways and disruption of bacterial membrane structure. Several antibiotics such as Penicillins and Cephalosporins bind to the proteins located within the bacterial cytoplasmic membrane. This action results in the inhibition of transpeptidase, which is needed for cell wall synthesis. Besides, antibiotics act to inhibit the synthesis of bacterial protein by binding to a portion of the bacterial ribosome. Inhibition of metabolic pathways in bacterium usually occurs through the use of competitive chemical analogs for bacterial enzymatic reactions. Polymyxins increase the bacterial membrane permeability and cause the leakage of cell components which would lead to the death of bacteria.

The development of new antimicrobial agents is not keeping pace with the emergence of bacteria that are resistant to currently used antimicrobial agents. According to Hancock (1997), although few new antibiotics are still in expansion, there are still no
new classes of antibiotic were developed in medical applications in more than 20 years. Hence, the medicinal chemists are intensely developing new and improved antimicrobial agents in order to remain a step ahead of antibiotic-resistant bacteria that has proceeded at an alarming rate.

2.2 Antibiotics-Resistant Bacteria

Bacteria are acquiring resistance to existing antibiotics used against them in direct correlation with the degree of application of the antibiotics to treat them (Lauterwein et al., 1995). This has resulted in strains such as methicillin-resistant *S. aureus*, penicillin-resistant *S. pneumonia*, vancomycin-resistant enterococci which limited the therapeutically active antibiotics for treatment of diseases (WHO, 2002). Consequently previously treatable diseases become untreatable again as the bacteria that were susceptible to antibiotics in the past have become resistant so the antibiotics become useless. Therefore, new sources of bioactive substances have been searched from medicinal herbs, fungi and lichen (Karaman et al., 2003).

According to Tenover (2006), bacteria develop resistance to antimicrobial agents through mutation and selection or acquisition of new genetic information that encode resistance from other resistant organisms. Mutation in bacterial DNA triggers genetic change in bacteria (Anderson, 2005). The bacteria may produce enzymes that destroy the antibiotic, alter the antibiotic’s target site and express efflux systems to prevent the antibiotic from reaching the target site (Tenover, 2006). Consequently, the bacteria become resistant to antibiotic and spread under the selective pressure of use of that antibiotic. Horizontal gene transfer between same or different bacterial species or genera resulted in the acquisition of new genetic material from the resistant bacteria by antimicrobial-susceptible bacteria.
The emergence of antibiotic-resistant bacteria has the potential to jeopardise advanced medical treatments (Baquero & Blázquez, 1997). Therefore, emphasis has been placed on the research to develop and discover new and novel antibiotics from exotic resources. It is crucial that the new sources will create a new diversity of usable bioactive substances to generate a new era of antibiotics in order to control the antibiotic-resistant strains of bacteria (Melgarejo et al., 2008). Lichens that showed antimicrobial activities in some of their secondary metabolites have significant value in antimicrobial research for development of bioactive substances to be used in pharmaceutical industries (Gulluce et al., 2006).

2.3 Lichens

Lichens are structures that consist of symbiotic association between mycobiont (fungus) and photobiont (alga) (Sharma, 1992). The mycobiont that makes up lichen is usually a member of the Ascomycotina and less commonly a member of the Basidiomycotina. On the other hand, the photobiont may be blue green alga (cyanobacteria) or green alga (chlorophyceae). The mycobionts parasitise the algae and shelter them from high light intensity as well as provide water and minerals to the algae. In contrast, the algae carry out photosynthesis to produce sugar for the mycobionts to create metabolic energy.

Lichens grow very slowly and must be protected in order to survive in extreme environmental conditions (Huneck & Yoshimura, 1996). The study carried out by Din et al. (1999) showed that 33 of the total of 36 lichen samples examined contained secondary metabolites. The secondary metabolites produced act as chemical protection to support continued lichens dominance (Cocchietto et al., 2002). The secondary metabolites produced by lichens have biological activities such as antibiotic, antitumoural, antimutagenic, antiviral, anti-inflammatory, plant growth inhibitor and enzyme inhibitor
Various bioactive secondary metabolites have been isolated from lichens and some of them are applied in pharmaceutical industry (Karthikaidevi et al., 2009). According to Sharnoff (1997), 50% of all lichens have antibiotic properties. Consequently, lichens are attracting much attention among researchers as significant new sources of antimicrobial agents.

Lichens have been used for medical purposes since ancient times (Karthikaidevi et al., 2009). For example, *Cetraria islandica*, (Iceland moss), *Lobaria pulmonaria* and *Cladonia* species have been used in treatment of pulmonary tuberculosis. According to Karthikaidevi et al. (2009), researchers have studied the antibacterial activity of several secondary metabolites of lichens against Gram positive and Gram negative bacteria. According to Lawrey (1986), usnic acid, evernic acid and vulpinic acid were reported to inhibit the growth of Gram positive bacteria *S. aureus*, *Bacillus subtilis* and *Bacillus megaterium*. Besides, studies also reported antifungal activity for some lichens. As a result of the antimicrobial activity of their secondary metabolites, lichens are attracting attention among researchers as new sources of bioactive substances.

One of the lichen metabolites that is extensively investigated and widely used is the usnic acid. Usnic acid is a yellowish pigment and occurs in two enantiomeric forms (Yilmaz et al., 2004). Cocchietto et al. (2002) summarised its biological active roles as antibiotics, antiprotozoal, antiviral, antiproliferative, anti-inflammatory, analgesic, antipyretic, antitumor activities and UV absorption and protection. According to Yilmaz et al. (2004), usnic acid showed antibacterial activity against *Streptococcus mutans*. The study carried out by Shahi and Patra (2003) demonstrated that the culture of mycobionts extracted from lichen *Usnea longissima* in specified medium under proper conditions produced usnic acid which is effective against human pathogenic fungi. Besides, usnic acid also showed antifungal activities against some plant pathogens (Halama & Haluwin, 2003).
Therefore, it is evident that the lichen metabolites represent a valuable source for discovery of new antimicrobial agents in combating infectious diseases.

2.3.1 Bioactive metabolites from mycobionts of lichens

According to Tay et al. (2004), most of the secondary metabolites in lichens are produced by the mycobionts alone. The secondary metabolites produced are deposited in either the cortex or medulla in amorphous or crystals forms on the outer surface of the hyphae. According to Karthikaidevi et al. (2009), the mycobionts of lichens produced antibiotic secondary metabolites to protect them against pathogenic microorganisms. Many studies have been conducted to study the secondary metabolites produced by the mycobionts isolated from lichen. Studies have proved that the secondary metabolites extracted from mycobionts isolated from lichen could be new sources of bioactive substances (Yukiko et al., 2001).

Culberson and Armaleo (1992) demonstrated that the mycobiont isolated from Cladonia grayi was able to produce depsida and depsidon. From the research of Russell et al. (1999), hybocarpone, a potent cytotoxin that is active against murine P815 mastocytoma cell line was successfully isolated from mycobiont of lichen Lecanora hybocarpa. The studies carried out by Yukiko et al. (2001) have proved that the mycobionts isolated from lichen were able to produce novel metabolites which can be applied in pharmaceutical industry.

2.4 Palm Trees

Palm trees are tropical plant in the family Arecaceae (Heatubun et al., 2009). A study showed the dry organic materials produced by palm trees are threefold more than other
species of trees (Teixeira et al., 2000). Hence, many studies were carried out to study the palm trees in forest ecosystem.

There were studies carried out for mycobionts of lichens collected from palm trees. Lücking and Matzer (2001) screened palm leaves for foliicolous lichen diversity. The result showed high diversity of foliicolous lichens on individual leaves. Moreover, Suprantini (1996) has studied the lichen species of *Chrysothrix candelaris* collected from palm trees. In her study, four mycobionts with different morphology were isolated from *C. candelaris*. The mycobionts isolated comprised of two genera, three from *Pestalotia* sp. and one from *Botryodiplodia* sp.. The result from the characterisation of the compounds extracted from the mycobionts revealed that most of them were belonged to the fatty acids group containing lactone and showed inhibitory effects.

### 2.5 Agar Overlay Technique

Agar overlay technique was used for preliminary and secondary antimicrobial activity testing because it is easier to perform and sharper edges of zones can be created so that the size of the zones can be measured precisely (Barry & Badal, 1982). The earlier study carried out by agar overlay technique showed that the inhibition zones produced were 1 to 2mm larger than those showed by Kirby-Bauer disc diffusion technique. Besides, a homogenous lawn of bacteria can be created within a layer of agar across the surface of the plate by agar overlay technique (Fankhauser, 2005).

According to Fankhauser (2005), the strength of an antimicrobial agent can be determined by agar overlay technique. The test bacteria spread on the plates through the top agar will grow and a homogeneously turbid lawn will be created. However, any inhibition of the bacterial growth will result in the reduction in the turbidity of the lawn.
near the antimicrobial agents. The size of the zone of inhibition formed around it reflected the strength of the antimicrobial agent. A wider zone of inhibition will be observed for a greater strength of antimicrobial agent.

2.6 Antimicrobial Susceptibility Testing

Kiska (1998) described that Kirby-Bauer disc diffusion method is commonly used as antibiotic susceptibility test to determine whether a bacteria is susceptible to a specific antibiotic. Kirby-Bauer disc diffusion test is a qualitative method for antimicrobial susceptibility testing. The paper discs which are impregnated with different concentration of antibiotic are placed on the surface of an agar plate that has been inoculated with test bacteria.

During incubation, the antibiotic diffuses outward into the medium and inhibits the growth of test microorganisms at certain concentration. The effectiveness of antibiotics toward test bacteria is analysed by the formation of inhibition zone around the paper disc. The diameter of the zone of inhibition is proportional to the degree of susceptibility of the test bacteria toward the antibiotic. The organism is characterised as susceptible, intermediate or resistant based on the diameter of the zone of inhibition formed.
3.0 Materials and Methods

3.1 Preparation of Media

3.1.1 Preparation of PDA media

A total of 19.5g of PDA powder was weighed in order to prepare 500ml of PDA media. The PDA powder was transferred into a 1000ml bottle and 500ml of distilled water was added. Then, the mixture was heated to boil on hot plate and stirred thoroughly for the PDA powder to dissolve completely. After that, the bottle was autoclaved.

Subsequently, the PDA media was poured into Petri dish for preparation of PDA plates used for culturing the sterilised lichen samples and the fungal isolates. About 13ml of the media was aseptically poured into each plate. After that, the plates were allowed to cool and solidify in the laminar flow hood before stored at 4°C for future use.

3.1.2 Preparation of V8 juice agar media

A total of 200ml V8 canned vegetable juice was filtered with filter cloth to eliminate the fibers in order to prepare 1000ml of V8 juice media. Then, 15.0g of Bacto agar and 2.0g of CaCO₃ was added to the filtered vegetable juice. The volume was made up to 1Liter with distilled water. Then, the media was heated to boil on hot plate and stirred thoroughly before autoclaved.

After that, the V8 juice media was poured into Petri dish for preparation of V8 juice agar plates used for culturing the fungal isolates. About 13ml of the media was aseptically poured into each plate. After that, the plates were allowed to cool and solidify in the laminar flow hood before stored at 4°C for future use.
3.1.3 Preparation of MHA media

A total of 18.0g of MHA powder was used to prepare MHA plates. The MHA powder was transferred into a 1000ml bottle and the volume was made up to 500ml by distilled water. Then, the mixture was heated to boil on hot plate and stirred thoroughly for the MHA powder to dissolve completely. After the MHA powder was completely dissolved, the bottle was autoclaved.

Subsequently, the MHA media was poured into Petri dish for preparation of MHA plates used for antimicrobial susceptibility test by Kirby-Bauer technique. Approximately equal volume of the media was aseptically poured into each plate. The MHA plates had been poured to an average depth of 4-5mm. After that, the plates were allowed to cool and solidify in the laminar flow hood before stored at 4°C for future use.

3.1.4 Preparation of PDA slant

A total of 19.5g of PDA powder was weighed and transferred into a 1000ml bottle in order to prepare PDA slant for storage of fungal isolates. The volume was made up to 500ml with distilled water. The mixture was heated to boil on hot plate and stirred thoroughly for the powder to dissolve completely.

Subsequently, the media was poured into the universal bottles until half of the total volume of the universal bottles. The universal bottles were then autoclaved at 121°C. After that, the universal bottles were allowed to stand at 45° in order to prepare PDA slant. The universal bottles were stored after the agar was solidified.
3.2 **Sampling Sites and Collections**

The lichen samples were collected randomly from palm trees at different areas in UNIMAS campus. Small section of lichen was cut off from the bark of the trees by using sterile scalpel and put into sterile plastic bag. The lichen samples collected were brought to the Virology Laboratory and immediately used for isolation of fungi. Ten lichen samples were collected for this study.

3.3 **Surface Sterilisation**

The collected lichen samples were scrapped off from the pieces of the bark of the trees. After that, the lichen samples were subjected to double sterilisation by using two sterilising agents. First, the lichen samples were immersed in 5% Clorox added with few drops of Tween 20 for 5 minutes. After dried-blot with sterile filter paper, the samples were immersed in 70% ethanol for 5 minutes. Then, the samples were rinsed with three-wash of autoclaved distilled water and dried-blot with sterile filter paper.

Alternatively, powdery type of lichen was sterilised by crushing it in sterile filter paper and filtered with 5% Clorox for 5 minutes. Subsequently, the lichen was filtered with 70% ethanol for 5 minutes. Then, the sample was rinsed with three-wash of autoclaved distilled water.

3.4 **Fungal Cultivation**

The surface-sterilised lichen samples were aseptically scraped out and then seeded onto PDA plates. The plates were incubated at room temperature for 3-4 days for fungal growth.