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Effects of different extracting conditions on antioxidant activities of *Pleurotus flabellatus*

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Abstract

The purpose of this study was to investigate the effect of different extracting conditions on antioxidant activity of *Pleurotus flabellatus*. Samples were extracted by using 2 different conditions at room temperature; shaking for 24 hours and sonicating for 1 hour and the extracting solvents were 65%, 80% and 95% ethanol (v/v). The antioxidant activities were analyzed in terms of total phenolic content, total flavonoid content, ABTS and DPPH assays. It was found that *P. flabellatus* extracted by shaking and sonicating with 65% ethanol gave the highest extraction yields of 32.33 and 33.86 % dry weight, respectively. The sample extracted by shaking method with 95% ethanol gave the highest total phenolic contents of 301.76 mg GAE/100 g sample. The total flavonoid contents were the highest when using shaking and sonicating methods with 95% ethanol from *P. flabellatus* extract of 30.84 and 31.17 mgQCE/100g sample ($p<0.05$). The results showed that the total phenolic and total flavonoid content of mushroom extracts had affected on antioxidant activities by DPPH and ABTS assay.

Introduction

Free radicals are defined as molecules having an unpaired electron in the outer orbit. They are generally unstable and very reactive (Fang *et al.*, 2002). Free radicals are known to be the major cause of various chronic and degenerative diseases, including aging, coronary heart disease, inflammation, stroke, diabetes mellitus and cancer (Wu and Ng, 2008). To prevent the free radicals within the body needed to make or obtain antioxidants that can prevent or delay the process that causes free radicals in the body (Keawsaard, 2012). Synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are commonly used in foods but they cause side effects and are carcinogenic (Pal *et al*., 2011). Thus, there has been an upsurge of interest in naturally-occurring antioxidants from vegetables, fruits, leaves, oilseeds, cereal crops, tree barks, roots, spices and herbs (Li *et al*., 2011).

Phenolic compounds are plant secondary metabolites, which are important determinants in sensory and nutritional quality of fruits and vegetables (Tomas *et al*., 2000). Importance of natural antioxidants for food application has been underlined by numerous works as reported by Spigno and Faveri (2007). There is an increasing interest in the substitution of synthetic antioxidant by natural ones. Mushrooms have received an increasing attention in recent years. It contains high protein, vitamin and mineral contents, very lower amount of carbohydrates, sugars and no or very less amount of cholesterol (Patel *et al*., 2012). The presence of some compounds, such as ascorbic acid, α-tocopherol, β-carotene and phenolic compounds is an indication that mushrooms have antioxidant properties (Vamanu, 2013). *Pleurotus flabellatus* is grouped in oyster mushrooms and its fruiting bodies are white to red color depending on the temperature of cultivation, when the cultivation temperature is cold the fruiting bodies grows slowly and the color is deep red. Adhiraj *et al*. (2014) reported *P. flabellatus* can be a potential source of natural antioxidant to treat various oxidative stress related diseases.

Extracting efficiency is commonly a fraction of process conditions. Previous finding have reported the influence of some variables (e.g., temperature, time contact, solvent-to-solid ratio, etc.) on the phenolic yields capable of being extracted from diverse natural product (Rubilar *et al*., 2003; Pinelo *et al.*, 2004). There is an increasing demand for new extraction techniques with shortened extraction time, reduced organic solvent consumption, and increased pollution prevention. Novel extraction method including ultrasound-assisted extraction (Vinatoru, 2001) is fast and efficient for extracting chemicals from solid plant matrixes. Ultrasound, in its most

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basic definition, refers to pressure waves with a frequency of 20 kHz or more. Generally, ultrasound equipment uses frequencies from 20 kHz to 10 MHz. (Piyasena *et al.*, 2003), it gives the solvent infiltrate into the material to be extracted better. In addition to the solvent used in the extraction, it also affects the amount of extract and antioxidant activity. The aim of this study was to investigate the effect of extracting conditions on antioxidant activity of from extracted *P. flabellatus* including, extraction yield, total phenolic content, total flavonoid content and antioxidant activities by ABTS assay DPPH were also determined.

Materials and Methods

Preparing mushrooms

The fruiting bodies of *P. flabellatus* were obtained from Phromphiram district, Phitsanulok, Thailand. The samples were cleaned, desiccated and dried with hot air oven at 60^0C for 4 hours or until the moisture content is less than 12% (w/w). The dried mushroom was ground and sieved 0.3 mm. and stored at -20^0C until analyzed.

Extraction of mushroom

The extracting solvents were 65%, 80% and 95% ethanol (v/v). The ratio of dried mushroom: solvent was 1 g: 10 ml. The extraction condition were shaking method by the shaker with a speed of 150 rpm for 24 hours and sonicating method by ultrasonic bath for 1 hour. The extract was filtered through Whatman No. 1 filter paper and the filtrate was evaporated to dryness using a rotary evaporator (BUCHI, R-200, BUCHI (Thailand) Ltd. Bangkok, Thailand). The extracts were stored in amber bottle at -20°C until analyzed.

Determination of extraction yield

The method was described by Leung *et al.* (2006) which the yield can be calculated from the following equation

$$\% \text{ yield} = \frac{\text{weight of extract (g)}}{\text{weight of mushroom powder (g)}} \times 100$$

Determination of total phenolic content

Total phenolic content was estimated using the Folin-Ciocalteu assay following a modified method of Miliauskas *et al*. (2004). For the preparation of calibration curve, 0.5 ml aliquots of 0.01-0.08 mg/ml ethanolic gallic acid solutions were mixed with 2.5 ml Folin–Ciocaleu reagent (ten-fold dilution) and 2 ml (75 g/L) sodium carbonate. The absorbance was read after 30 min at room temperature at 756 nm using the spectrophotometer and the calibration curve was drawn. Mushroom extract was mixed with the same reagents as described above, and after 30 min the absorbance was measured for the determination of mushroom phenolics. Results were expressed as mg of gallic acid equivalent (GAE) per 100 g of dry weight (DW).

Determination of total flavonoid content

The aluminium chloride colorimetric method was modified the procedure reported by Chang *et al*. (2002). Quercetin was dissolved in 80% ethanol and then diluted to 25, 50, 75 and 100 µg/ml. The diluted standard solutions (0.5 ml) were separately mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm. The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank. Similarly,0.5 ml of mushroom extracts were reacted with aluminum chloride for determination of flavonoid content as described above. Results were expressed as mg of quercetin equivalent (QCE) per 100 g of dry weight (DW).

Measurement of antioxidant activity by ABTS assay

Antioxidant activity was estimated using the ABTS assay following a modified method of Jeong *et al.* (2010). Briefly, 2.45 mM potassium persulfate solution was added into 7 mM ABTS (1:1, v/v) and kept for 12-16 h at room temperature in the dark to make ABTS solution. The ABTS solution was diluted with ethanol to an absorbance of less than 0.700±0.002 at 734 nm before analysis. The mushroom extracts were diluted with ethanol (500, 1,000, 1,500, 2,000 and 2,500 mg/L). Then, 0.1 ml of the diluted sample were mixed with 2.9 ml of the diluted ABTS solution. After 20 min, the absorbance was measured at 734 nm using the spectrophotometer. The calculation of the percent inhibition of antioxidant activity as follows.

$$\% \text{ inhibition} = \frac{(Ac - As) \times 100}{Ac}$$

Where Ac is the absorbance of control and As is the absorbance of sample.

The relationship between % inhibition and on concentration of extract was calculated in term of IC_{50} where ml/L.

Measurement of antioxidant activity by DPPH assay

Antioxidant activity was estimated using the DPPH (1, 1-diphenyl-2-picryl hydrazine) assay described previously (Nimmi and George, 2012) with

Table 1. Extraction yield, Total phenolic content and Total flavonoid content of *Pleurotus flabellatus*

Extraction method	Extracting solvent	Extraction yield (% dry weight)	Total phenolic (mgGAE/100g)	Total flavonoid (mg QCE/100g)
Shaking	65% Ethanol	32.33 ± 0.80^{a}	215.26 ± 1.07^{e}	14.59 ± 0.36^{d}
	80% Ethanol	23.52 ± 2.18^{b}	281.77 ± 1.45^{b}	23.97 ± 0.39^{b}
	95% Ethanol	13.90 ± 2.75^{c}	301.76 ± 0.32^{a}	31.17 ± 1.05^{a}
Sonicating	65% Ethanol	33.86 ± 1.09^{a}	253.30 ± 2.56^{d}	14.57 ± 0.74^{d}
	80% Ethanol	23.07 ± 2.40^{b}	276.44 ± 2.13^{c}	22.03 ± 0.35^{c}
	95% Ethanol	12.36 ± 1.38^{c}	282.93 ± 0.85^{b}	30.84 ± 0.36^{a}

Mean±SD from three determinations.
Different superscripts in the same column under the same fermentation time indicate significant differences ($p<0.05$)

a some modification. The DPPH solution 0.1 mM was prepared and 1.0 mL of this solution was added to 1.0 ml of extract solution (or standard) at different concentrations (500, 1,000, 1,500, 2,000 and 2,500 mg/L). Sixty minutes later, the absorbance was measured at 517 nm using the spectrophotometer, calculate the %inhibition and IC_{50} value as same as ABTS assay.

Statistical analysis

Data are reported as mean of three determinations. Analysis of variance by the general linear models (GLM) procedure and mean comparisons by the Duncan to determine statistically significant differences at the $p<0.05$. All analysis was conducted using SPSS for Window Version 11.5. Pearson correlation coefficient was used to determine the relationship between total phenolic content, total flavonoid content and antioxidant activity.

Results and Discussion

Extraction yield

The extraction yield from fruiting bodies of P. flabellatus by shaking and sonicating methods are shown in Table 1. As can be seen, the extraction method affected the extraction yield which varieds from 13.90 to 32.33 and 12.36 to 33.86 % dry weight in case of shaking and sonicating methods, respectively. The highest of extraction yield found in 65%, 80% and 95% ethanol (v/v), respectively ($p<0.05$). The higher extraction yield of 65% ethanol (v/v) might be due to the fact that *P. flabellatus* contained more water-soluble substance and also the polaritie of solvents, the solvent which have a polarity similar to the solute are able to dissolve more than those with differing polarities (Maisuthisakul, 2008). Considering the extraction method it was found that shaking and sonicating methods were not significantly different of statistically ($p<0.05$). From Table 1 found that the extraction by using 65% ethanol (v/v) of shaking and sonicating methods had the highest extraction yields of 32.33 and 33.86 % dry weight, respectively ($p<0.05$).

Total phenolic content

Phenolics are considered to be beneficial antioxidants as they exhibit scavenging activity of harmful active oxygen species (Jiang *et al*., 2015). It had been reported that the antioxidant activity of mushrooms are well correlated with the content of their total phenolic content (Mau *et al*., 2002; Barros *et al*., 2007; Lee *et al*., 2008).

The total phenolic content of different extraction of *P. flabellatus* is shown in Table 1 which the mgGAE/100 g sample of the extraction by using 95% ethanol (v/v) of shaking method was the highest ($p< 0.05$). Most of the phenolics are soluble in organic solvents with high polarity and little soluble in water (Walter and Purcell, 1979). The solubility of polyphenols, which have polar character, was the highest in methanol, a little lower in ethanol and the lowest in water (Lapornik *et al*., 2005). In the present study, the total phenolic content of *P. flabellatus* (215.26 to 301.76 mgGAE/100 g sample) was found to be much higher than that of the ethanolic extract of *F. velutipes* which was reported to be 90 mgGAE/100 g sample (Wong *et al*., 2013), *S. commune* that being of 175.71 mgGAE/100 g sample (Yim *et al*., 2009) and *G. gargal* that of 51.3 mgGAE/100 g sample (Bruijn *et al*., 2009), while it was found to be a little lower than that of *L. edodes* which was 345 mgGAE/100 g sample (Woldegiorgis *et al*., 2014).

Total flavonoid content

From Table 1, total flavonoid contents in the

Table 2. IC_{50} values (mg/ml) of *Pleurotus flabellatus* in ABTS and DPPH radical scavenging activity

Extraction method	Extracting solvent	DPPH assay (IC_{50}, mg/ml)	ABTS assay (IC_{50}, mg/ml)
Shaking	65% Ethanol	3.15 ± 0.04^{a}	13.33 ± 0.18^{a}
	80% Ethanol	2.43 ± 0.01^{d}	11.70 ± 0.45^{bc}
	95% Ethanol	2.29 ± 0.03^{e}	11.11 ± 1.02^{c}
Sonicating	65% Ethanol	2.88 ± 0.06^{b}	12.30 ± 0.73^{ab}
	80% Ethanol	2.54 ± 0.04^{c}	11.91 ± 0.42^{bc}
	95% Ethanol	2.13 ± 0.01^{f}	11.19 ± 0.67^{c}

Mean±SD from three determinations.
Different superscripts in the same column under the same fermentation time indicate significant differences ($p<0.05$)

mushrooms extract varied from 14.57 to 31.17 mgQCE/100g sample. The highest of total flavonoid contents found in 95%, 80% and 65% ethanol (v/v), respectively ($p<0.05$). This is because the flavonoids with structures containing –OH and –COOH functional groups are easily extracted by the polar solvent in the sample (Gan *et al*., 2013). Bruijn *et al*. (2009) reported a positive correlation was found between the flavonoid contents and solvent relative polarity thus the flavonoids are rather polar compounds. Arbaayah and Umi (2013) stated that antioxidant properties in mushroom were mainly in the form of phenolic acids and flavonoids. Moreover, in mushrooms there were some main compounds with antioxidant effect such as gallic acid, ascorbic acid, β-carotene, lycopene, α-tocopherol and L-ergothioneine (Vamanu, 2014; Vangkapun *et al*., 2011).

Antioxidant activity by DPPH and ABTS assay

The IC_{50} of a compound is inversely related to its antioxidant capacity, as it expresses the amount of antioxidant required to decrease the DPPH and ABTS concentration by 50%. At lower value of IC_{50} show that the extract had higher antioxidant activity (Samruan *et al*., 2012).

DPPH has been widely used to test the free radical scavenging ability of various samples (Sribusarakum *et al*., 2004). The DPPH assay is best on the ability of 1-1-diphenyl-2-picrylhydrazyl, is a stable free radical to decolorize in the presence of antioxidants (Vithya *et al*., 2012). In this study, the IC_{50} values in DPPH assay varied from 2.13 mg/ml to 3.15 mg/ml for all samples tested (Table 2). The highest of antioxidant activity found in 95%, 80% and 65% ethanol (v/v), respectively ($p<0.05$). The extract by using 95% ethanol (v/v) of sonicating method was assumed to be the strongest inhibitor which showed IC_{50} of DPPH assay at the lowest concentration (2.13 mg/ml) among all sample extracts ($p<0.05$). These results, compared to the extract from *P. ferulae* (IC_{50}: 4.55 mg/ml) (Tsai *et al*., 2009), *P. pulmonarius* (IC_{50}: 6.00 mg/ml) (Arbaayah and Umi, 2013), *P. eryngii* (IC_{50}: 8.67 mg/ml) (Reis *et al*., 2012) and *L. edodes* (IC_{50}: 9.8 mg/ml) (Woldegiorgis *et al*., 2014) reveals that *P. flabellatus* extract has significant antioxidant activities.

Experimental results are often influenced by radical systems, and therefore it is worthwhile to analyze the extracts for free radical scavenging activity by ABTS assay (Rawat *et al*., 2013). For the IC_{50} values in ABTS assay from Table 2 found that the IC_{50} value varied from 11.11 mg/ml to 13.33 mg/ml. The extract by using 95% ethanol (v/v) of shaking and sonicating methods were assumed to be the strongest inhibitor which showed IC_{50} of ABTS assay at the lowest concentration (11.11 mg/ml and 11.19 mg/ml, respectively) among all test extracts ($p<0.05$).

Correlation between total phenolic content, flavonoid content and antioxidant activity of P. flabellatus

The Pearson's correlation coefficients between total phenolic content, total flavonoid content, IC_{50} value of ABTS assay and IC_{50} value of DPPH assay were calculated and shown in Table 3. There were very strong negatively correlated between total phenolic content in mushroom extracts with ($r = -0.899$, $p = 0.001$) and IC_{50} value of ABTS assay ($r = -0.941$, $p = 0.000$). Total flavonoid content in mushroom extracts strong and very strong negatively correlated with IC_{50} value of DPPH assay ($r = -0.756$, $p = 0.018$) and IC_{50} value of ABTS assay ($r = -0.905$, $p = 0.001$). This study found the total phenolic and total flavonoid contents of mushroom extracts had affected

Table 3. Pearson's correlation coefficient between total phenolic content, total flavonoid, IC_{50} value of ABTS assay and IC_{50} value of DPPH assay of *Pleurotus flabellatus*

Antioxidant		Pearson's correlation	
		IC_{50} of DPPH assay	IC_{50} of ABTS assay
Total phenolic content	r value	-0.899**	-0.941*
	p value	0.001	0.000
Total flavonoid content	r value	-0.756*	-0.905**
	p value	0.018	0.001

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).
Mean±SD from three determinations.
Different superscripts in the same column under the same fermentation time indicate significant differences ($p<0.05$)

on antioxidant activities. Mushrooms containing high phenolic compound are normally well correlated with higher antioxidant activity (Ahmad *et al*., 2014). The total phenolic content are among the phenolic compounds found in mushrooms, such as phenolic acids, gallic acid, caffeic acid, quercetin, flavonoids and tannins, in which the phenolic hydrogen is a major contributor to antioxidant activity (Gursoy *et al*., 2009; Siu *et al*., 2014).

Conclusions

The results of this study demonstrated that using of 65% ethanol (v/v) provided the highest extraction yield ($p<0.05$). *P. flabellatus* extracted by shaking method with 95 % ethanol gave the highest total phenolic content ($p<0.05$). The total flavonoid content were the highest when using shaking and sonicating methods with 95% ethanol ($p<0.05$). The total phenolic and total flavonoid contents of mushroom extracts had affected on antioxidant activity. This result indicates the different condition have effect on antioxidant activity of extracted.

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