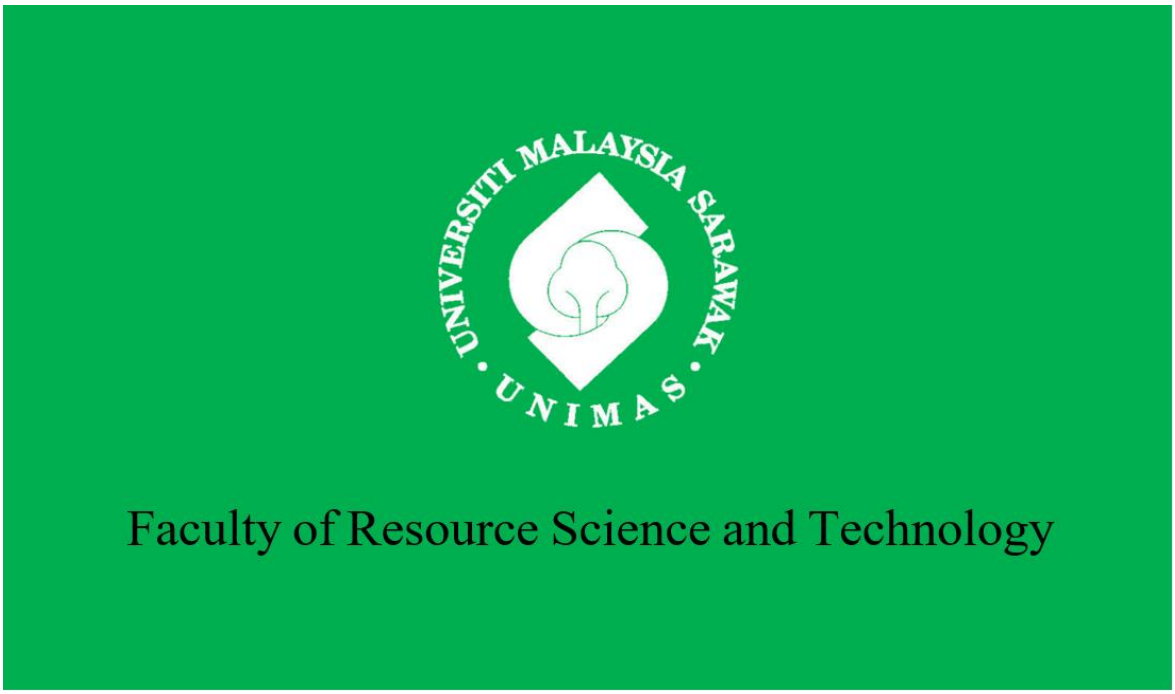

Antibacterial Activity of Endophyte *Marasmius cladophyllus* UMAS MS8

Dayang Nur Amira binti Abang Halim Badar Sham (72179)

Bachelor of Science with Honours

(Resource Biotechnology)

2022

UNIVERSITI MALAYSIA SARAWAK

Grade: 3rd Year

Please tick (√)

Final Year Project Report	√
Masters	
PhD	

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This declaration is made on the **14th** day of **June** 2022.

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I, **DAYANG NUR AMIRA BINTI ABANG HALIM BADAR SHAM (72179) from FACULTY OF RESOURCE SCIENCE AND TECHNOLOGY,** hereby declare that the work entitled, **Antibacterial Activity of Endophyte *Marasmius cladophyllus* UMAS MS8** is my original work. I have not copied from any other students' work or from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.

14th June 2022
Date submitted

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I**,_Dr Ngieng Ngui Sing ,** hereby certify that the work entitled, **Antibacterial Activity of Endophyte *Marasmius cladophyllus* UMAS MS8** was prepared by the above named student, and was submitted to the "FACULTY" as a * partial fulfillment for the conferment of Bachelor of Science with Honours (Resource Biotechonology), and the aforementioned work, to the best of my knowledge, is the said student's work

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Table of Contents

Antibacterial Activity of Endophyte *Marasmius cladophyllus* UMAS MS8

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ABSTRACT

The antibacterial activity of *Marasmius cladophyllus* was evaluated with different species of bacteria which consists of gram-negative bacteria and gram-positive bacteria. Different solvents; ethyl acetate and ethanol, were used to extract the bioactive compounds from supernatant and fungal biomass. Extracts from the two solvents showed different antimicrobial. Disc diffusion assay method was used to screen the antibacterial activity of *M. cladophyllus* against *Esherichia coli* and *Staphyloccoccus aureus*. Supernatant and fungal biomass that were extracted with ethyl acetate did not show any inhibition zone while both of the samples that were extracted with ethanol did show the inhibition zone. Once done, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were done in order to know the concentration of the fungal extract that can inhibit the bacterial growth and kill the bacteria, respectively. Broth microdilution method was used for MIC using microtiter plate. As a results, 14.55 mg/mL (fungal biomass) and 119.70 mg/mL (supernatant) were found to be the exact concentration of MIC for both samples of ethyl acetate extraction while 7.275 mg/mL (fungal biomass) and 59.85 mg/mL (supernatant) were the best for ethanol extraction to inhibit the bacterial growth. MBC was determined by observing the presence of the bacterial growth on the nutrient agar. The concentration that can kill both of the bacterial species or MBC was 14.55 mg/mL for fungal biomass and 119.70 mg/mL for supernatant of ethyl acetate extraction meanwhile for ethanol extraction supernatant and fungal biomass required 29.10 mg/mL and 239.40 mg/mL, respectively. Besides that, Gas Chromatography-Mass Spectrophotometry (GC-MS) was performed to identify the active compounds present in the fungal extract. The major constituents found in the fungal is hexadecanoic acid (16.68% to 23.46%), octadecenoic acid (2.22% to 13.05%) and bis(2-ethylhexyl) phthalate (0.39% to 10.96%). In conclusion, fungal extract from *M. cladophyllus* has the antimicrobial properties as it showed the antimicrobial activity against *E. coli* and *S. aureus*.

Key words: *Marasmius cladophyllus*, antibacterial activity, MIC, MBC

ABSTRAK

Aktiviti antibakteria Marasmius cladophyllus telah dijalankan dengan spesis bakteria yang berbeza terdiri daripada gram-negatif bakteria dan gram-positif bakteria. Dua pelarut berbeza; etil asetat dan etanol, digunakan untuk mengkstrak sebatian bioaktif daripada biojisim supernatan dan kulat. Ekstrak daripada dua pelarut menunjukkan antimikrob yang berbeza. Kaedah ujian resapan cakera telah digunakan untuk menyaring aktiviti antibakteria M. cladophyllus *terhadap* Esherichia coli *dan* Staphylococcus aureus. *Supernatan kulat dan biojisim kulat yang diekstrak dengan etil asetat tidak menunjukkan sebarang zon perencatan manakala kedua-dua sampel yang diekstrak dengan etanol menunjukkan zon perencatan. Setelah selesai, ujian nilai perencatan minima atau MIC dan nilai kepekatan bakterisidal minima atau MBC dilakukan untuk mengenalpasti kepekatan ekstrak kulat yang boleh menghalang pertumbuhan bakteria dan membunuh bakteria masing-masing. Teknik pencairan mikrokaldu digunakan untuk MIC menggunakan plat mikrotiter. Hasilnya, 14.55 mg/mL (biojisim kulat) dan 119.70 mg/mL (supernatan) didapati kepekatan MIC untuk kedua-dua sampel pengekstrakan etil asetat manakala 7.275 mg/mL (biojisim kulat) dan 59.850 mg/mL (supernatan) adalah terbaik untuk pengekstrakan etanol dalam menghalang pertumbuhan bakteria. MBC ditentukan dengan memerhatikan kehadiran pertumbuhan bakteria pada agar nutrien. Kepekatan yang boleh membunuh kedua-dua spesies bakteria atau MBC ialah 14.55 mg/mL untuk biojisim kulat dan 119.70 mg/mL untuk supernatan pengekstrakan etil asetat manakala supernatan pengekstrakan etanol dan biojisim kulat diperlukan 29.10 mg/mL dan 239.40 mg/mL, masing-masing. Selain itu, kromatografi gas-jisim spektrometri jisim atau GC-MS dilakukan untuk mengenal pasti sebatian aktif yang terdapat dalam* M. cladophyllus. *Juzuk utama yang terdapat dalam kulat ialah asid heksadekanoik (16.68% hingga 23.46%), asid oktadesenoik (2.22% hingga 13.05%) dan bis(2-ethylhexyl) ftalat (0.39% hingga 10.96%). Kesimpulannya, ekstrak kulat daripada M. cladophyllus mempunyai sifat antimikrob kerana ia menunjukkan aktiviti antimikrob terhadap* E. coli *dan* S. aureus.

***Kata kunci**: Marasmius cladophyllus, aktiviti antibakteria, MIC, MBC*

List of Tables

List of Figure

List of Abbreviations

Abbreviations	Description
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
TIC	Total Ion Chromatogram
GC-MS	Gas Chromatography-Mass Spectrometry
DEHP	di(2-ethylhexyl) phthalate

CHAPTER 1: INTRODUCTION

1.1 Background

Antimicrobial agent has been used widely in the world to cure and treat infectious diseases that mainly caused by bacteria. Antibiotics and other modern drugs are the examples of antimicrobial agent that have been labeled a mode of treatment. However, the use of antibiotics against pathogenic bacteria has cause many adverse complications or in other words is side effects. According to Zhang *et al.*, 69% of the medicine drugs have between 10 to 100 negative side effects, 22% have more than 100 side effects and only 9% of the drugs have less than 10 side effects as shown in **Figure 1.1**. In the study, the dataset contained 61,102 association between medicine drugs and side effect, with each drug having an average of 68.8 side effects. It shows that modern drugs development nowadays have many side effects in response to the treatment of the disease itself. Thus, we need an alternative to treat the disease instead of consuming modern drugs and antibiotics.

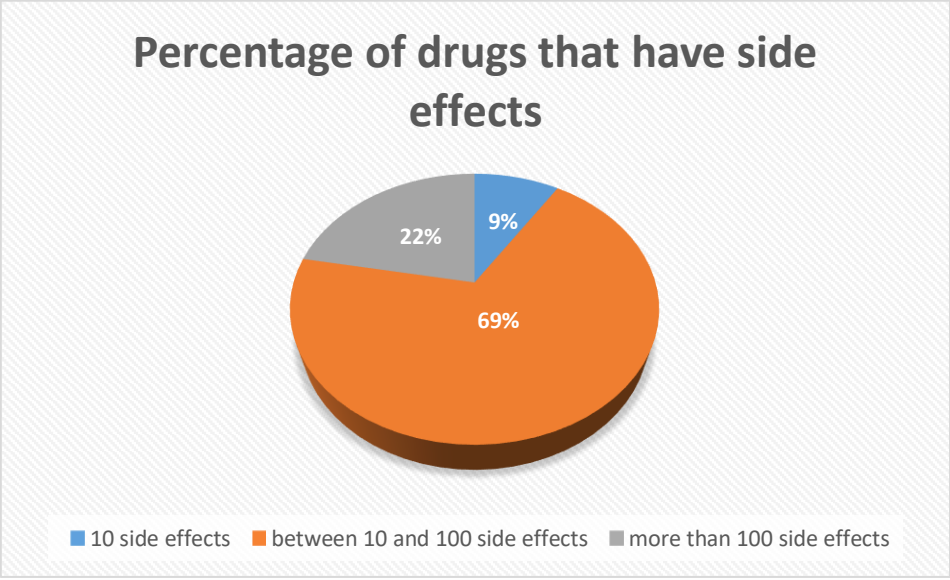

Figure 1.1 A statistic of percentage of drugs that have side effects. (Adapted from Zhang *et al.*, 2013)

Besides that, the emergence of antimicrobial resistance, along with a scarcity of newly produced antimicrobial medications and treatments, poses a severe threat to human health. There are fewer or even no effective antibacterial agents that are available to use in treating infection caused by the pathogenic bacteria or microorganisms (Manandhar *et al*., 2019). Thus, we need an alternative to treat the disease instead of consuming modern drugs and antibiotics.

One of the largest sources in higher plants that can be useful as antibacterial agent derived from mushroom such as, *Marasmius cladophyllus*. *M. cladophylllus* is a species of fungus that belongs to the family Tricholontataceae which has a close related genera that have been known in producing novel and active secondary metabolites (Meng *et al*., 2011). Usually, mushroom is preferable to be eaten as food in all over the world. It can be grilled, roasted or even fried due to the benefits in the food itself. Mushrooms have a dry matter concentration of 50 to 65% total carbohydrate, 19 to 35% proteins with biological and therapeutic activity such as lectins, and 2 to 6% fat content (Rathore *et al.*, 2017).

Despite the benefits of mushroom, little however is known of the antibacterial potential of *M. cladophyllus*. Hence, this study was conducted to determine and identify the antibacterial activity of *M. cladophyllus* against pathogenic bacteria such as *Staphylococcus aureus* and *Esherichia coli*. Two different kind of fungal extract which are the liquid broth containing fungus excretion and also the fungal biomass were used for testing of antibacterial activity. Last but not least, mushroom or *M. cladophyllus* might be an effective antibacterial agent in killing pathogenic bacteria.

1.2 Objectives

The objectives of this study are:

a) To screen for antibacterial activity of *M. cladophyllus* with different solvent

b) To determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the fungal extract.

c) To identify the active compounds in the extract of *M. cladophyllus*

CHAPTER 2: LITERATURE REVIEW

2.1 Overview of Mushroom

Mushroom has been known in all over the world as a medicine or therapeutic food especially among rural people. Along with its chemical composition, this plant source can prevent people from diseases and illnesses including hypertension, hypercholesterolemia, atherosclerosis, and cancer (Waktola & Temesgen, 2018). Furthermore, mushroom has a unique ability of which is high in nutrition and low in calories. This ability has been recognized and proved by the medical profession throughout the world. Mushrooms have been reported to contain eight different amino acids, polyunsaturated fatty acids and also a small amount of saturated fatty acids (Waktola & Temesgen, 2018). Comparing mushroom with common protein food such as fish and beef, mushroom has a higher nutritional values than both protein food (Waktola & Temesgen, 2018). Few examples of medicinal mushrooms together with their medicinal use can be referred in **Table 2.1**.

Table 2.1 Examples of medicinal mushroom with its medicinal use (Adapted from Pardeshi, 2016).

Common name of medicinal mushroom	Scientific Name	Medicinal use
Button mushroom	*Agaricus bisporus*	Reduce the breast cancer risk and prostate cancer risk.
Oyster	*Pleurotus ostreatus*	For cancer, cholesterol reduction immune-modulators
Shitake	*Lentinus edodes*	For cancer, cholesterol reduction, hypertension, arthritis, heart diseases
Phellinus, sanghuang	*Phellinus linteus*	Anti-tumor, antibacterial, liver-protecting
Hexagonia	*Hexagonia vesparia*	Diarrhea, immune stimulation
Reishi Gaanoderma	*Ganoderma lucidum*	For cancer, hypertension, asthma, heart diseases, immune stimulation
Gyanocillium	*Ganoderma lucidum*	For cancer, hypertension, asthma, heart diseases, insomnia

2.2 Taxonomy of *Marasmius cladophyllus*

One of the largest sources in plants that has been used as antibacterial compound is mushroom which belongs to a kingdom of fungi. Mushroom has been used and practiced widely all over the world as medicine especially the ethnic groups of south and southwest Ethiopia (Waktola & Temesgen, 2018). As shown in **Figure 2.1**, *M. cladophyllus*, is a species of fungus that belongs to the order of Agaricalesis. It is from the phylum of Basidiomycota and belongs to class Agaricomycetes. To be specific, *M. cladophyllus* belongs to family of Marasmiaceae and genus *Marasmius* (Kirk, 2020).

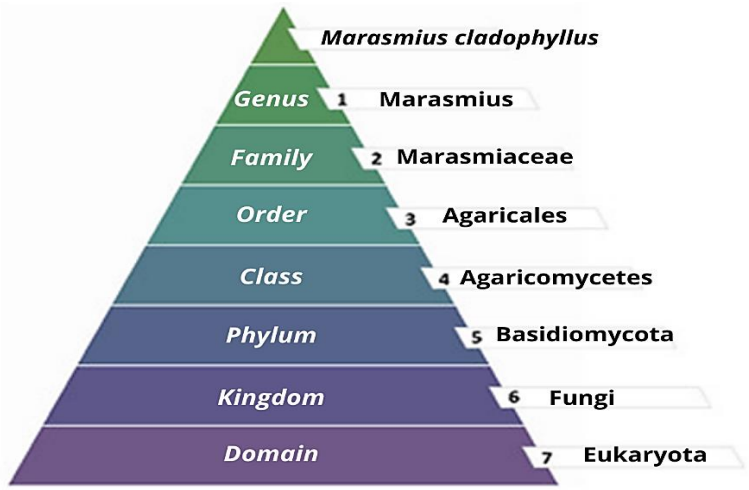

Figure 2.1 A taxonomy of *M. cladophyllus* (Adapted from Kirk, 2020).

2.3 Mushroom usage in medications and treatments

There are numerous therapeutic applications for medicinal mushroom due to their antimicrobial activity, antiviral activity, antiaging activity, anticancer activity, antidiabetic activity, antiasthmatic activity and hepatoprotective effect as shown in **Figure 2.2** (Chaturvedi *et al*., 2018). Mushroom also helps in enhancing immunogenicity, preventing cardiovascular diseases and enhancing bone health (Chaturvedi *et al*., 2018).

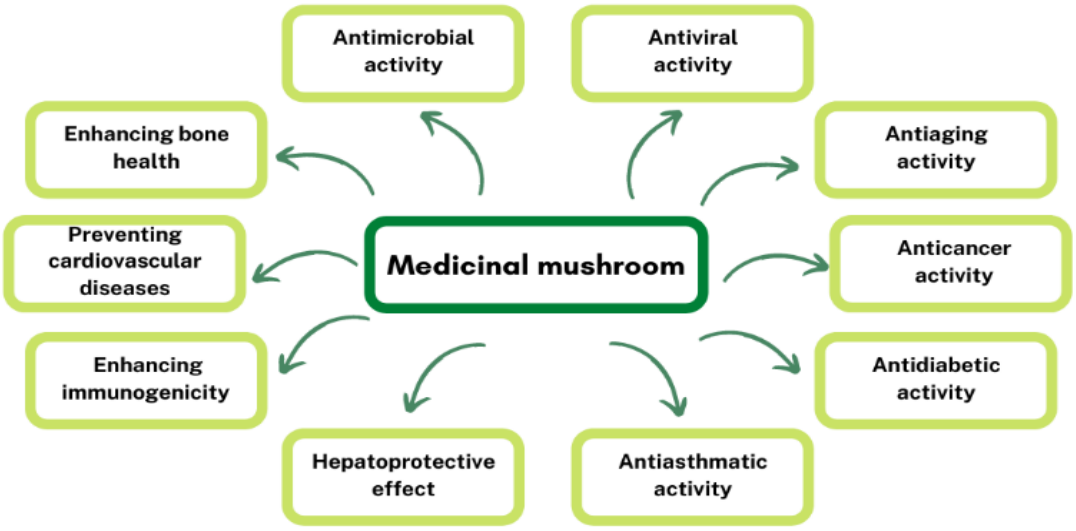

Figure 2.2 Various therapeutic applications for medicinal mushroom (Adapted by Chaturvedi *et al.*, 2018)

Due to the existence of few types of secondary metabolites, mushroom have these therapeutic potential. Polysaccharides, which belong to the 1,3- β-glucans family, are the most important secondary metabolites in medicinal mushrooms, with anticancer properties that are accomplished by boosting and inhibiting the cellular immune system (Chaturvedi et al., 2018). For instance, *Pleurotus ostreatus* is an edible mushroom that block and inhibit breast and colon cancer cell proliferation by up-regulating a cell cycle regulatory protein (Chaturvedi *et al.*, 2018).

Furthermore, large amount of bioactive compound in the mushroom like α–β-unsaturated polysaccharides, glycoproteins, peptides, phenolics derivatives, hydrolytic, lipids and oxidative enzymes showed therapeutic application in it (Chaturvedi *et al.*, 2018). All these compounds are derived from a mycelia crude extract and medicinal mushroom fruiting body (Chaturvedi *et al.*, 2018).

2.4 Mushroom as antibacterial agent

Antibacterial activity is a term that refer to all the active principles or agents that are used to inhibit the bacteria from growing and prevent the formation of bacterial colonies. These agents are also sometimes used to kill the microorganisms. According to Alves *et al.*, (2012), compounds that have been identified as an active compounds from *Albatrellus fletti* are confluentin, grifolin & neogrifolin. These compounds showed an antibacterial activity against *Bacillus cereus* and *Enterococcus faecalis*. Furthermore, ganomycin A and B are the compounds that have been isolated from *Ganoderma pfeifferi* and these showed an inhibitory effect against *Bacillus subtilis*, *Micrococcus flavus* and *Staphylococcus aureus*. The active compounds from these mushrooms can be seen in **Figure 2.3**.

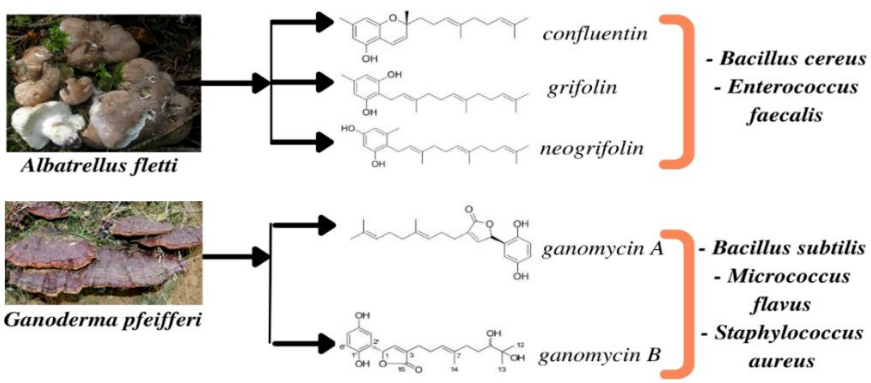

Figure 2.3 Several compounds that has been isolated from different species of mushroom (Adapted from "ChemFaces", n.d.).

Ethanol extract was the most effective against the bacteria. In term of extraction solvent, hot water extraction helps in extracting more compounds with a higher antimicrobial activity against all of the species tested and examined (Gebreyohannes *et al.*, 2019). Using water for extraction is much preferable than alcohol due to the polarity of the components used and the way penetrates the mushroom cell wall.

2.5 Antibacterial activity of *Marasmius spp*.

Research has been done and it demonstrated that the ethanol extract of *Marasmius oreades* showed antibacterial activity against gram-positive bacteria such as *Enterococcus faecium* and *Staphylococcus epidermidis* (Shomali *et al.*, 2019). However, it does not show any antibacterial activity against gram-negative bacteria. Besides that, there is an antibacterial activity of *M. oreades* methanol extract against various gram-positive and gram-negative bacteria including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *S. epidermidis* and *Escherichia coli* (Shomali *et al.*, 2019).

Furthermore, methanol extracts of some wild mushroom were discovered to have a higher antibacterial activity and give the effect on nosocomial pathogens than typically used antibiotics. These findings show that other mushroom products can be used instead of antibiotics in the treatment of infectious disease (Shomali *et al.*, 2019). Antibacterial activity screening of *M. cladophyllus* therefore might enable a better understanding of the antimicrobial potential of the *cladophyllus* species that has not been studied.

2.6 Antibacterial effects of Ethanol, Methanol and Ethyl Acetate extract against bacteria

As mentioned by Shahraki-Mojahed et al., (2021) in their study, it aims to investigate the antimicrobial effects of ethanol, methanol, ethyl acetate *Teucrium polium* and *Citrullus colocynthis* extract on *Pseudomonas aeruginosa*. As a results, MIC or the inhibitory concentration of ethanolic extract of *C. colocynthis* against *P. aeruginosa* was 2.5 mg/mL while for methanolic extract of *C. colocynthis* was 1.25 mg/mL and for 0.62 mg/mL was the concentration for the ethyl acetate extract of *C. colocynthis*.

Besides that, the minimum inhibition concentration for ethanolic extract, methanolic extract and ethyl acetate extract of *T. polium* against *P. aeruginosa* was 5 mg/mL, 2.5 mg/mL and 0.62 mg/mL respectively (Shahraki-Mojahed *et al.*, 2021).

To conclude, ethanol extract required the highest concentration to inhibit the bacteria from growing while ethyl acetate extract was determined to have the lowest concentration inhibitory. Different solvents extract have different inhibitory level in inhibiting the bacteria which was *P. aeruginosa* at different concentration. This is due to the efficiency of ethyl acetate in extracting the compound from the organism. The better the efficiency of extraction, the more bioactive compound that will be extracted and therefore, better antimicrobial activity will be shown.

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

The materials used for this study include 96-well microtiter plates, ampicillin, centrifuge tube, cuvette, ethanol, ethyl acetate, falcon tube, filter paper, inoculation loop, Mueller Hinton Agar (MHA), Mueller Hinton Broth (MHB), mushroom, nutrient agar, sterile cotton swab, sterile distilled water.

3.2 Microorganisms

Mushroom or *Marasmius cladophyllus* was obtained from Molecular Genetic Laboratory of Faculty of Resource Science and Technology, UNIMAS. *Escherichia coli* (gram-negative bacteria), and *Staphylococcus aureus* (gram-positive bacteria) were used to screen the antibacterial activity of the fungus.

3.3 Microorganisms Preparation and Culture Maintenance

Marasmius cladophyllus was grown in Malt Extract Agar (MEA) for a period of 7 to 10 days at 34°C (room temperature) to have the fungal source. Several fungal agar plugs from the nutrient agar were taken and grown in the 300 mL of Mueller Hinton Broth (MHB) for 7 days at room temperature with shaking 150 rpm.

3.4 Bioactive Compound Extraction

After 7 days, the liquid broth was filtered in order to separate the fungal mycelium from the media. Upon filter, two samples from filtered fungus were produced which are broth containing the fungal excretion compounds or supernatant (Sample A) and fungal biomass

(Sample B). Sample A was placed in the fume hood for 7 days while Sample B was placed in the oven with 50°C for 24 hours for drying purposes. Sample A was weighed together with the beaker before and after dried. Once sample B was fully dried, the sample was weighed as well and grinded using pestle and mortar. The grinded biomass and the dried supernatant were extracted with ethyl acetate. Both samples were extracted by using conventional extraction method where the samples were placed in the water bath at 70°C for 2 hours. The extraction method was repeated by using different solvent which was ethanol.

3.5 Antibacterial Assay – Disc Diffusion Assay

Disc diffusion assay was used in this study as an initial screening for antibacterial activity. A filter paper discs with a diameter of 6 mm were cut by using a punch machine and were used in this method. These filter paper discs were sterilized in an autoclave and stored for later use at room temperature. A sterile cotton swab was used to make a lawn of each bacteria tested (*E. coli* and *S. aureus*) on the Mueller Hinton Agar (MHA) plates. Four MHA plates were prepared for each bacterial species in order to determine the antibacterial activity for both supernatant (Sample A) and biomass extract (Sample B). All assays were performed in duplicate to get an accurate result. For the preparation of the bacterial suspension, overnight bacterial culture was adjusted to the turbidity of 0.5 McFarland standard with the help of spectrophotometer. If the reading was higher than 0.5, the bacterial suspension was diluted with the Mueller Hinton Broth (MHB) and vice versa.

The filter paper discs soaked in both fungal extracts were placed on the bacterial lawn prepared on MHA. Ampicillin discs (50 mg/mL) was utilized as a positive control. Filter paper discs soaked in the ethyl acetate and ethanol and were utilized as a solvent control. Then, those filter paper discs were aseptically placed over the swabbed MHA plates and

incubated for 24 hours at 37°C. The presence of antibacterial activity of the tested extract after 24 hours was interpreted by observing the presence of halo or clear zone formation.

3.6 Determination of MIC and MBC

3.6.1 Minimum Inhibitory Concentration (MIC) Determination

Broth microdilution method was used to determine the MIC of the fungal extracts. Test bacterial or inoculum was prepare by aseptically transferring colony of the bacteria grown on nutrient agar surface into a universal bottle containing 14 mL of Mueller Hinton Broth (MHB). Next, the concentration of an extracted fungal excretion compound was prepared into a range from 239.4 mg/mL to 1.87 mg/mL at a volume of 100 µL. Both of the bacterial species were diluted 1/150 times in MHB and the extracted compounds was diluted by two-fold concentration ranged from 29.1 mg/mL to 0.227 mg/mL in a sterile MHB as well. An aliquot of 50 µL of sterile MHB was transferred to the well A until well H of the 96-well microtiter plates. Then, 50 µL of the diluted fungal extract were added into the 1st well and 2nd well of each column of the plates. The compounds were diluted by transferring the mixture from the 2nd well until the 10th well of well A till well F. In well G and well H, the compounds were exchanged with ampicillin as it was utilized as a positive control. The ampicillin were diluted similar to the extraction compounds. 50 µL of *E. coli* inoculum was added into each of the well A until well C while 50 µL of *S. aureus* inoculum was added from well D to well F. For the positive control, well G and H was specifically for negative control containing only broth and bacteria. 1st well was left without adding any extraction compounds and ampicillin as it was utilized as a negative control.

3.6.2 Minimum Bactericidal Concentration (MBC) Determination

For each well that did not show any precipitation or bacterial growth after MIC determination, an inoculation loop was used to take a small amount of the mixture which was then streaked onto nutrient agar and checked for any bacterial growth. Each bacterial species was done in triplicate in order to get a precise result. All the agar plates were further incubated at 37°C for 24 hours. The minimum concentration of both supernatant and biomass extract that showed no growth of bacteria on the agar were recorded and deduced as the MBC.

3.7 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS analysis was performed using Shimadzu GCMS-QP2010 Plus in Universiti Malaysia Sarawak (UNIMAS). The BPX-5 column with 30.0 m x 0.25 µL x 0.25 mm measurements were used. The initial oven temperature was set at 50.0°C with 1 minute of sampling time. The injection and detector were adjusted to 260°C while the pressure was set to 53.6 kPa. Helium was used as a carrier gas with a column flow of 1.0 mL/min. Linear velocity was chose to be flow control mode and total flow was identified to be 24.0 mL/min with a linear velocity of 36.3 cm/sec. Split ratio was 20.0. The chemical constituents of both biomass and supernatant of *M. cladophyllus* were identified by comparing their area percentage with the mass spectral library incorporated in data system to ascertain its name, molecular weight, chemical formula and the structure as well.

CHAPTER 4: RESULTS

4.1 Determination of Fungal Extract Concentration

Firstly, the amount of fungal biomass ethyl acetate extract that have obtained was 22.531 g while the amount of supernatant ethyl acetate extract was 4.788 g. Besides that, the amount of fungal biomass and supernatant ethanol extract that have obtained were 31.007 g and 1.348 g, respectively.

Next, the concentration of fungal biomass for both ethyl acetate extract and ethanol was adjusted to 29.1 mg/mL while the concentration of supernatant for both extract was 239.4 mg/mL. **Table 4.1** shows the amount of fungal extract obtained and the concentration for both fungal extract.

Table 4.1 Amount and concentration of fungal extract obtained

Solvent extract	Fungal extract	Amount of fungal extract obtained (g)	Concentration of fungal extract (mg/mL)
Ethyl acetate extract	**Fungal biomass**	22.531	29.1
	Supernatant	4.788	239.4
Ethanol extract	**Fungal biomass**	31.077	29.1
	Supernatant	1.348	239.4

4.2 Disc Diffusion Assay

In disc diffusion assay method, no inhibition zones was observed in both supernatant and fungal biomass ethyl acetate extracts of *Marasmius cladophyllus*. In contrast, ethanol