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Investigation of the White-rot Fungus Biomass Extraction Conditions and Their Impact on Phenolic, Flavonoids Content and Antioxidant Activities

Ahmad Anas Nagoor Gunny[1,2*], Shahizatul Emilia Jahidin[1], Zulkarnain Mohamed Idris[1], Gidado Muhammad Junaid[1], Safa Senan Mahmod[1,2], Norhidayah Bohari[3]

[1]Faculty of Chemical Engineering & Technology, Universiti Malaysia Perlis (UniMAP), 02600, Arau, Perlis, Malaysia

[2]Centre of Excellence for Biomass Utilization (CoEBU), Universiti Malaysia Perlis, 02600 Arau, Perlis, Malaysia

[3]FGV Innovation Centre (Beneficial Microbes) PT35377 Lengkuk Teknologi, 71760 Bandar Enstek, Negeri Sembilan, Malaysia

ABSTRACT

White-rot fungus are known as natural source of antioxidant compounds such as flavonoids, phenolic, and other enzymes through extraction processes. In this study, Phanerochaete chrysosporium and Panus tigrinus biomass were subjected into different extraction conditions to determine the best optimal level for the extraction of phenolic and flavonoids compounds. P. chrysosporium produced high yield of phenolic (0.7041 mg/mL) and flavonoids (0.0286 mg/mL) compared to P. tigrinus; 0.3755 mg/mL and 0.0109 mg/mL of phenolic and flavonoids compounds, respectively. P. chrysosporium has higher antioxidant activity (70.31%) compared with P. tigrinus (25.30%). Therefore, P. chrysosporium was selected to determine the optimum condition for extraction method of phenolic and flavonoids compounds using aquoues-two phase system. The conditions used in the extraction was time within the range of 30 min to 180 min and temperature range from 250 ℃ to 650 ℃. The conditions were optimized using Response Surface Method (RSM) under Central Composite Design. The best optimum condition was 105 min and 450 ℃, this condition produced the highest concentrations of phenolic, flavonoids, and antioxidant activity (AA%) at 0.4269 mg/mL, 0.0695 mg/mL, and 76.13% respectively. Thin Layer Chromatography (TLC) was used to determine the presence of phenolic and flavonoids in the sample. The Rf value from P. chrysosporium was 0.98 closed to the Rf values of gallic acid and 3,4-hydroxybenzoic acid at 0.95 and 0.97, respectively. Fourier Transform Infrared (FTIR) spectroscopy shows that P. chrysosporium have gallic acid, 3,4 hydroxybenzoic acid and small amount of quercetin.

Keywords: White-rot Fungus, Biomass, Natural Antioxidants, Aqueous-two Phase Extraction.

*Corresponding author: ahmadanas@unimap.edu.my

1. INTRODUCTION

Free radicals are molecules that have odd electron number in the outer shell, they affect the human health if its production is uncontrolled because they can damage the cellular degeneration and finally fatal. In order to prevent the excessive production of free radicals, antioxidants are produced by human body in small quantity. However, natural sources of antioxidants are widely discovered by fungus and plants. For many years, fungus is widely used as valuable source in the development of medicines and nutraceuticals including antimicrobial, antioxidant and immune-potentiating (Chang and Buswell, 1996; Wasser, 2002). The bioactive compounds of fungal can affect the human immune system and used as a treatment to the disease (Xu et al, 2011). Bioactive compounds that were formed from white rot fungus in food and pharmaceutical industries generated a new finding of natural bioactive sources from fungus (Wong et al, 2010).

The products derived from shikimate and acetate pathways are simple phenolic acids, phenylproponoids, flavonoids and highly polymerized molecules such as melanin, lignin and tannin, the most common was flavonoids with higher antioxidant activity (Bravo, 1998; Ferriera et al, 2009; Liu et al, 2014). Flavonoids has the ability to scavenge free radicals, and shown positive results in protecting organisms from oxidation reaction (Jaszek et al, 2013). For years, 8000 natural phenolic compounds have been identified (Balasundram et al, 2006). Phenolic compounds are widely extracted from various types of plants and fungus. In the past years, attention has been drawn on phenolic compounds because of their antioxidant activities and free radical scavenging abilities that are beneficial to human health (Imeh and Khokhar, 2002).

According to Jung et al. (2002), isolation of new bioactive substances from white-rot fungus are very useful because of their antioxidant activity. Li et al. (2006) applied ethanol for the simple extraction of phenolic compounds from various citrus peels. The parameters used were the conditions of the peel samples, effect of repeated extraction, types and concentrations of organic solvent and the temperature of the extraction. Conventional method is another popular extraction method used in the extraction of phenolic compound such as soxhlet extraction, maceration extraction, and hydrodistillation extraction. Aqueous-two phase method extraction (ATPE) is suitable and effective method for using in biomolecules downstream processing (Wu et al., 2011). This type of extraction results in high extraction yield and product purity (Zhang et al., 2013) and widely used in the proteins, enzymes and antibiotics separation (Wu et al., 2011).

In this study, different extraction conditions were subjected and investigated to determine the optimum condition for the extraction method of phenolic and flavonoids from selected white-rot fungus biomass using aqueous-two phase extraction. The aim was to screen the potential white-rot fungus for the highest antioxidant activity, determine the optimum extraction conditions for phenolic and flavonoids using aqueous-two phase extraction, and evaluate the bioactive constituents naturally presence in the white-rot fungus.

2. MATERIALS AND METHODS

2.1 Material

Ethanol, di-potassium hydrogen phosphate, acetic acid buffer, methanol, potato dextrose agar, gallic acid, sodium carbonate, Follin-Ciocalteu reagent, aluminium chloride, potassium acetate and quercetin were all purchased from Sigma Aldrich (M) Sdn. Bhd. White-rot fungi; *P. chrysosporium* and *P. tigrinus* were obtained from culture collection stock at Faculty of Chemical Engineering & Technology, Universiti Malaysia Perlis (UniMAP).

2.2 Preparation of Media

First, 19.5 g of potato dextrose agar (PDA) powder was added in 500 mL distilled water and the mixture was heated while stirring to fully dissolved all the components. The mixture was autoclaved at 121°C for 15 minutes.

2.3 Preparation of Fungal Strain and Inoculum

The white rot fungi *P. chrysosporium* was obtained from initial culture plate by transferring four plugs (5 mm in length) of active mycelia into new potato dextrose agar (PDA) plate. Then, the PDA plate was incubated at 32°C for 5 days (Jamal et al, 2014). The inoculum was prepared by washing four PDA plates cultured with 100 mL of sterile water. Next, the PDA plates cultured spore suspension were rubbed gently and transferred into 250 ml Erlenmeyer flasks.

2.4 Preparation of Fermentation Medium and Biomass

The preparation of fermentation media was adapted from El-Naggar et al (2014) with modification. The fermentation was prepared by using Malt extract broth. Then, 50 mL of the sample poured into 100 mL Erlenmeyer flasks and autoclaved at 121°C for 30 min. The sterile media was cooled down at room temperature. The inoculum was prepared by adding 20 mL sterile distilled water into one petri dish of the fungus. Then, 2 mL from the inoculated with spore suspension *P. chrysosporium* was added into the 100 mL Erlenmeyer flasks. The incubation time of the fermentation was 5 days, 4% (v/v) of inoculum size, inoculum age of 7 days, pH 6, agitation speed of 100 rpm, and fermentation temperature of 25°C. All fermentations were carried out in triplicates. The mycelia biomass of *P. chrysosporium* was collected after the fermentation by filtration process using filter paper. The filtrated was rinsed with distilled water. The biomass was dried in oven at 60°C for 24 h and grinded into powder by using mortar and pestle.

2.5 Aqueous-two Phase Extraction

Aqueous-two phase extraction was modified by Tan (2013). The extraction was prepared by using 15 mL centrifuge tube with 50% of 80% (v/v) ethanol, 20% of 40% (w/v) di-potassium hydrogen phosphate and 0.028 g of sample powder. Then, 14 g of the final mass was obtained by adding distilled water to the mixture. The mixture consists of 7 g of ethanol, 2.8 g of salt and 0.028 g of sample powder and topped up until 14 g with distilled water. The parameters used in the extraction process were temperatures (range from 25°C to 65°C) and extraction time (range from 30 min to 180 min). The mixture was mixed evenly by gentle agitation and centrifuged for 10 min at 3000 rpm (25 °C) to induced phase separation. After the extraction process, the solution separated into two phase layers (top and bottom). Extract of the desired compounds at the top phase of the solution and dried overnight at 60 °C to removed ethanol from the extract. The total phenolic, flavonoids, and antioxidant activity (AA %) were determined.

2.6 Optimization Studied Based on Extraction Method Selected

Response Surface Method (RSM) was used in the optimization studies to determine the optimal level of parameters studied and their interactions (Mansor et al, 2001). The extraction conditions were optimized and the Response Surface Method (RSM) was calculated using the following equation. The extraction time and extraction temperature were the two parameters studied in this extraction method.

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2$$

2.7 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) Radical Scavenging Assay

DPPH assay was used to determine the antioxidant activities of the samples as described by Jouki et al (2014). The reaction mixture for the antioxidant activity was 0.03 mL of extract sample, 3 mL methanol and 0.3 mL of DPPH solution (0.5 mM in methanol). Spectrophotometric measurements were done at 517 nm using UV-VIS spectrophotometer after 60 min. The blank was prepared by mixing 0.03 mL sample and 3.3 mL methanol. The control was prepared by mixing 3.5 mL methanol and 0.3 mL DPPH solution. The percentage of antioxidant activity (AA %) was calculated using the following formula according to Garcia et al (2012).

$$AA\% = [\frac{Abs517nm\ sample - Abs517nm\ blank}{Abs517nm\ control}] \times 100$$

2.8 Determination of Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

Total phenolic content (TPC) and total flavonoids content (TFC) were determined by Do et al (2014) with slight modification. For TPC the calibration curve was established by using gallic acid (0-1.0 mg/mL). Total phenolic content (TPC) of each extract was determined by adding 0.03ml of distilled water (blank) or extract in 2.37 mL distilled water and 0.1 5mL Follin-Ciocalteau (FC) reagent in 15 mL test tube. The mixture was vortexes for 1 min before 0.45 mL of 20% (w/v) saturated sodium carbonate (Na_2CO_3) were added. The mixture was incubated in water bath for 30 min at 40 °C. The absorbance of the mixture was measured at 750 nm using a UV–VIS spectrophotometer. TPC was expressed as milligram of gallic acid equivalent per liter (GAE mg/mL).

For TFC, the calibration curve was prepared by diluting quercetin in methanol (0-0.12 mg/mL). 0.03 mL diluted extract of quercetin mixed with 0.1 mL of 10% (w/v) aluminum chloride solution and 0.1 mL of 0.1 mM potassium acetate solution. The mixture was incubated at 25 °C for 30 min. Then the maximum absorbance of the mixture was measured at 415 nm using a UV–VIS spectrophotometer.

2.9 Thin Layer Chromatography (TLC) Method

This method was used to analyzed the phenolic and flavonoid contents in the samples. TLC silica plate was used as described by Bipin et al (2012) with modification. The plate length used in this method was 8.5 cm and width 5 cm. Three controls and the sample were performed in one silica plate and this method was run triplicate. The controls used were gallic acid (mixed with ethanol and distilled water), 3, 4- hydroxybenzoic acid (mixed with ethanol and distilled water) and quercetin (mixed with ethanol and distilled water) for determination of phenolic (gallic acid and 3, 4-hydroxybenzoic acid) and flavonoid (quercetin). The sample used was the extract from *P. chrysosporium*. The mobile phase used in this method was ethanol and distilled water with ratio 60:40. After the plate was observed under UV light, the Rf value was calculated using the formula below;

$$Rf = \frac{Distance\ traveled\ by\ compound}{Distance\ traveled\ by\ solvent\ front}$$

2.10 Fourier Transform Infrared (FTIR) Spectroscopy Analysis

A PerkinElmer FTIR was used to record the absorbance between 4000 and 650 cm^{-1} based on method described by Mahajan and Master (2010) with slight modification.

3. RESULTS AND DISCUSSION

3.1 Screening of White-rot Fungus

Two types of white-rot fungus (*P. Chrysosporium* and *P. Tigrinus*) were selected and screened based on the antioxidant activity, Total Phenolic Content (TPC), and Total Flavonoid Content (TFC). The screening process was performed to select the highest antioxidant activity. The result shows that *P. chrysosporium* has higher antioxidant activity compared to *P. tigrinus*. The percentage of antioxidant activity was determined using 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) Radical Scavenging Assay at 517 nm absorbance. The antioxidant activity of the two types of white-rot fungus were calculated and presented in Table 1.

Table 1. Antioxidant Activity (AA %) of Two Types of White-rot Fungus

Types of White-rot fungus	Abs517nm Sample	Abs517nm Blank	Abs517nm Control	AA% (%)
P. chrysosporium	0.365	0.012	0.502	70.31
P. tigrinus	0.206	0.079	0.502	25.30

Table 1 shows that *P. chrysosporium* has higher antioxidant activity (70.31%) than *P. tigrinus* (25.30%). *P. chrysosporium* was selected because of its higher antioxidant activity and proceed to determined the total phenolic and flavonoids contents. This oservation inline with Liu, et al 2014, where they found *P. chrysosporium* extracts possessed remarkable antioxidant activity compared to other fungal.

The selection based on Total Phenolic Content (TPC) and Total Flavonoids Content (TFC) were considered. The result for TPC and TFC determination were presented in Table 2. Table 2 shows that *P. chrysosporium* has the highest values of Total Phenolic Content (TPC) and Total Flavonoids content (TFC) than the *P. tigrinus*. Therefore, *P. chrysosporium* was selected for the extraction of phenolic and flavonoids compounds using Aqueous-two phase extraction.

Table 2. TPC and TFC determination of Two Types of White-rot Fungus

Types of White-rot fungus	TPC Determination		TFC Determination	
	Abs760nm	TPC (mg/mL)	Abs415nm	TFC (mg/mL)
P. chrysosporium	0.6370	0.7041 + 0.0002	0.1935	0.0286 + 0.0001
P. tigrinus	0.3515	0.3755 + 0.0001	0.087	0.0109 + 0.0002

3.2 Extraction of Phenolic Compounds using Aqueous-two phase extraction

The extraction was conducted at different temperatures range from 25˚C to 65˚C and different time range from 30 min to 180 min to determined the best optimum parameters for the separation process using aqueous-two phase system. After the extraction process, the solution was separated into two layers as seen below in Figure 1.

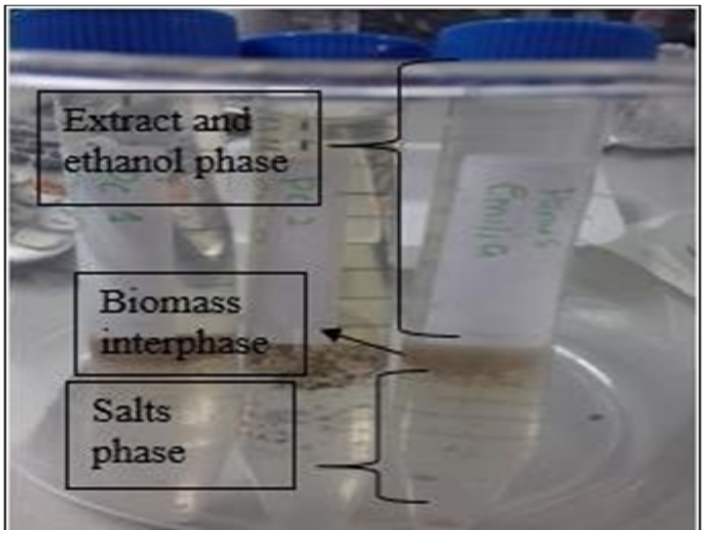

Figure 1. Formation of two phase layer in aqueous-two phase extraction

The extract phase at the top were the desired phenolic and flavonoids compounds mixed with ethanol and the lower phase was the salt solution. The extract phase was extracted and dried in oven at 60˚C overnight to removed the ethanol from the compounds. After the dried process of the extract, total phenolic and flavonoids contents were determined. Gallic acid was used as standard reference for the determination of total phenolic contents.

3.3 Statistical Optimization of Aqueous-two Phase Extraction

The optimization design of aqueous-two phase extraction was carried out using Central Composite Design (CCD) under Response Surface Methodology (RSM). The parameters used for the extraction process were analysed to obtain the optimized condition for optimum extraction of phenolic, flavonoids and antioxidant activity of *P. chrysosporium*. The optimization processes of the experiment were presented below in Table 3, Table 4 and Table 5.

3.3.1 Optimization process of Total Phenolic Content (TPC) of *P. chrysosporium*

As seen in Table 3, the total phenolic content were in the range of 0.0890 mg/mL to 0.4269 mg/mL. The run with the highest total phenolic content was run 4 (0.4269 mg/mL) with the extraction time of 105 min and extraction temperature of 45 ˚C. The lowest total phenolic content was in run 3 (0.0890 mg/mL) with the extraction time of 180 min and extraction temperature of 65 ˚C. The results show that the highest TFC activity was shown at time and temperature for the extraction process was 105 min and 45 ˚C respectively. The results also show that the increased in time and temperature the decreased in the total phenolic compound in the *P. Chrysosporium*, because the extraction of phenolic compound slowed down during the extraction processes due to high temperature. Generally, higher temperature increases the phenolic extraction but not always favoured the extraction after 60 ˚C. According to Ruenroengklin et al (2008), the temperature decreases the production as it increases. The longest extraction time increased the phenolic compound but at certain point of time degraded the phenolic. Similar to Réblová (2012), the production of phenolic decreased as the temperature increased and the decreased in the phenolic was typical although it does not have universal validity. Ruenroengklin et al (2008) reported that significant differences appear from temperature among 30, 40, 50 and 60 ˚C but did not appear after 60 ˚C.

Table 3. Total Phenolic Content (TPC) of *P. chrysosporium*

Run	Time (min)	Temperature (˚C)	Average Abs760nm	TPC (mg/mL)
1	105	65	0.196	0.1964
2	180	45	0.144	0.1366
3	180	65	0.103	0.0890
4	105	45	0.396	0.4269
5	105	25	0.215	0.2183
6	30	65	0.174	0.1712
7	105	45	0.333	0.3540
8	105	45	0.268	0.2994
9	30	45	0.257	0.2367
10	180	25	0.137	0.1286
11	105	45	0.304	0.2712
12	30	25	0.215	0.2183
13	105	45	0.284	0.3586

3.3.2 Optimization process of Total Flavonoid Content (TFC) of *P. chrysosporium*

As shown in Table 4, the total flavonoid content were in the range of 0.0094 mg/mL to 0.0695 mg/mL. The run with the highest total flavonoid content was run 4 (0.0695mg/mL) with the extraction time of 105 min and extraction temperature of 45 ˚C. The lowest total flavonoid content was in run 3 (0.0094 mg/mL) with the extraction time of 180 min and extraction temperature of 65 ˚C. The results show that the highest TFC recorded at time and temperature for the extraction process were 105 min and 45 ˚C respectively. The results also show that the increased in time and temperature the decreased in the total flavonoid compound in the *P. chrysosporium* because some of the flavonoids compound denatured during the extraction process due to high temperature. Generally, higher temperature increases the flavonoid extraction but not always favoured the extraction after 60 ˚C. Ruenroengklin et al (2008) stated that the temperature decreases the production as it increases. The longest extraction time increased the flavonoid compounds but at certain point of time degraded the flavonoid. Sharma et al (2015) reported that the decreased in the total flavonoid at higher temperature depends on the structure of particular flavonoids.

Table 4. Total Flavonoid Content (TFC) of *P. chrysosporium*

Run	Time (min)	Temperature (˚C)	Average Abs415nm	TFC (mg/mL)
1	105	65	0.1978	0.0293
2	180	45	0.1767	0.0258
3	180	65	0.0777	0.0094
4	105	45	0.4404	0.0695
5	105	25	0.2938	0.0452
6	30	65	0.2083	0.0310
7	105	45	0.3220	0.0499
8	105	45	0.3800	0.0595
9	30	45	0.2997	0.0462
10	180	25	0.1936	0.0286
11	105	45	0.3887	0.0609
12	30	25	0.2737	0.0419
13	105	45	0.4267	0.0672

3.3.3 Optimization process for Antioxidant Activity (AA %)

Based on Table 5, the highest antioxidant activity (AA %) value was obtained in run 4 (76.13%) at 105 min and 45 ˚C, while the lowest antioxidant activity (AA %) value was in run 3 (28.81%) at 180 min and 65 ˚C.

Table 5. Antioxidant Activity (AA %) of *P. chrysosporium*

Run	Time (min)	Temperature (˚C)	Antioxidant Activity % (AA %)
1	105	65	42.96
2	180	45	35.98
3	180	65	28.81
4	105	45	76.13
5	105	25	54.55
6	30	65	33.29
7	105	45	75.45
8	105	45	73.34
9	30	45	55.00
10	180	25	37.40
11	105	45	73.94
12	30	25	50.69
13	105	45	75.05

3.4 Thin Layer Chromatography (TLC) Method

This method was used to identified the bioactive constituents presented in *P. chrysosporium*. The mobile phase used in this process was ethanol and distilled water at ratio 60:40. Gallic acid (phenolic), 3, 4- hydroxybenzoic acid (phenolic) and quercetin (flavonoid) chemical were used as controls for the process to determined the bioactive constituents of the *P. chrysosporium*.

Table 6. Thin Layer Chromatography Analysis

No	Sample	Distance travelled by compound (cm)	Distance travelled by solvent front (cm)	Rf value
1	Gallic acid	6.2	6.5	0.95
2	3, 4-Hydroxybenzoic acid	6.3	6.5	0.97
3	*P. chrysosporium*	6.4	6.5	0.98
4	Quercetin	6.7	6.5	0.87

From the Table 6, the Rf value of *P. chrysosporium* was 0.98 closed to the Rf values of gallic acid and 3, 4- hydroxybenzoic acid. The result shows that *P. chrysosporium* has gallic acid and 3, 4-hydroxybenzoic acid contents due to the closeness of Rf values between *P. Chrysosporium*, gallic acid and 3, 4 hydroxibenzoic acid. *P. chrysosporium* has small amount of quercetin from control due to the distance between their Rf values. The Rf value was used to identified the movement of the materials used along the TLC plate.

3.5 Fourier Transform Infrared (FTIR) Spectroscopy Analysis

Fourier Transform Infrared (FTIR) Spectroscopy Analyses was used to identify the functional groups that present in *P. chrysosporium*. FTIR determined the presence of compound through the appearance of their similar functional groups. Himmelsbach et al (2006) stated that infrared (IR) spectroscopy was one of the effective methods for the identification of molecular functional groups, such as methyl (C-H), hydroxyl (O-H), and carbonyl (C=O) groups. Figure 2 shows the result from FTIR analysis.

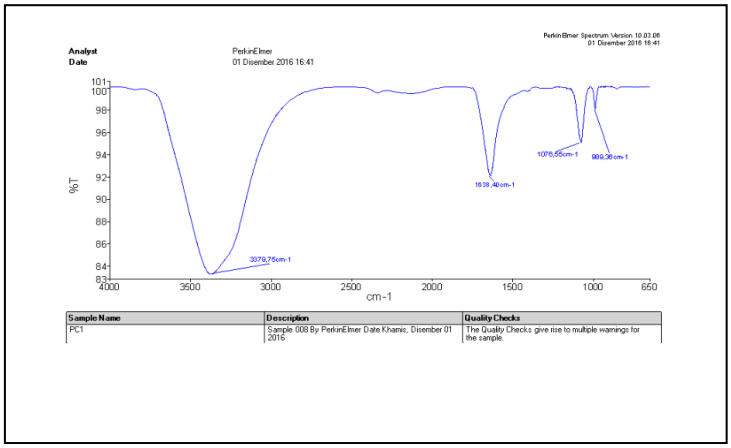

Figure 2. Fourier Transform Infrared (FTIR) for *P. chrysosporium*

A broad band at 3379.75 cm^{-1} belongs to stretching vibration of phenolic hydroxyl group (-OH) which represent the hydrogen bonding present in *P. chrysosporium*. Appearance of strong and stretch band at wave number 1638.40 cm-1 and 1076.55 cm^{-1} indicates the presence of (C=O) group. From the functional group obtained from FTIR, the result shown that *P. chrysosporium* has gallic acid, 3, 4- hydroxybenzoic acid and quercetin contents.

4. CONCLUSION

This research was successful in selecting the highest antioxidant activity (AA %) between two types of fungi, *P. chrysosporium* and *P. tigrinus*. *P. chrysosporium* was selected as the potential antioxidant after the screening process through extraction method using aqueous-two phase due to its high contents of phenolic, flavonoids and antioxidant activity than *P. tigrinus*. The selected fungus was used to determine the optimum condition in aqueous-two phase extraction to produce high phenolic and flavonoid contents. The parameters used in this research were time (between 30 min to 180 min) and temperature (between 25 ˚C to 65 ˚C). Based on the Response Surface Method (RSM) under Central Composite Design (CCD), the highest condition recorded for TFC, TPC and antioxidant activity were at 105 min and 45 ˚C. These conditions produced the highest concentrations of phenolic, flavonoids and antioxidant activity at 0.4269 mg/mL, 0.0695 mg/mL, and 76.13% respectively. The condition proved that the extraction method used was less energy and less time consuming as the time and temperature of the condition was low. The presence of phenolic and flavonoid in the samples were determined using Thin Layer Chromatography (TLC) method based on their Rf values. The result from Fourier Transform Infrared (FTIR) spectroscopy proved that the extraction method using aqueous-two phase system was able to extract the phenolic and flavonoids at the optimal time and temperature.

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