CHAPTER 5

DISCUSSIONS

5.1 Isolation and Morphology Identification of THR2 Fruiting Bodies

Mushrooms are a group very distinct from plants, animals and bacteria. Mushrooms are large enough to be seen with naked eye and can be picked by hand, they are considered as macro-fungus with a distinctive fruiting body. There are about 2 000 edible mushrooms out of 69 000 known mushroom species in this world. People have been collecting and consuming edible mushrooms for over thousand years (Gameli, 2020). This study aimed to grow the *Termitomyces* (THR2) mushroom mycelium using submerged liquid fermentation and to analyse the compound of mushroom to study its antimicrobial, antifungal and antioxidant.

In this study THR2 mushroom was collected during Southwest monsoon in September 2019. The mushroom was dried after it was collected in order to remove its water content and to store it for further usage. There are two ways in which drying can be done which are through natural or artificial processes. One of the natural processes is sun- drying which may take couple of weeks for completely drying as the time relies upon dampness and temperature. One of the artificial processes is oven drying. According to Gameli, (2020) oven drying of mushrooms gives the best result compared to the rest of processing method since it maintains the nutrient composition whereas sun drying method gives a negative effect on the nutrient content of the mushroom despite able to maintain the mushroom colour. In this research, the mushroom was dried in an oven for 5 days at 50°C.

At the sample location, THR2 fruiting bodies were discovered scattered across the soil's surface under conditions of high humidity and low range of temperature. According to weather analysis data, Kuala Pilah experienced two thunderstorms at midnight and noon the day before the isolation, which resulted in high humidity of 82–87% and an average temperature range of 25–28°C (Source: weather.atlas.com). High humidity along with heavy rain the day before isolation created an ideal environment for stipe growth. The mentioned environmental circumstances are found in the studies done by Froslev et al., 2003, which claimed that termite combs' fungus mycelium develops during wet seasons, which eventually infiltrate termite nests and soil to spread their spores.

In this study, the soil cross section (Figure 4.1B) clearly demonstrated how the fruiting body stipe erupted on the earth's surface after growing from the termite nest, as shown in Figure 4.1A. According to Figure 4.1C, the termite nest had a diameter of 10 to 15 cm. According to research by Wisselink et al., (2020), the *Termitomyces* mushroom grows as basidium inside the nest rather than on the exterior layer. While Figure 4.2B depicts the morphological phases of hyphal developing onto the termite comb, Figure 4.1E demonstrates the mutualistic symbiosis between the *Termitomyces* mushroom and termite colony. According to Figure 4.2 A and B, the mycelium that has accumulated on the termite comb and the growing tips of the hyphal that have pinholes demonstrate that the mycelial has absorbed the nutrients and substrate from the termite's nest and has grown until it has matured stipes as seen in Figure 4.2 C.

From figure 4.2 D, it can be seen that the morphology of wild THR2 fruiting bodies may be distinguished by its long, white-yellowish stipe, which is linked to the stipes at the centre and has a small, conspicuous perforatorium (pileus) on the tip. Figure

4.2 E depicts the varying heights of the isolated wild THR2 mushroom fruiting bodies, which range from 10 to 30 cm.

5.2 Cultivation of Mycelium onto Agar Medium

The isolated THR2 fruiting bodies were subsequently grown after being collected from the sample site in order to preserve the freshness of the interior tissue of the fruiting bodies. The agar is further manipulated in this study to grow the THR2 mycelium by the addition of yeast and malt extract in the potato dextrose agar (PDA) to obtain a healthy dense mycelium without sign of contamination. For fungal cell cultivation, PDA is considered as standard medium for many years to support high cell growth and usually considered as the first choice for mushroom cell cultivation. However, to enhance the mushroom growth and shorten the time needed for preparation of inoculum this medium is not fully optimized. Thus, the PDA agar was manipulated by supplementing yeast and malt extract; it was observed that the supply of yeast and malt extract to PDA agar gave a positive impact on THR2 mushroom mycelium growth. Since malt extract is a very rich supply of sugars with a variety of different chemical structures (sucrose, maltose, and other sugars), adding it to PDA agar has a good effect on cell development. Additionally, yeast extract is one of the best sources of the nutrients, vitamins, growth factors, and trace elements needed for the development and growth of many types of primary and secondary metabolites (Maftoun et al., 2017). As shown in the results Table 4.1 and Table 4.2, combination of PDA+YE+ME is the most suitable agar composition as the growth of mycelium was seen to be dense and compact. Not only that the mycelium grew to full plate in day 14 of cultivation unlike other agar compositions.

5.3 Molecular Identification of Wild *Termitomyces* sp. Mushroom

Molecular characterisation of the collected wild *Termitomyces* sp. RFES 230662 (THR2) mushroom was done to determine the species of the mushroom. In the past few years molecular techniques have been widely increased since they are the most reliable and accurate tools for various purposes such as evaluation of genetic structure and molecular phylogeny. Gel electrophoresis is a method for sorting DNA fragments into different sizes. DNA samples are placed into wells at one end of a gel, and an electric current is used to drag them through the gel. Since the DNA fragments are negatively charged, it flows towards the positive electrodes (Avin et al., 2013). In order to check the quality of extracted *Termitomyces* sp. DNA, agarose gel electrophoresis method was used to determine the quality of extracted nucleic acid in this study. In a gel documentation system, the gel was viewed by exposing it to UV light and to show the presence of DNA bands. As illustrated in Figure 4.4, the base pairs were estimated using agarose gel electrophoresis under UV light which was 300 bp.

The obtained gDNA sequence was then fed into BLAST. The Basic Local Alignment Search Tool, also known as BLAST, identifies regions of similarity between biological sequences. The tool calculates the statistical significance by comparing nucleotide or protein sequences to the sequence databases. After sequencing, the results were aligned with top ten closely related species found using NCBI BLAST; *Termitomyces* sp. RFES 230662 was found to be 99% similar to other *Termitomyces* species based on BLAST reference databases, and a comprehensive phylogenetic analysis (Figure 4.5) revealed that it was closely related to *Termitomyces heimii* (THR2).

Termitomyces heimii is a seasonal, rare edible fungus with excellent nutritional value. Termite hills are where this fungus typically makes its home. *T. heimii* is one of

the *Termitomyces* species that are frequently found in Malaysia. It is typically found in the wild and is prized for its delicacy. *T. heimii* ingestion would be advantageous for health reasons and may have chemo preventive characteristics for certain human diseases (Sri et al., 2012). *T. heimii* is used as a therapy for fever, colds, and fungal infections in India. Additionally, it functions as a blood tonic during blood coagulation and wound healing (Hsieh & Ju, 2018).

5.4 Submerged Liquid Fermentation of THR2

According to Papaspyridi et al., (2012), the typical method of acquiring commercial mushroom products is from the labour- and time-intensive fruiting bodies of field-cultivated mushrooms. Submerged liquid fermentation is one of the greatest techniques used to obtain adequate amount of mycelium. Submerged growing of culinary and medicinal mushrooms has drawn increasing attention globally as it is seen as a promising alternative for the efficient production of biomass and important metabolites. Particularly, submerged liquid fermentation has the benefit of more quickly producing metabolites and mycelia biomass over a shorter time span in a smaller volume of space with less potential for contamination. In addition, it helps in overcoming challenges associated with THR2 seasonal harvesting activities, allowing the bioactive chemicals from the fruiting bodies to be harvested without encountering seasonal problems.

In this study, a liquid medium was supplemented with a carbon source, a nitrogen source, and other micronutrients in order to successfully apply the SLF method to create the bioactive components of THR2. Glucose serves as the carbon source in the medium that has been created, supporting the growth of mycelial biomass up to 8.55 g/L. Steluti et al., (2004) claim that carbon and nitrogen sources operate as a source of nutrients for

the mycelial to erect membranes and cell walls, resulting in the production of bio-polysaccharide. As a result, the confirmed -glucan in this investigation is actually a bio-polysaccharide that is part of the cell wall or membrane of the fungus.

In order to obtain ENS, the fruiting bodies of THR2 were removed using hot and cold water. Since water has no deleterious effects for human cells, it was used as the extraction solvent in both the hot and cold water extraction processes. Hot water is frequently used in the extraction of mushroom fruiting bodies because it aids in breaking down the chitin in the cell walls, while cold water extraction is also recommended since it may not completely destroy the bioactive components in the extract (Abd Razak et al., 2020). Additionally, water extraction is one of the most popular mushroom preparation techniques because the bulk of the active ingredients in the great majority of mushrooms are water soluble, such as β-glucans.

Figure 4.6 shows that crude polysaccharides generated by fruiting bodies (HW-ENS & CW-ENS) were higher than those produced by SLF mycelium (IPS & EPS); HW-ENS showed a value of 3.20 g/L while CW-ENS displayed a value of 1.36 g/L. Meanwhile, 0.80 g/L of IPS and 1.44 g/L of EPS were created. According to Wan-Mohtar et al. (2016), SLF is a potential technique for obtaining a high concentration of polysaccharide from mushroom mycelial. The mycelium pellet was depicted in Figure 4.7, and it can be seen that it is a small, dense pellet. Due to the mycelium pellet's greater surface area, the small, rounded pellet used in this study allowed for a high concentration of EPS (1.44 g/L) (Supramani et al., 2019).

5.5 β-glucan Determination

The vibrational characteristics of amino acids and cofactors, which are susceptible to minute structural changes, are investigated using Fourier transform infrared (FTIR) spectroscopy. The position and anomeric configuration of the glycosidic linkage in the glucan-targeted HW-ENS, CW-ENS, IPS, and EPS extracts were characterised and confirmed in this investigation using FTIR.

The peaks from the HW-ENS, CW-ENS, IPS, and EPS extracts are displayed in Figure 4.8. According to Figure 4.8, the presence of β-glucan polysaccharides in the extracts was confirmed by the FTIR peaks of HW-ENS, CW-ENS, IPS, and EPS. Mushrooms β-glucans offer such a wide range of health advantages hence, they could be used as functional foods, dietary supplements, or nutraceuticals (Giavasis, 2014). The sample was identified as polysaccharide when the displayed peaks were compared to those of standard laminarin, as shown in Table 4.3, because absorption was evident in the range between 1250 cm^{-1} and 1650 cm^{-1} (Chen et al., 2013). Laminarin is also frequently referred to as -1,3-glucan storage. A class of 1,3-glucans are polysaccharides with a 1,3-linked glucose main chain and 1,4-, 1,6, or 1,8-linked glucose branches.

According to Table 4.3, the prominent, broad peak in the absorption area between 3370 cm^{-1} that represents the stretching vibration of O-H groups served as evidence of the presence of poly-hydroxylic compounds (Wan-Mohtar et al., 2016). Figure 4.8C and D demonstrate how the absorption peaks for IPS and EPS were at 2924 cm^{-1}, which is associated with the stretching vibration of C-H indicating the existence of the methylene group, or -CH2. Usuludin et al. (2020) state that the water bending vibration in the polysaccharide is indicated by the absorption peak of 1641 cm^{-1} displayed by all of the crude extracts (CW-ENS, HW-ENS, IPS, and EPS), whereas the absorption peak of 1076 cm^{-1} displayed by IPS and EPS denotes the presence of C-O-C and C-O bonds

stretching vibrations. The C-O, C-O-C, and O-H stretching vibration absorptions approximated the features of a polysaccharide structure. According to Figure 4.8, the absorption peak of HW-ENS, IPS, and EPS appeared in the range of 860 cm^{-1}, 890 cm^{-1}, and 889 cm^{-1}, respectively. Usuldin et al., (2020) reported that the anomeric configuration within this peak range was the configuration of the polysaccharide and the glycosidic linkage was in the -configuration. Based on the absorption peaks of the three samples, it can be said that the structural characterization of HW-ENS, IPS, and EPS polysaccharide from THR2 was a β-glucan. A study made by Bhanja et al., (2012) showed that the extracted β-glucan exhibited high immune stimulating properties.

5.6 Antimicrobial and Antifungal Analysis

5.6.1 Disk Diffusion Assay

In this study, the antibacterial and antifungal effects of THR2 crude extracts (HW-ENS, CW-ENS, IPS and EPS) were evaluated against four different types of bacteria: three Gram-negative bacteria (*Ralstonia* sp., *Salmonella* sp., and *E. coli*), two Gram-positive bacteria (*S. aureus* and *Streptococcus* sp.), and one fungus (*A. niger*). Based on Table 4.7, all the crude extracts showed antimicrobial activity against the tested bacteria as a clear zone was formed except IPS and EPS towards *E. coli*. It was observed that the HW-ENS, IPS and CW-ENS extract of THR2 showed largest zone of inhibition against *Ralstonia* sp, which was 6.5 ± 0.1 mm, 6.0 ± 0.1 mm, and 4.5 ± 0.1 mm. whereas the EPS extract of THR2 exhibited a large zone of inhibition (4.0 ± 0.1 mm) against *S. aureus.* HW-ENS exhibited a large zone of inhibition against *E. coli* and *Streptococcus* sp. In contrast to other crude extracts, IPS exhibited a significantly larger zone of inhibition against *S. aureus* (5.7 ± 0.1 mm). However, there was not any formation of clear zone in all the extracts against *A. niger* and this may be due to the extract used in

this study which was water as a study made by Kim et al., (2022) showed that the methanol extract of *T. camphoratus* exhibited a promising antifungal and antibacterial activity.

The HW-ENS THR2 extract, which had a greater zone of inhibition against all the tested bacteria, is the most effective THR2 extract of the four studied extracts, followed by the IPS THR2 extract from mycelium. *Termitomyces* sp. hot water extracts exhibit good antibacterial activity against *S. aureus* and *E. coli* with a minimum inhibitory concentration of 0.67 ± 0.29 mg/ml, according to a prior study reported by Gebreyohannes et al., (2019). It was hypothesised that the broad HW-ENS zone of inhibition was caused by the hot water extraction method's ability to extract more bioactive chemicals at a high temperature than CW-ENS, which was extracted at room temperature (Mahamat et al., 2018). As a result of EPS being secreted into the broth from mycelium pellets during the fermentation process itself, a reduced average zone of inhibition was seen in THR2 EPS (extracted from the SLF broth).

The effect of the antimicrobial activities from other *Termitomyces* sp. have been reported previously, for example, the aqueous extracts (hot water extraction process) from *Termitomyces clypeatus* fruiting bodies was reported to highly inhibited the growth of several bacteria such as *E. coli*, *S. aureus*, *Salmonella typhi* and *E. aerogenes* in the range of 2.6–10.55 mm zone of inhibition (Mahamat et al., 2018). Apart from water extracts, *T. hemii* acetone extract had demonstrated antibacterial effectiveness against *S. aureus* and *Klebsiella pneumoniae* (Singha et al., 2017). Different concentration of crude methanolic extracts were also reported antibacterial activity against five pathogenic bacteria including *S. aureus, E. coli, K. pneumoniae* and *Pseudomonas* sp. (Kumar et al., 2019).

In this study, it was found that polysaccharides taken from fruiting bodies, mycelium, and SLF culture broth had a notable antibacterial effect. According to Friedman et al., (2016), under certain circumstances, mushroom β-glucan with β-linkage can stimulate the human immune system and modulate the immunological response, which is why β-glucans are referred to as biological response modifiers (BRM). The ENS (HW-ENS & CW-ENS), IPS, and EPS from this study were confirmed as β-glucan. According to Villares et al., 2012 significant antibacterial capabilities were demonstrated by the β-glucan as a result of immune system activation in the human body

5.7 Phytochemical and Antioxidant Analysis

5.7.1 Phytochemical Identification

As shown in the Table 4.9, flavonoids, saponins and terpenoids was found in all the extracts of THR2 whereas tannins and phenols were not detected. Flavonoids, glycosides, saponins and terpenoids was observed to be present in HW-ENS, CW-ENS extracts and IPS mycelium extracts of THR2 whereas flavonoids, saponins and terpenoids is observed to be presents in EPS extracts of THR2. According to Wandati et al., (2013) phytochemicals such as flavonoids, saponins terpenoids, and glycosides are important phytochemical compounds that are responsible for various biological activities which are important in health research. It has been reported by Concepta et al., (2021) that phytochemical compounds such as saponins and flavonoids possess antibacterial activities. Glycosides was found to be present in all the THR2 extracts except in EPS. Organic compound formed of a non-sugar group (aglycon) and sugar (glycon) are glycosides which are linked together by a glycosidic bond. It was reported

that it can be used as an antirheumatic, cardiotonic, analgesic, antibiotic and purgative agent.

In a study made by Adejumo et al., (2015) concluded that *Termitomyces* contains flavonoids which acts as radical scavengers in terminating radical chain reactions which occurs during the oxidation of triglycerides in the food system. As mentioned by Wandati et al., (2013) saponins involve a huge group of primarily related compounds containing a triterpenoid or steroid. A wide range of pharmacological properties were reported which exert different advantages such as anti-diabetic and anti- inflammatory properties. Terpenoids belongs to the terpenes group which possesses beneficial function when consumed (Wu, 2013). Furthermore, terpenoids are secondary metabolites that shows a molecular structure which have carbon backbones comprised of isoprene. A wide range of pharmacological benefits have been reported by this compound which includes anti-inflammatory, anti-cancer and anti- malarial among others (Moazzem, 2021).

Besides that, Jagadish et al., 2010 stated that the consumption of wild edible mushroom act as a good dietary supplement (taken as whole or entire mushroom) since all extracts of the wild edible mushroom *Termitomyces reticulatus* showed potent antioxidant activities and antioxidant components such as total phenol, flavonoid, β-carotene and lycopene were also determined. Not only that polyphenol-rich fraction from edible mushroom, *Termitomyces microcarpus*, was tested by Mitra et al., 2016 for its antioxidant capacity and estimated for the presence of bioactive components. The extract was found to contain maximum amount of phenols followed by flavonoids, ascorbic acid, β-carotene and lycopene consecutively which contributed for the antioxidant properties.

Based on the results shown in Table 4.9, it can be concluded that THR2 contains phytochemicals which are beneficial that contains potential medicinal value. The compound detected in this research will depend on the method used, pH and interaction among other compounds. There is no reported data on phytochemicals from mycelium.

5.7.2 Total Flavonoid Content (TFC)

According to Johnsy et al., (2014) flavonoid compound can be considered as a good antioxidant compound. Flavonoids are reported as free radical scavengers besides phenols by disrupting the chain reaction during the oxidation of triglycerides (Dapkevicius et al., 1998). Consumption of foods high in flavonoid content can increase human health as a whole in which it was reported in many studies that showed it may reduce the risk of heart diseases by slowing the progression of atherosclerosis, because they act as antioxidants (Johnsy et al., 2014).

Based on Table 4.10, it was observed that all the extracts found to contain certain number of flavonoids, however higher level of flavonoids content was found in THR2 HW-ENS extracts which are 2.86 ± 0.15 followed by CW-ENS, IPS and EPS. Jonhsy et al., (2014) found TFC ranging from 1.30 – 2.80 mg/g. Hence, the results obtained from this study are in agreement with the latter study by Johnsy et al, (2014). Therefore, the finding of this studies suggests that the various extracts of THR2 mushroom might reduce the oxidative damage in human body and promotes health protection from the oxidative stress.

5.7.3 DPPH Assay

Free radical scavenging ability of various sample was evaluated widely by using stable free radical DPPH by measuring hydrogen donating or radical scavenging ability

(Ansari et al., 2013). Radical scavengers or antioxidants are substances which are able to perform this reaction. In this assay ascorbic acid was used as the standard to compare the results obtained. A widely used and available antioxidant which is water- soluble is ascorbic acid; mainly due to its potential of donating hydrogen molecule to the free radical compounds and has a high compatibility in comparison to other antioxidants (Ibrahim, 2012).

Based on the findings shown on Figure 4.13, it was observed that with increasing concentration of various THR2 extracts the radical scavenging activities increased; HW-ENS showed the highest percentage of inhibition which was 90.83% whereas EPS showed the lowest percentage of inhibition which was 66.44%. A study made by Abarca et al., (2019) suggested that high temperatures (Hot extraction) improve extraction efficiency by increasing the diffusion rate and the solubility of the solvent below a certain limit providing higher DPPH radical scavenging activity in mushrooms similar to this findings HW-ENS gave a high percentage of inhibition. However, a study made by Hafizah et al., (2014) on *P. niruri* plant showed that high temperature can damage the existing phytochemicals, this may be contradicting with this research as the study was focused on plant instead of mushroom unlike this research which is focused on *T. heimii* mushrooms.

Based on the Table 4.13, HW-ENS sample of THR2 extract shows the least IC_{50} value which is 4.44 mg/ml whereas EPS showed the highest IC_{50} value which is 6.71 mg/ml and this result is in the agreement of Johsny et al., (2014) that HW-ENS sample shows the least IC_{50} value. According to Jagadish et al., (2010) sample with highest antioxidant contents show higher antioxidant activity with lowest IC_{50} values. From the results, it was observed that high DPPH assay result was shown in the extracts which contained high flavonoids content. Thus, HW-ENS THR2 extracts can be considered as

extract with high potential of antioxidant activity as the IC_{50} value was the lowest with high percentage of inhibition at 10 mg/ml (90.83%) compared to the other extracts.