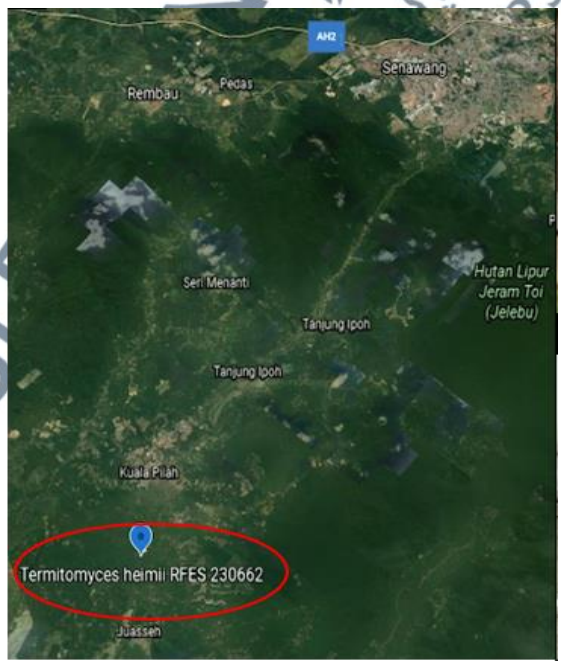
CHAPTER 3

MATERIALS AND METHODS

3.1 Collection of *Termitomyces* sp. Wild Fruiting Bodies

Wild fruiting bodies of *Termitomyces* sp. (2 kg) was collected during October to December from Kampung Sungkak, (Senaling- Latitude 2.7069° N, Longitude 102.2484° E), located in Negeri Sembilan. The fruiting bodies protruding from the soil is considered as matured. These mushrooms were picked up from the wild based on morphological identification and was brought to the laboratory in which the mycelium of the fruiting bodies collected was grown onto agar plate immediately after collected. The remaining fruiting bodies was dried under the oven at 50°C for 5 days to a stable moisture content to ease the grinding process.



Source: Google Earth

Figure 3.1: The locality of wild termite mushroom Termitomyces heimii strain RFES 230662 (THR2) found at Kg. Kuala Sungkak, Negeri Sembilan, Malaysia (red circle indicates the coordinates (2.7069° N, 102.2484° E)

3.2 Morphological Characterization of Wild Termite Mushroom

As mentioned in Hsieh et al., (2017) the isolated mushroom strain was morphologically identified based on its stipe, pileus, and the mycelia budding from the termite nest.

3.3 Molecular Species Identification of Wild *Termitomyces* sp.

3.3.1 Mycelium Preparation for DNA Extraction

The fruiting bodies was cleaned, cut into small pieces and then dried in the oven at 50°C for 5 days. Once the samples were fully dried it was powdered by using a blender, weighed and stored in the fridge at 4°C for further usage.

3.3.2 gDNA Extraction

Mycelium fine powder (50 mg) was transferred into a reaction tube (2.0 ml). Then, it was lysed with 400 µL of Lysis Solution SLS and 20 µL of Proteinase K which was mixed vigorously for 5 seconds through pulsed vortexing. After vortexing, at 65°C incubation was done for 30 minutes. Before centrifugation at 11000 rpm, the sample was first transferred to a Prefilter which was in a receiver tube. After centrifugation, Prefilter was discarded and to the lysed sample 200 µL of Binding Solution SBS was added; mixing was done through pipetting it up and down several times. The spin filter was placed into a new receiver tube after discarding the receiver tube with the filtrate. Before centrifugation at 11000 rpm for 1 minute the spin filter was added with 650 µL of washing solution MS. The spin filter was placed into a new receiver tube after a minute of centrifugation as the receiver tube with the filtrate was discarded (this step was repeated again). Then, for 2 minutes the sample was centrifuged at 11000 rpm in order to remove all traces of ethanol and the receiver tube was discarded. Before the

incubation at room temperature for 1 minute, the spin filter was placed into an elution tube which was added with 100-200 µL of Elution Buffer. It was then centrifuged at 11000 rpm after 1 minute. (Hennicke et al., 2016).

3.3.3 PCR Amplification

In this process the universal primers ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') forward primer and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') was used. The solution (400 µL) was added into the PCR tubes in order to amplify the fungal internal transcribed spacer (ITS) gene. Then, 0.5 pmol of ITS1 and ITS2 primers was added following by MyTaq red mix bioline (MyTaq red reaction buffer contains dNTPs, MgCl2 and enhancers at optimal concentrations) supplied with water. The following process was done for performing PCR: for initial denaturation 1 cycle (98 °C for 2 min); for annealing and extension 25 cycles (98 °C for 15 secs; 60 °C for 30 secs; 72 °C for 30 sec) and for final extension of the amplified DNA 1 cycle (72 °C for 10 min).

3.3.4 PCR-Amplified Product Purification and Sequencing

An innuPREP DOUBLE pure Kit (Applied Biosystem, ABI) was used according to the manufacturer's protocol. Using 96-capillary 3730xl DNA Analyser, the PCR products were purified and directly sequenced.

3.3.5 Gel Electrophoresis

Agarose gel electrophoresis used to estimate the base pair of wild *Termitomyces* sp. under UV light. The PCR product was mixed with loading dye (bioline) before

loaded into the wells of the prepared agarose gel, and 100 bp ladder was used. As the running buffer, TBE buffer was used and the electrophoresis was run for an hour.

3.3.6 Data Analysis

gDNA sequence that are obtained was entered into BLAST. The query was submitted after the NCBI Nucleotide Collection (nr/nt) database selected. Sequences producing significant alignment were identified, and the top 10 hit blast was selected for Multiple Sequencing Alignment (MSA) using Clustal Omega (Park, et al., 2004).

3.3.7 Phylogenetic Analysis

In Molecular Evolutionary Genetic Analysis (MEGA-X), evolutionary distance (K_{nuc}) of identical fungal species was calculated by using the neighbouring-joining (NJ), and a phylogenetic tree was generated. It was considered as the same species if have closest K_{nuc} (Park et al., 2004).

3.4 Growing of Mushroom Mycelium

3.4.1 Tissue Culture Technique

The fresh wild fruiting bodies was cleaned with 70% ethanol and sterile distilled water for about two or three times repeatedly. The cleaned fresh wild fruiting bodies was cut using sterile scalpel and the inside fleshy fresh tissue was placed in the middle of Potato Dextrose Agar (PDA) (30g/L) + 2% (w/v) yeast extract (YE). Then, it was sealed and incubated at 28°C for a week. After one week it was sub-cultured onto PDA plate and maintained onto another fresh PDA agar plate and keep at 4°C refrigerator for long term storage.

3.4.2 Manipulation of Agar Medium Preparation

Manipulation of agar medium was carried out by changing the composition of the medium as shown in Table 3.1. The mixture of the medium composition was dissolved in distilled water and subsequently autoclaved at 121°C for 15 minutes at 15 psi. Once it is cooled down, 30 ml poured into petri plates and left to harden. The plates were stored at 4°C in refrigerator for further usage. Manipulation of agar medium was carried out by changing the composition of the medium as shown in Table 3.1.

Table 3.1: Type of medium with its composition

Medium	Composition	Amount (g/L)
Medium 1	PDA	PDA = 39
Medium 2	PDA + YE	PDA = 39, YE = 4
Medium 3	PDA + ME	PDA = 39, ME = 4
Medium 4	PDA + YE + ME	PDA = 39, YE = 4, ME = 4

*PDA= Potato Dextrose Agar, YE = Yeast Extract, ME = Malt Extract

3.5 Submerged Liquid Fermentation (SLF)

From a 10-day old plate 10 plugs mycelial (5 mm) was obtained by using a cork-borer and inoculated in 500 ml Erlenmeyer Flask that contains 100 ml of standard medium which consist of 24 g/L glucose, 1.6 g/L yeast extract, 0.4 g/L of $MgSO_4$ and KH_2PO_4. The standard medium containing the mycelial was incubated at 28°C in incubator shaker for seven days in 170 rpm to obtain the mycelium biomass. By using a Buchner funnel filter the sample was filtered after the tenth day of fermentation, and with distilled water the mycelial biomass was washed three times. By using an oven at 35°C, the filtered mycelial was dried to a constant weight. The weight of pre-dried filter paper before filtering was subtracted from the weight of filter paper with mycelial biomass to calculate the mycelial biomass (Wan- Mohtar et al., 2016).

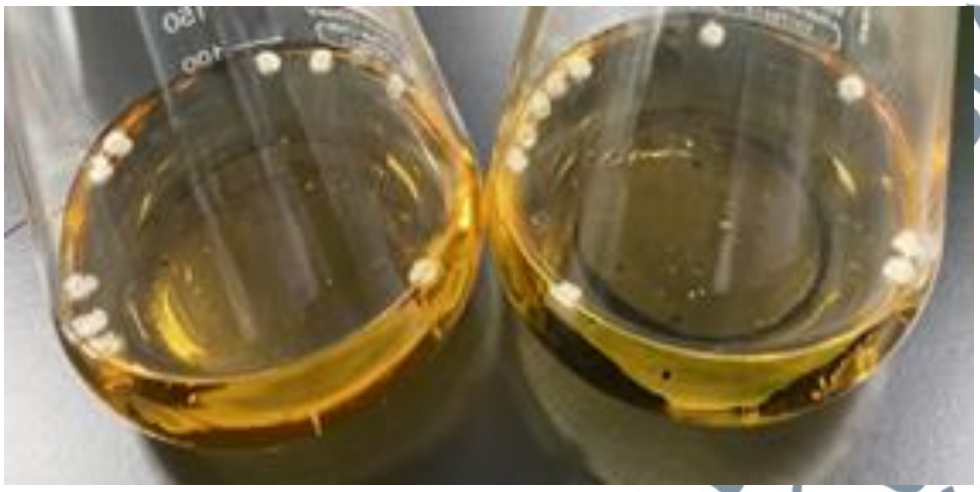


Figure 3.2: Inoculation of mycelium plugs in fermentation media

3.6 Extraction Process

3.6.1 Hot Water Extraction (HWE)

As described in Ubaidillah et al., (2015), dried mushroom powder (40 g) of fruiting bodies and mycelium was extracted twice at 80°C with 600 ml of distilled water in a water bath for 3 hours. The cooled filtrate solution was mixed with 4 times absolute ethanol (1:4 ratio) after filtering using Whatman No. 1 filter paper. The solution was kept overnight at 4°C. The formed precipitate was collected and dried in an oven at 50°C yielding hot extraction endo- polysaccharide from the fruiting bodies (HW-ENS) and IPS from mushroom mycelium.

3.6.2 Cold water Extraction (CWE)

According to Ahmad et al, (2014), 5 g of dried fruiting bodies powder was mixed with 500 ml of distilled water and by using a magnetic plate stirrer for 3 hours vigorously at room temperature. Before filtering using Whatman No. 1 filter paper, the extract was cooled and then the filtrate solution was mixed with 4 times absolute ethanol

(1:4 ratio). The solution was kept overnight at 4°C. The formed precipitate was collected and dried in an oven at 50°C yielding cold extraction endo- polysaccharide from the fruiting bodies (CW-ENS).

3.6.3 Ethanol Precipitation for EPS

By using 4 volumes of absolute ethanol (1:4 ratio), the supernatant from submerged liquid fermentation was mixed with ethanol. The mixture was maintained at 4°C overnight. Then the formed precipitate was placed in an oven at 50°C yielding EPS (Wan-Mohtar et al., 2016).

3.7 β-glucan Determination

Fourier- Transform Infrared Spectroscopy (FTIR) analysis was performed to determine the presence of β-glucan in various THR2 extracts. The sample holder was cleaned by 70% ethanol with Kimwipes on the instrument. The extracts (HW-ENS, CW-ENS, IPS, EPS) was placed on the sample holder and the frequency range was measured as wave numbers in the range of 4000 - 650 cm^{-1}. The spectrum was collected and the obtained results were compared with laminarin as stated in Chen et al., 2013.

3.8 Antimicrobial and Antifungal Activity

3.8.1 Preparation of Nutrient Agar (NA)

Fourteen grams of Nutrient Agar (NA) was weighed using an electronic weighing balance and it was then dissolved in 500 ml of distilled water and autoclaved at 121°C for 15 min at 15 psi. Once it is cooled down, 30 ml was poured into petri plates and left to harden before use for further antimicrobial activity. The prepared plates were stored at 4°C in refrigerator (Farhana and Nadzir, 2017).

3.8.2 Preparation of Nutrient Broth (NB)

Nutrient Broth (NB) was prepared by adding 1.3 g of NB into 100 ml of distilled water. The broth was autoclaved at 121°C for 15 minutes at 15 psi and then it was stored at 4°C in refrigerator (Farhana and Nadzir, 2017).

3.8.3 Preparation of Sabouroud Dextrose Agar (SDA) and Sabouroud Dextrose Broth (SDB)

Sabouraud Dextrose Agar (SDA) powder weighing 65 g was dissolved in 1 L of distilled water. The mixture was dissolved and subsequently autoclaved at 121°C for 15 minutes at 15 psi. Once it is cooled down, it was poured into petri plates and left to harden before use for further antifungal activity; the plates were stored at 4°C in refrigerator for further usage (Farhana and Nadzir, 2017).

Sabouraud Dextrose Broth (SDB) was prepared by dissolving 30 g of SDB into 1 L of distilled water. The mixture was dissolved and subsequently autoclaved at 121°C for 15 min at 15 psi and then it was stored at 4°C in refrigerator (Farhana and Nadzir, 2017).

3.8.4 Optical Density (OD) Standardization for Bacteria and Fungi

Nine millilitres of Nutrient Broth (NB) were poured into 5 falcon tubes and single colony of all 5 types of bacteria [*Escherichia coli* (*E. coli*), *Ralstonia* sp., *Salmonella* sp. *Staphylococcus aureus* (*S. aureus*) and *Streptococcus* sp.] was inoculated into each falcon tube containing NB. The tubes were incubated for 24 hours at 37°C. After 24 hours, OD for each bacterium was checked by taking 1 ml of the broth containing the inoculum and placed in cuvette with 1 ml of NB broth as the blank and the OD was read using Spectrophotometer at 625 nm (Farhana and Nadzir, 2017).

Nine millilitres of Sabouraud Dextrose Broth (SDB) were poured into a falcon tube. Ten millilitres of distilled water were poured on the grown *Aspergillus niger* plate and 1 ml of the distilled water containing the fungi spore was taken and poured into the falcon tube containing SDB; it was incubated for 5 days at 28°C. After five days incubation, the OD was checked using the Spectrophotometer at 625 nm by using 1 ml of SDB as the blank (Farhana and Nadzir, 2017).

3.8.5 Antimicrobial and Antifungal Analysis

Disk diffusion method was used to carry out this analysis in which three Gram-negative bacteria; *Escherichia coli* (*E. coli*), *Ralstonia* sp., *Salmonella* sp., two Gram-positive; *Streptococcus* sp. and *Staphylococcus aureus* (*S. aureus*), was used for antimicrobial analysis and one fungal strain; *Aspergillus niger* (*A. niger*) was used for antifungal analysis. In this method, Nutrient Agar (NA) was used as culture medium and the filter disc was placed aseptically over the bacterial culture plate. For fungal strain, the same steps was done but Sabouraud Dextrose Agar (SDA) was used as the culture medium. The discs plate was prepared by using Whatmann filter paper No. 1 approximately with a size of 6 mm and autoclaved. A 100 µl of the bacteria was transferred on the agar and spread using 'L' shaped hockey stick (spread plate technique). Six discs were placed on the agar surface; one with hot water extracts from fruiting bodies (HW-ENS), cold water extract from fruiting bodies (CW-ENS), EPS, IPS from mycelium, antibiotic as standard, distilled water as positive and negative control. Then the bacteria plates were incubated at 37°C for 24 hours whereas fungi were incubated at 28°C for a week. The zone of inhibition was measured in millimetre, mm (Ramesh et al., 2010). Penicillin- streptomycin solution ATCC® 30- 2300™ (Pen-Strep 10 µg) was used as standard for the Gram- positive and Gram- negative bacteria

whereas for the fungi Penicillin- streptomycin- Amphotericin B solution PCS® 99-002™ (Pen- Strep- Amp B 25 µg) was used as standard.

3.9 Qualitative Analysis of Phytochemicals

3.9.1 Flavonoids Detection

Few drops of $FeCl_3$ were added to 1 ml of various crude extract of THR2. The existence of flavonoids can be confirmed when a blackish red precipitate appeared in the test sample (Mahadeva et al., 2016).

3.9.2 Glycosides Detection

By using the Keller Killani test, 1 ml of glacial acetic acid was added to 1 ml of various different crude extract of THR2 and cooled. After cooling, 2 drops of $FeCl_3$ were added followed by careful addition of concentrated H_2SO_4 along the walls of the test tube. A reddish-brown colour ring formation at the junction of two layers indicates the existence of glycosides (Mahadeva et al., 2016).

3.9.3 Saponin Detection

Two millilitres of distilled water were added to 2 ml of various THR2 crude extracts and shaken vigorously for 15 min. A layer of 1 cm or more thick foam confirms the existence of saponin (Mahadeva et al., 2016).

3.9.4 Tannin Detection

A volume of 2 ml of 5% $Fecl_3$ were added to 1 ml of various THR2 crude extracts samples. Appearance of greenish-black or dark blue colour confirms the presence of tannins (Mahadeva et al., 2016).

3.9.5 Terpenoids Detection

To 5 ml of various THR2 crude extracts samples, 2 ml of chloroform, $CHCL_3$ and 3 ml of concentrated H_2SO_4 were carefully added. A reddish-brown colouration signifies the presence of terpenoids (Mahadeva et al., 2016).

3.9.6 Phenols Detection

A small amount of various THR2 crude extracts samples was taken with 1 m; of water in test tube and few drops of $FeCl_3$ were added. A blue, green, red or purple colour indicates the existence of phenols (Mahadeva et al., 2016).

3.10 Total Flavonoid Content, TFC

According to the method of Ahmad et al., (2014) 1 ml of sample from THR2 crude extracts solution (2 mg/ml) was diluted with 80% of aqueous ethanol (4.3 ml) to determine the total flavonoid content. Before incubating at room temperature for 40 min, 0.1 ml aqueous potassium acetate (1M) and 0.1 ml of 10% aluminium nitrate was added to the mixtures. Then, the absorbance was read using spectrophotometer at 415 nm. Quercetin was used as a standard; data was depicted as mg of Quercetin equivalents per gram of extracts, total flavonoid content was calculated. Table 3.2 below shows the preparation of different concentration of Quercetin solution.

Table 3.2: Quercetin solution preparation for standard curve

Final Concentration (mg/ml)	Stock (µL)	Distilled Water (µL)	Total (ml)
0	0	1000	1
1	100	900	1
2	200	800	1
4	400	600	1
6	600	400	1
8	800	200	1
10	1000	0	1

3.11 Quantitative Analysis - DPPH Assay

Stable radical DPPH reagent was used to carry out this analysis; from the bleaching of the purple colour methanol solution of DPPH the ability of the hydrogen or electron donation of the extracts was measured. The mixtures were incubated in the dark for 30 min at room temperature after various crude extract of THR2 (1 ml) added to 4 ml of methanol solution of DPPH. Then, at 517 nm the absorbance was read against a blank (Ahmad et al., 2014). By using the following way free radical inhibition by DPPH in percent (%) was calculated:

$$\text{Percentage of Inhibition (\%)} = \left(\frac{Absorbance\ control - Absorbance\ sample}{Absorbance\ control}\right) \times 100 \quad (3.1)$$

In which the absorbance of the test compound is absorbance sample whereas absorbance control is the absorbance of the control reaction that contains all reagents except the test compound. Tests was carried out in triplicates. In the graph plotted percentage of inhibition against concentration crude extracts sample, the value of 50% inhibition (IC_{50}) was determined Table 3.3 shows the preparation of different concentration of ascorbic acid to plot the standard curve.

Table 3.3: Ascorbic acid solution preparation for standard curve

Final Concentration (mg/ml)	Stock (µL)	Distilled Water (µL)	Total (ml)
0	0	1000	1
1	100	900	1
2	200	800	1
4	400	600	1
6	600	400	1
8	800	200	1
10	1000	0	1

3.12 IC_{50} Value Determination

The obtained linear equation was used to calculate the IC_{50} value by replacing the Y as 50%. The result was calculated using the gradient calculation method for various THR2 crude extracts.

$$Y = mx + c \quad (3.2)$$

Y = y-value

m = Gradient

x = x-value

c = y-intercept