CHAPTER 4

RESULTS

4.1 Isolation and morphology observation of THR2 fruiting bodies

This study successfully isolated the wild termite mushroom (THR2) during the September 2019 Southwest Monsoon. At the sampling site, THR2 fruiting bodies were discovered strewn across the soil's surface under conditions of high humidity and low range of temperature. The interior of the termite nest that is growing fungus resembles a comb when viewed through a cross section of the soil (Figure 4.1B), which has accumulated mycelium on it (Fig 4.2A). White termites were also seen (Figure 4.1E), and it was clearly demonstrated that the fruiting body stipe was growing from the termite nest (Figure 4.1C), passing through the soil, and emerging on the soil's surface (Figure 4.1A). Termite nests were between 10 and 15 cm in diameter, white-brownish in colour with immature stipe, and black in colour with mature stipe (Figure 4.2C). The fruiting bodies were seen to be of varying heights (Figure 4.2B), and Figure 4.2D depicted the morphological structure of the white gills beneath the pileus with centrally attached stipes.

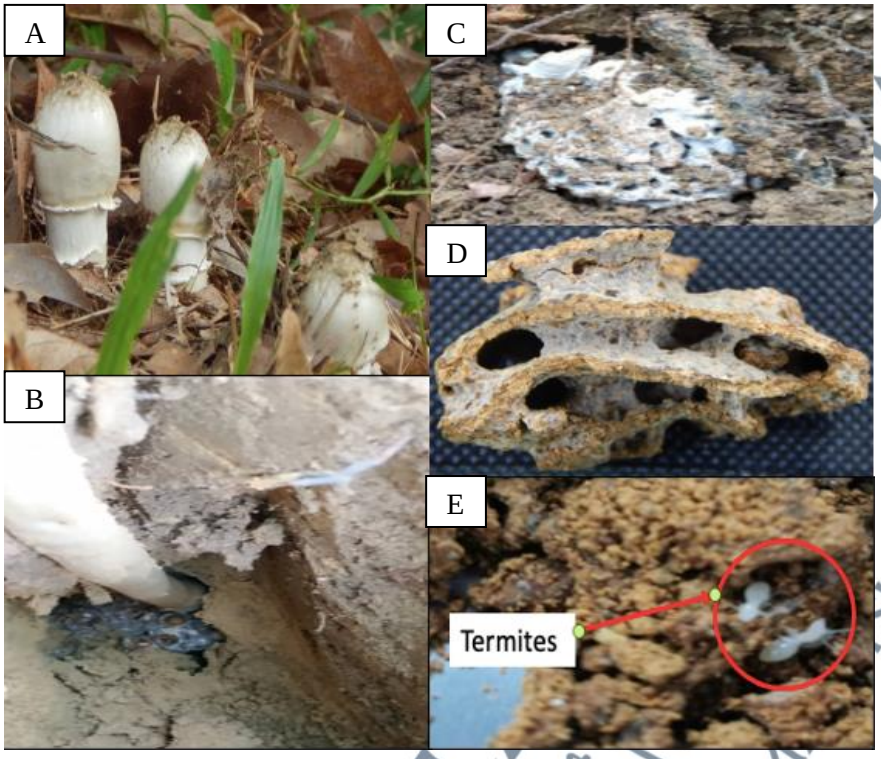


Figure 4.1: [A] Fruiting bodies of wild *T.heimii* mushroom emerged on the surface of the soil; **[B]** Cross-section of soil-wild *T.heimii* mushroom was observed growing from the termite nest; **[C]** Termite nest; **[D]** Inner comb of termite nest; **[E]** Soils and cracked termite nest with white termites.

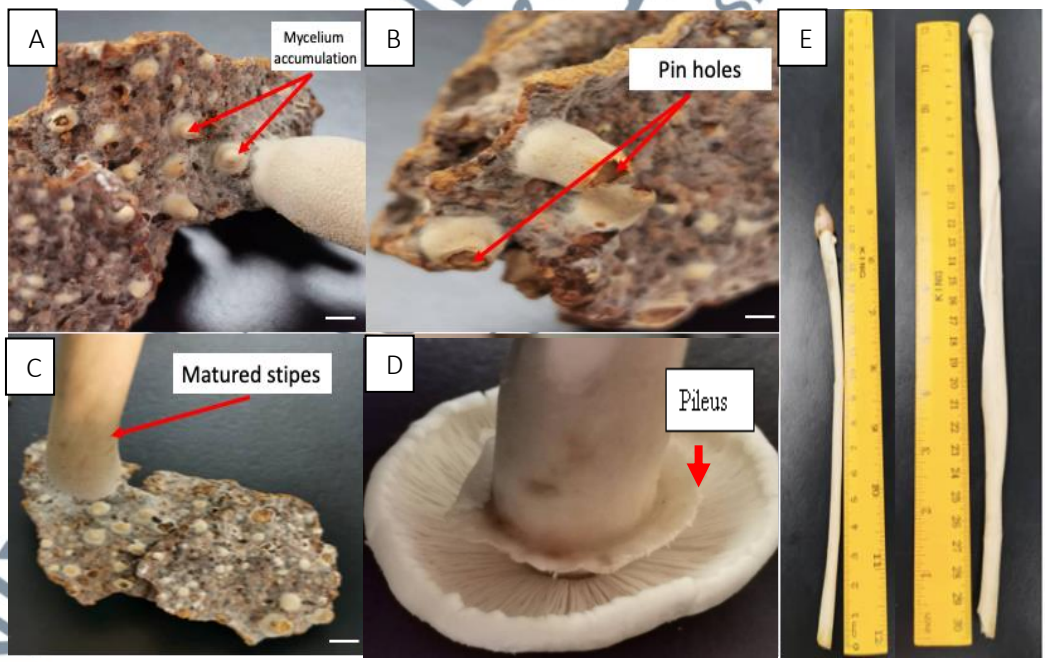


Figure 4.2: [A]Accumulation of budding mycelium on top of the termite nest; **[B]** Hyphal with pin-holes; **[C]** Matured stipes of wild termite mushroom; **[D]** Morphological structure of the prominent perforatorium (pileus), white gills under the pileus and centrally attached stipes (bar = 1 cm); **[E]** Different height of matured stipes.

4.2 Tissue Culture Technique

The mycelium was successfully cultivated on the PDA agar containing 2% of yeast and malt extract after 14 days of incubation under 28°C in the incubator. The morphology of the mycelium is seen to be fluffy, white, and soft for THR2 (Figure 4.3).

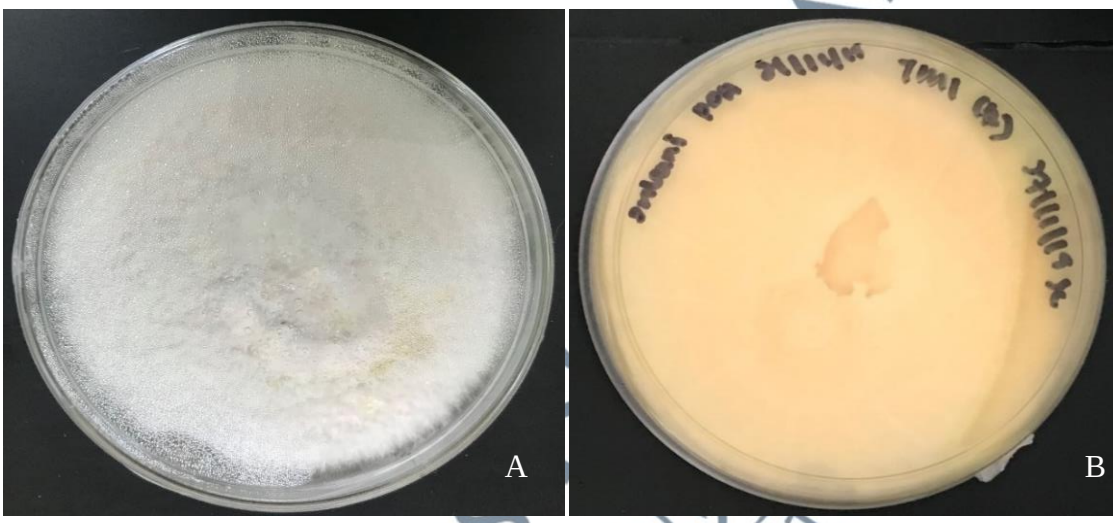
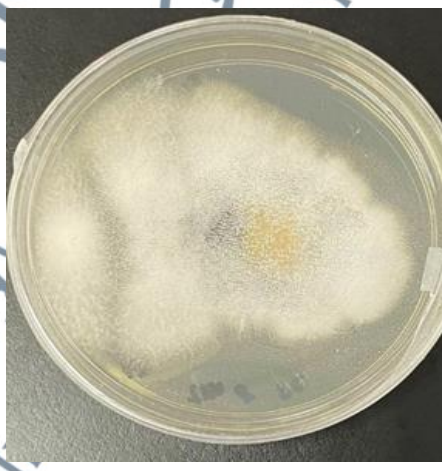


Figure 4.3: Successfully grown mycelium of THR2 by tissue culture technique, **[A]** front of the mycelium **[B]** back of the mycelium

4.3 Cultivation of Mycelium onto Agar Medium

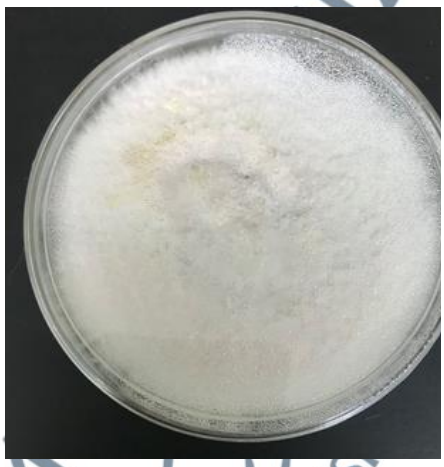
Growth of THR2 mycelium was enhanced by manipulating the agar medium composition and it was observed that medium 4 promotes the greatest growth of the mushroom mycelium, in Table 4.1 where the morphology of the mycelium shown. Table 4.2 shows the diameter of the mycelium with different cultivation period and composition of agar medium.

Table 4.1: Morphology of THR2 mycelium on different medium compositions

Medium	Composition	Plate Morphology	Mycelium Plate
Medium 1	PDA	Thin white mycelium	
Medium 2	PDA + YE	Thin white mycelium	
Medium 3	PDA + ME	Somewhat compact white mycelium	

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

Table 4.1: (continued)

Medium	Composition	Plate Morphology	Mycelium Plate
Medium 4	PDA + YE + ME	Compact and dense white mycelium	

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

Table 4.2: Diameter of THR2 mycelium on different cultivation period and medium composition

Days	Agar Composition	Diameter (mm)
9	PDA	5 ± 0.8
	PDA + YE	17 ± 1.0
	PDA + ME	20 ± 1.0
	PDA + YE + ME	29 ± 1.3
11	PDA	5 ± 0.7
	PDA + YE	19 ± 1.0
	PDA + ME	25 ± 1.2
	PDA + YE + ME	33 ± 1.0
14	PDA	5 ± 0.6
	PDA + YE	25 ± 1.2
	PDA + ME	30 ± 1.0
	PDA + YE + ME	FP
16	PDA	5 ± 0.2
	PDA + YE	29 ± 1.0
	PDA + ME	FP
	PDA + YE + ME	FP

*FP = Full plate. Values are the average of triplicates

Based on Table 4.2 above, the highest diameter of THR2 mycelium achieved on PDA medium was 5 mm from day 9 to day 11. The combination of PDA + YE showed

highest diameter of 29 mm at day 16 whereas for PDA + ME the diameter of mycelium increased to 20 mm (day 9) and reached full plate (45 mm) in day 16 only. It was observed that THR2 had the greatest growth on agar with the combination of PDA + YE + ME without any sign of contamination and the diameter of mycelium increased from 29 mm (day 9) to 33 mm (day 11) and reached full plate (45 mm) in day 14 itself.

4.4 Molecular Identification of Wild *Termitomyces* sp. Mushroom

4.4.1 Gel Electrophoresis

Molecular identification of a wild fungal sample is important to determine the species of the sample. The base pairs of wild THR2 RFES 230622 were estimated using agarose gel electrophoresis under UV light (Figure 4.4). Lane 1 resembles to 100 bp marker, Lane 2 resembles no template control (NTC) as the negative control (-ve), Lane 3 resembles to positive control (+ve) and Lane 4 resembles to the sample (THR2) with a base pair of 300.

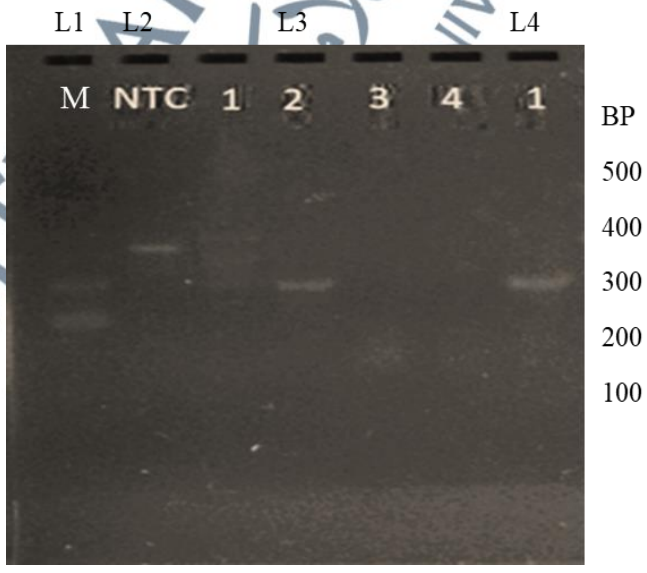


Figure 4.4: Band of DNA isolated from THR2

4.4.2 Phylogenetic Tree

RFES 230662 was found to be 99% similar to *Termitomyces* sp. Detailed phylogenetic analyses (Figure 4.5) using showed the evolutionary distances (K_{nuc}) values. Clade A showed that *Termitomyces* sp. RFES 230662 (THR2) was closely related to *Termitomyces heimi*.

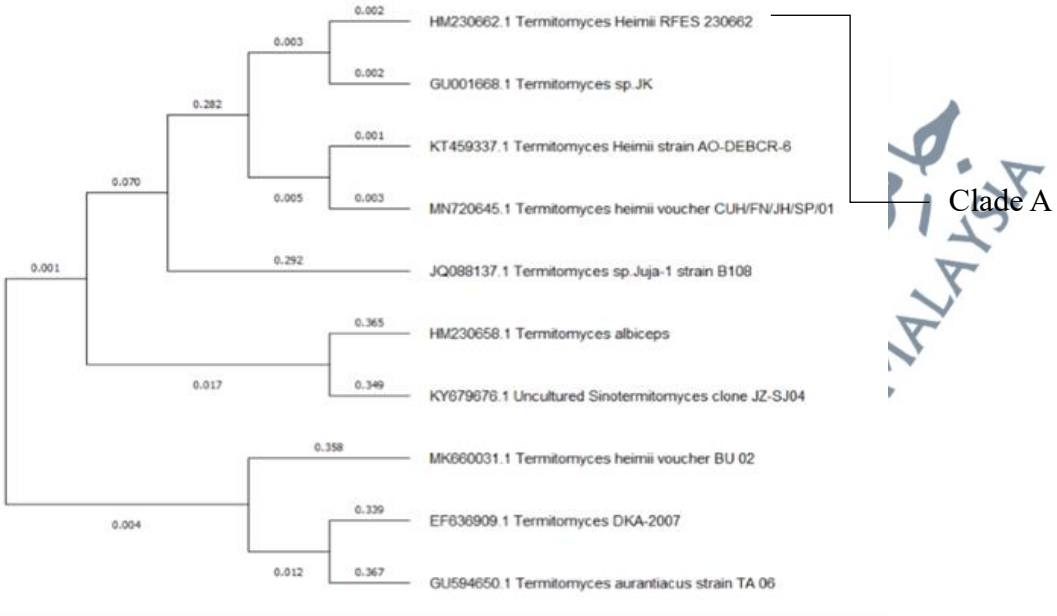


Figure 4.5: Phylogenetic tree of *Termitomyces heimii* strain RFES 230662

4.5 Submerged Liquid Fermentation (SLF) of THR2

Batch SLF was successfully carried out and it produced 8.55 g/L of mycelium biomass after 7 days of fermentation. THR2 fruiting bodies was extracted using two extraction method. As shown in Figure 4.6, 3.20 g/L of HW-ENS and 1.36 g/L of CW-ENS was obtained. The dried mycelium of THR2 was further extracted by hot water for IPS which was 0.80 g/L. The EPS was obtained from the supernatant of liquid fermentation medium and the yield obtained was 1.44 g/L. Crude polysaccharides from fruiting bodies (HW-ENS & CW-ENS) were higher than SLF mycelium (IPS & EPS).

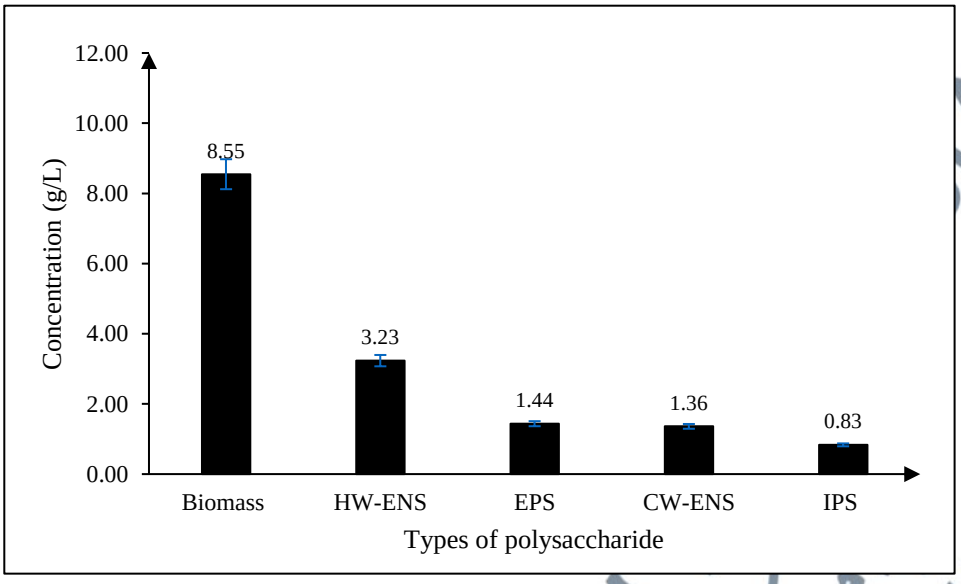


Figure 4.6: Concentration of ENS from THR2 mushroom fruiting bodies, IPS and EPS in g/L obtained from SLF

Figure 4.7 below shows the mycelium pellet of THR2 in 7 days cultivation. It was observed that the pellet is small and dense.

Figure 4.7: Mycelium pellet of THR2 after 7 days fermentation

4.6 β-glucan determination

The existence of the position and anomeric configuration of the glycosidic linkage in extracts (CW-ENS, HW-ENS, EPS and IPS) were characterized and confirmed using FTIR analysis. Figure 4.8 below shows the peak observed in the graph from FTIR and it was confirmed that β-glucan polysaccharides existed in HW-ENS, EPS and IPS of THR2 crude extracts. The component of the extracts was also compared with laminarin stated by Chen et, al. 2013. Table 4.3 shows comparison between the peak formed from the various extracts and laminarin.

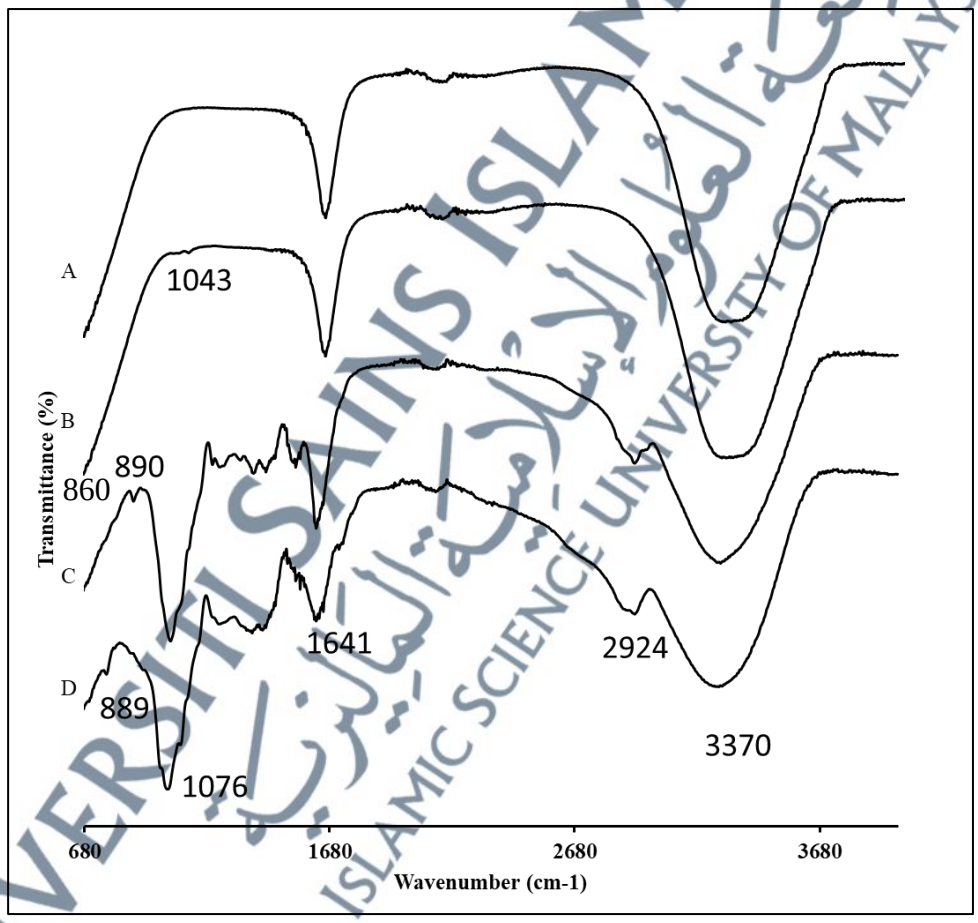


Figure 4.8: [A] FTIR peak of CW-ENS of THR2 fruiting bodies, **[B]** HW-ENS of THR2 fruiting bodies, **[C]** IPS (mycelium) of THR2 mycelium crude extract, **[D]** EPS crude extract of THR2 culture broth (supernatant)

Table 4.3: Spectral characteristics from various extracts of THR2

Group	Vibration Mode	Types of Crude Extracts				Standard
		CW-ENS	HW-ENS	IPS	EPS	Laminarin
O-H	O-H stretching vibration	3370	3370	3370	3370	3370
$-CH_2$	C-H stretching vibration	-	-	2924	2924	2924
C=O	Symmetric and asymmetric stretching vibration	1641	1641	1641	1641	1641
C-O	C-O stretching vibration	-	1043	1076	1076	1043, 1076
-	Anomeric configuration of glycosidic linkage (β-configuration)	-	860	890	889	889

Based on the Table 4.3 above all the crude extracts (CW-ENS, HW-ENS, IPS and EPS) showed the presence of both O-H and C=O stretching vibrations. The $-CH_2$ stretching vibration was exhibited by IPS and EPS whereas the C-O stretching vibration was exhibited by HW-ENS, IPS and EPS.

4.7 Antimicrobial and Antifungal Activity

4.7.1 Bacterial Cultures on Nutrient Agar, NA

The bacteria were grown on NA plate at 37°C in the incubator for 24 hours. The grown bacteria culture shown in Figure 4.9. At a wavelength of 625 nm, OD was as follow; *Ralstonia* sp. (0.893), *Salmonella* sp. (0.884) *Escherichia coli* (0.859), *Staphylococcus aureus* (0.862), *Streptococcus* sp. (0.871) and the test organism were adjusted the concentration at 1.0 OD.

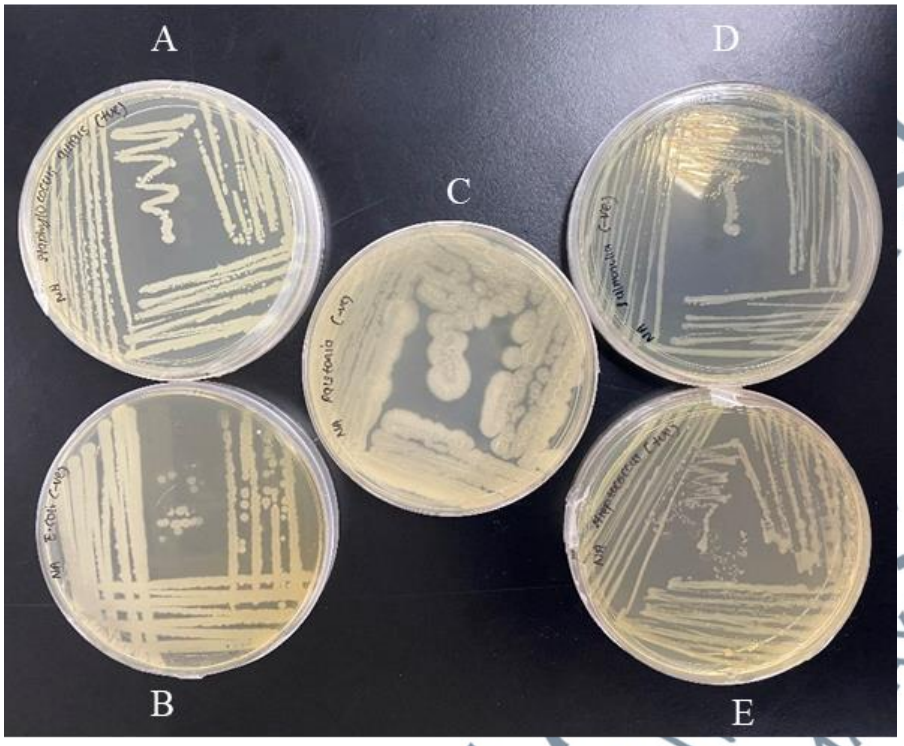


Figure 4.9: [A] *Staphylococcus aureus,* **[B]** *Escherichia coli,* **[C]** *Ralstonia* sp., **[D]** *Salmonella* sp., **[E]** *Streptococcus* sp.

4.7.2 Fungal Culture on Sabouraud Dextrose Agar, SDA

Aspergillus niger grew fully after one week of incubation at the temperature of 28°C in the incubator. Figure 4.10 shows the grown *A. niger*. The OD was 0.783 at a wavelength of 625 nm.

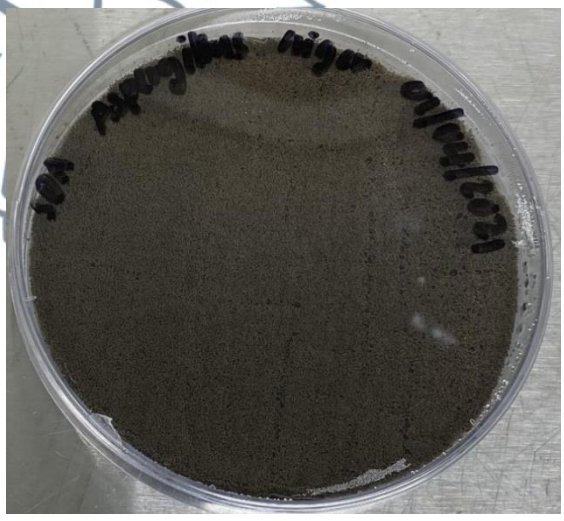
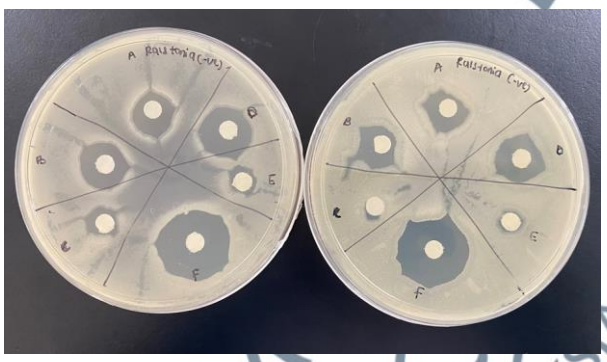
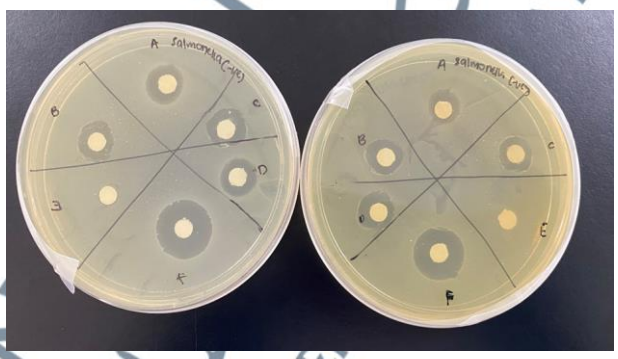


Figure 4.10: *Aspergillus niger*

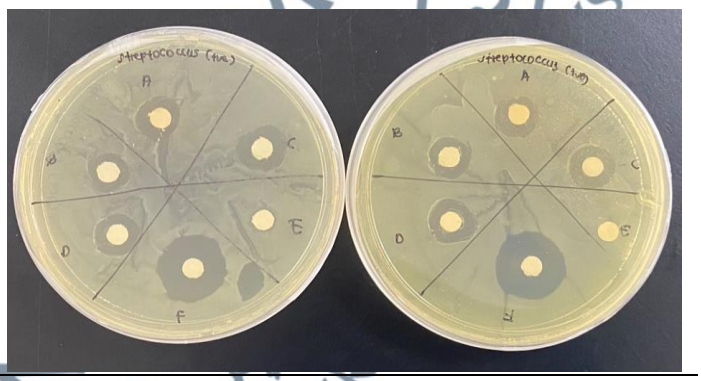
4.7.3 Antimicrobial and Antifungal Activity

Table 4.4: Antimicrobial test obtained from various extracts of THR2 against Gram-negative bacteria

Gram- negative Bacteria	Formation of Zone of Inhibition
Ralstonia sp.	
Salmonella sp.	
Escherichia coli	

Based on Table 4.4, disc diffusion test was done in duplicates for each Gram-negative bacteria with various crude extracts obtained from THR2 (A: HW-ENS, B: CW ENS, C: EPS, D: IPS), Penicillin- streptomycin solution ATCC® 30- 2300™ (F: Pen- Strep 10 µg) used as positive control, distilled water (E) was used as standard and negative control.

Table 4.5: Antimicrobial test obtained from various extracts of THR2 against Gram-positive bacteria

Gram- positive Bacteria	Formation of Zone of Inhibition
Staphylococcus aureus	
Streptococcus sp.	

Based on Table 4.5, disc diffusion test was done in duplicates for each Gram-positive bacteria with various crude extracts obtained from THR2 (A: HW-ENS, B: CW ENS, C: EPS, D: IPS). The positive control, standard and negative control remains the same as in Table 4.4.

Based on Table 4.6, disc diffusion test was done in duplicates for fungi with various crude extracts obtained from THR2 (A: HW-ENS, B: CW ENS, C: EPS, D: IPS),, Penicillin- Streptomycin- Amphotericin B solution PCS® 99- 002™ (F: Pen-Strep- Amp B 25 µg) used as positive control whereas distilled water (E) was used as the standard and negative control.

Table 4.6: Antifungal test obtained from various extracts of THR2 against fungi

Fungi	Formation of Zone of Inhibition
Aspergillus niger	

Table 4.7: Average zone of inhibition of various extracts from THR2 against five bacteria and a fungi

Samples	Tested Bacteria					Tested Fungi
	Gram-Negative Bacteria (-ve)			Gram-Positive Bacteria (+ve)		
	Ralstonia sp. (mm)	*Salmonella* sp. (mm)	*E. coli* (mm)	*S. aureus* (mm)	*Streptococcus* sp. (mm)	*A. niger* (mm)
HW-ENS	6.5 ± 0.1	5.3 ± 0.1	4.0 ± 0.1	4.5 ± 0.1	5.0 ± 0.1	-
CW-ENS	4.5 ± 0.1	3.5 ± 0.2	2.5 ± 0.1	3.0 ± 0.1	4.0 ± 0.1	-
EPS	3.0 ± 0.2	3.8 ± 0.1	-	4.0 ± 0.1	2.7 ± 0.1	-
IPS	6.0 ± 0.1	5.0 ± 0.1	-	5.7 ± 0.1	4.5 ± 0.1	-
Distilled Water	-	-	-	-	-	-
Pen- Strep	10 ± 0.1	10 ± 0.1	10 ± 0.1	10 ± 0.1	10 ± 0.1	-
Pen-Strep-Amp B	-	-	-	-	-	-

*(-) shows no zone of inhibition. Each value is the mean ± SD of triplicates.

THR2 extracts could inhibit both Gram- negative and Gram- positive bacteria as shown in Table 4.7. For the HW-ENS from fruiting bodies *Ralstonia* sp. showed the

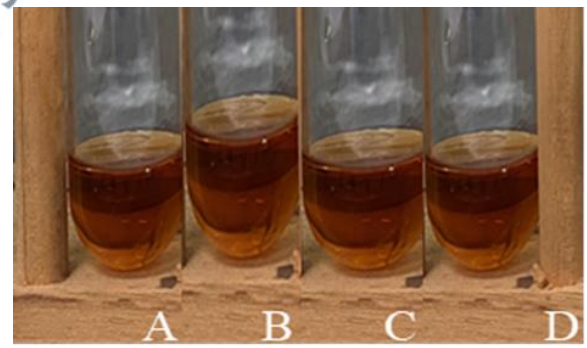
highest zone of inhibition which is 6.5 mm followed by *Salmonella* sp. (5.3 mm), *Streptococcus* sp. (5.0 mm) *S. aureus* (4.5 mm) and *E. coli* (4.0 mm). For the CW-ENS from fruiting bodies *Ralstonia* sp showed the highest zone of inhibition which is 4.5 mm whereas *E. coli* showed the least which is 2.5 mm. For hot water intracellular polysaccharide (IPS) from mycelium sample, *Ralstonia* sp showed the highest zone of inhibition which is 6 mm whereas *Streptococcus* sp showed the least which is 4.5 mm. *Salmonella* sp. showed the highest zone of inhibition which is 3.8 mm whereas *Streptococcus* sp. showed the least which is 2.7 mm for extracellular polysaccharide (EPS) sample. There was no zone of inhibition formed for *A.niger* because the extracts don't have the capability to inhibit the growth of fungi.

4.8 Qualitative Analysis of Phytochemicals and Antioxidant Analysis

4.8.1 Phytochemicals Detection

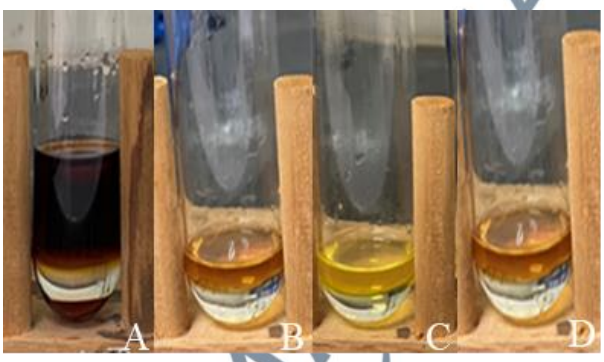
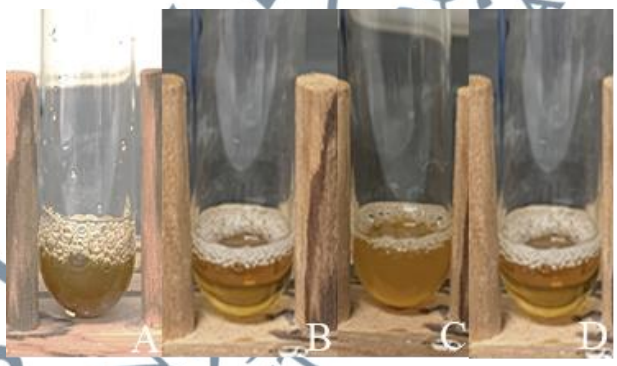
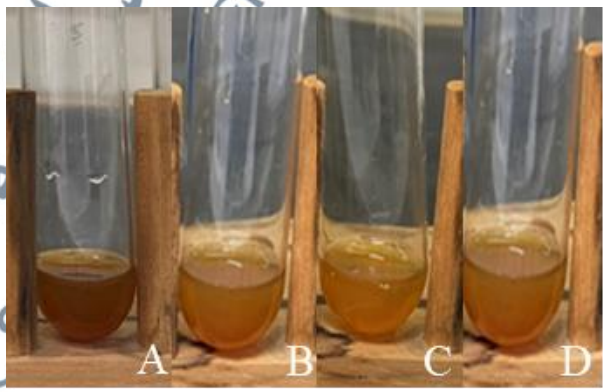
The Table 4.8. below shows the results of phytochemical test observed from various crude extracts obtained from THR2.

Table 4.8: Phytochemical test observation of various extracts from THR2

Test	Observation	Figure
Flavonoids	Formation of blackish red precipitate indicates the presence of flavonoids A - Present B - Present C - Present D - Present	

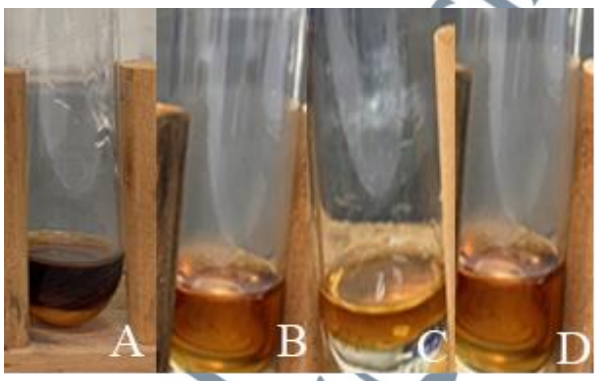
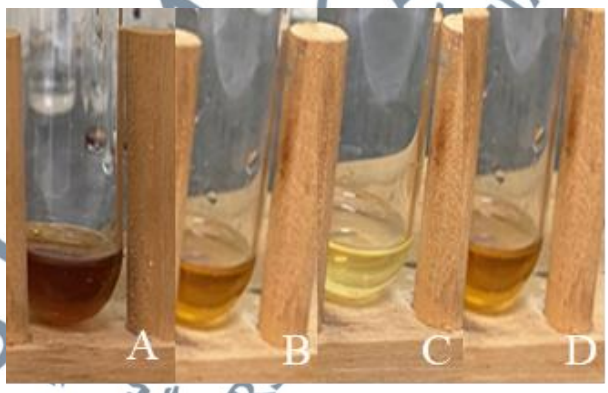
*A = HW-ENS, B = CW-ENS, C = EPS, D = IPS

Table 4.8: (continued)

Test	Observation	Figure
Glycosides	Formation of reddish-brown colour ring at the junction of two layers proves the presence of Glycosides; A - Present B - Present C - Absent D - Present	
Saponins	Formation of foams indicates the presence of saponins; A - Present B - Present C - Present D - Present	
Tannin	Appearance of greenish black or dark blue colour confirms the presence of tannins; A - Absent B - Absent C - Absent D - Absent	

*A = HW-ENS, B = CW-ENS, C = EPS, D = IPS

Table 4.8: (continued)

Test	Observation	Figure
Terpenoids	Reddish-brown colouration signifies the presence of terpenoids; A - Present B - Present C - Present D - Present	
Phenols	A blue, green, red or purple colour indicates the presence of phenols; A - Absent B - Absent C - Absent D - Absent	

*A = HW-ENS, B = CW-ENS, C = EPS, D = IPS

Table 4.9: Detected phytochemical compounds in various extracts of THR2

	Phytochemical Compounds					
Crude Extracts	Flavonoids	Glycosides	Saponin	Tannin	Terpenoids	Phenols
HW-ENS	+	+	+	-	+	-
CW-ENS	+	+	+	-	+	-
EPS	+	-	+	-	+	-
IPS	+	+	+	-	+	-

*(+) = Presence, (-) = Absence

Based on the Table 4.9 above it can be observed that flavonoids, glycosides, saponins and terpenoids are present in HW-ENS, CW-ENS extracts and IPS mycelium extracts of THR2 whereas in EPS extracts of THR2 only flavonoids, saponins and

terpenoids are present. Flavonoids, saponins and terpenoids was found in all the extracts of THR2 whereas tannins and phenols were not detected.

4.8.2 Quantification of Antioxidant Compound

4.8.2.1 Quercetin Standard Curve

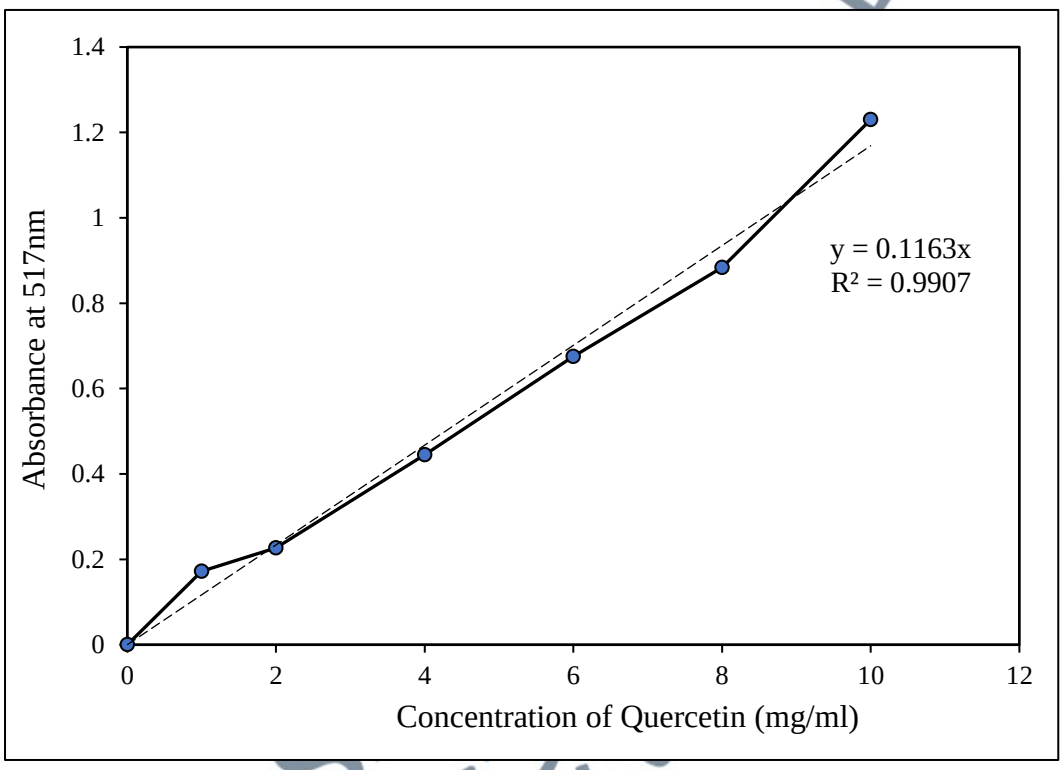


Figure 4.11: Standard curve of Quercetin for flavonoid determination

A standard curve using quercetin was created as shown in Figure 4.11, in order to calculate the concentration of flavonoid compound by associating their absorbance with quercetin absorbance. It can be observed from the figure above that as the concentration of the sample increases, the absorbance also increases steadily and the correlation coefficient of the standard graph is 0.9907.

4.8.2.2 Ascorbic Acid Standard Curve

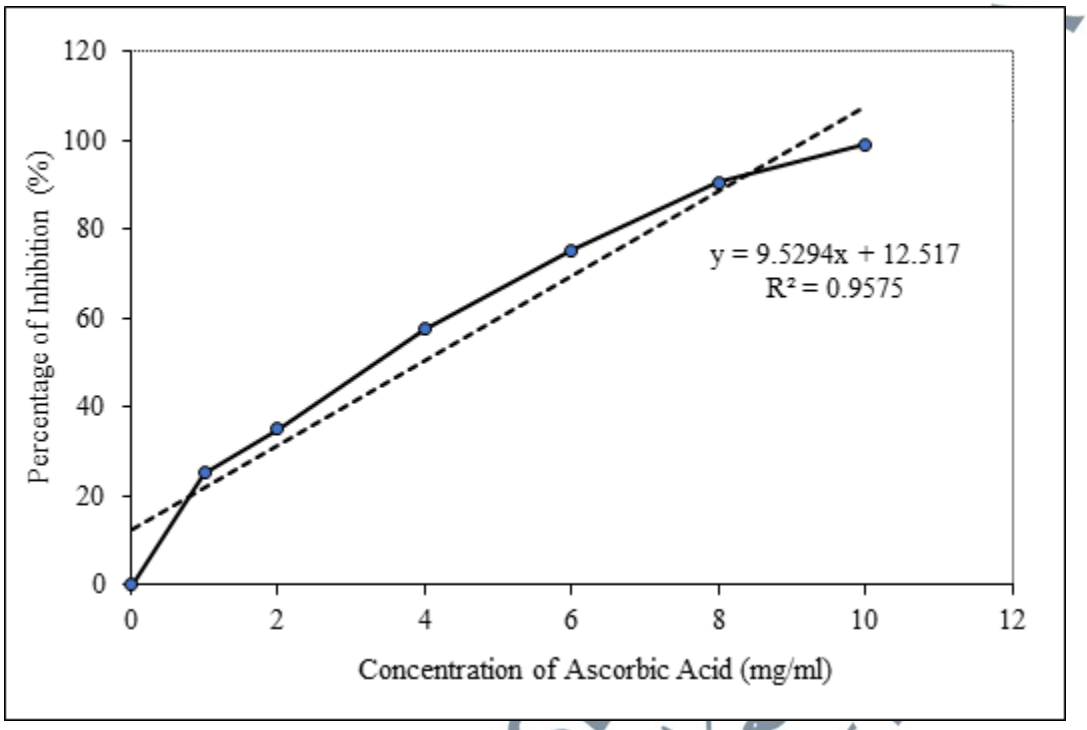


Figure 4.12: Standard curve of Ascorbic Acid for DPPH analysis

A standard curve using ascorbic acid was created as shown in Figure 4.12. It can be observed from the figure above that the standard graph is directly proportional to the percentage of inhibition; as the concentration of the sample increases, the percentage of inhibition (%) also increases steadily and the correlation coefficient of the standard graph is 0.9575.

Table 4.10: TFC content in various extracts of THR2

THR2 Extracts	TFC (mg/g)
HW-ENS	2.86 ± 0.15
CW-ENS	2.57 ± 0.09
EPS	2.16 ± 0.06
IPS	2.52 ± 0.07

*Each value is the mean ± SD of triplicates.

The total flavonoids are expressed as QE (Quercetin equivalent). It was observed that TFC was in the range of 2.16 – 2.86 mg/g from the Table 4.10, in all the THR2 extracts. However, a higher concentration of flavonoid content was found in HW-ENS THR2 extract followed by CW-ENS and IPS whereas EPS was found to contain the least flavonoid content compared to other THR2 extracts as demonstrated in Table 4.10. Based on the statistical analysis using SPSS (One-Sample Proportions Tests) the reading obtained are significant as the p-value is less than 0.5.

4.8.3 DPPH Assay

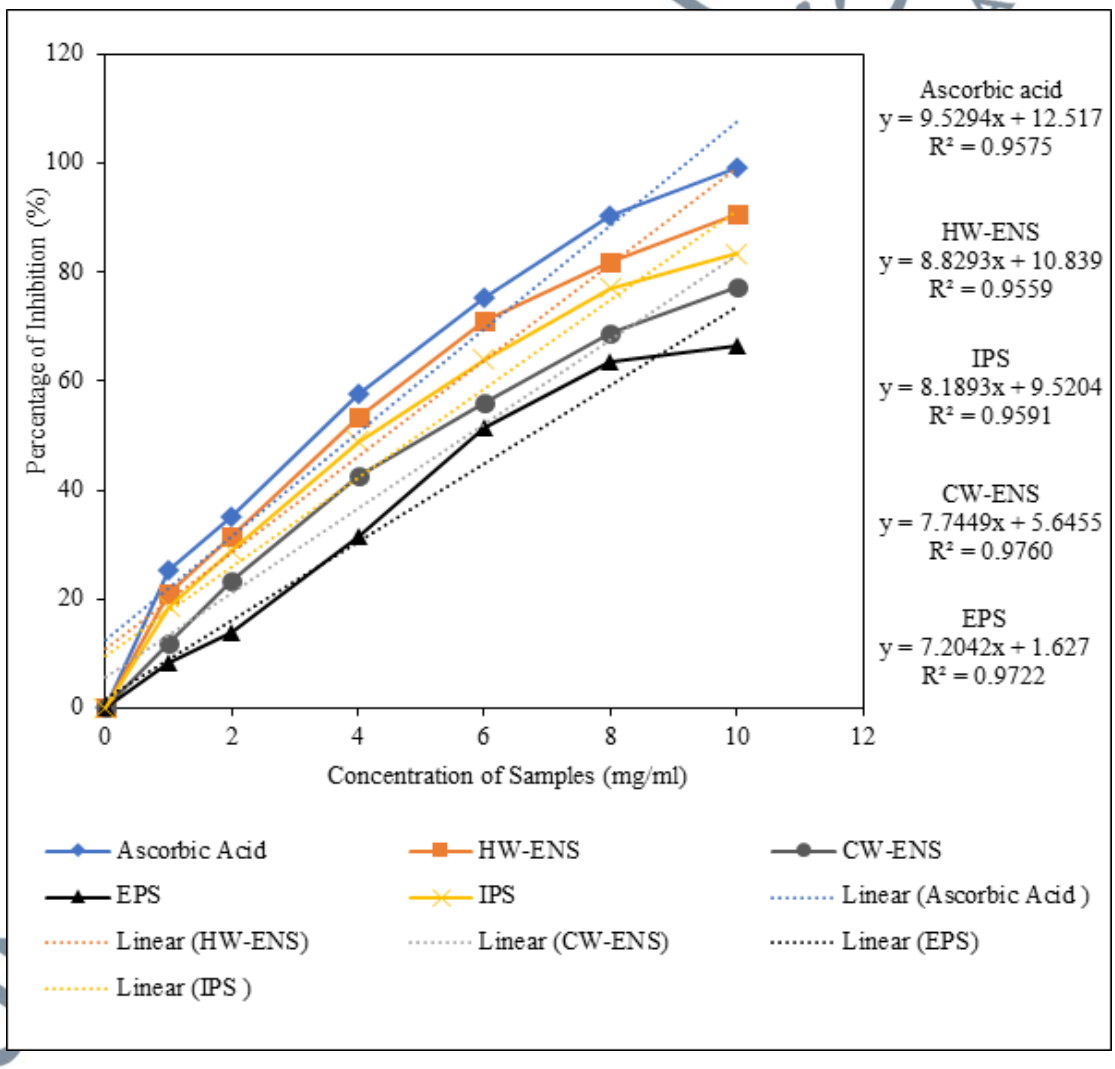


Figure 4.13: Percentage inhibition in DPPH assay

Ascorbic acid standard curve was plotted as the standard graph and the percentage of inhibition (%) was obtained using DPPH assay via comparing their absorbance with the standard ascorbic acid absorbance. It can be observed from the Figure 4.13, that the magnitude of the curve is positive and all the graphs represent various extracts of THR2 are directly proportional to the percentage of inhibition (%). The correlation coefficient of HW-ENS, IPS, CW-ENS and EPS is 0.9559, 0.9591, 0.9760 and 0.9722 respectively. At 10 mg/ml concentration, highest percentage of inhibition was showed by HW-ENS which was 90.83% followed by IPS (83.39%), CW-ENS (77.21%) whereas the lowest percentage of inhibition was showed by EPS which was 66.44% as demonstrated in Figure 4.13.

The IC_{50} values of all extracts lies between 4.44 mg/ml to 6.71 mg/ml and the IC_{50} value of ascorbic acid is 3.93 mg/ml. A low IC_{50} value signifies a high antioxidant activity. As shown in the Table 4.11, HW-ENS sample of THR2 extract shows the least IC_{50} value which is 4.44 mg/ml followed by IPS (4.94 mg/ml) and CW-ENS (5.73 mg/ml) whereas EPS showed the highest IC_{50} value which is 6.71 mg/ml.

Out of this four THR2 extracts HW-ENS 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 which was 90.83% compared to the other extracts.

Table 4.11: IC_{50} value of various extracts of THR2

THR2 Extracts	IC_{50} (mg/ml)
HW-ENS	4.44
CW-ENS	5.73
IPS	4.94
EPS	6.71
Ascorbic acid	3.93