INTRODUCTION

Phellinus is a genus of mushrooms which belongs to the Hymenocataceae family in the Fungi kingdom (Ruggiero et al., 2015). Many species of Phellinus have been used for their medicinal properties for a long time. The most well-known Phellinus; Phellinus linteus, has been ethnomedically used to prevent many ailments such as gastroenteric dysfunctions, diarrhoea, haemorrhages and cancer (Chen et al., 2016). Other Phellinus such as Phellinus gilvus, P. rimosus and P. pini also have been traditionally used for various purposes including antitumor treatments, improving immunity, treating rheumatism and promoting digestion. P. igniarius has been used for promoting blood circulation and hemostasis (Dai et al., 2010).

Phellinus linteus has been shown to have suppressive effects on the growth and metastasis of tumour cells and is also known to stimulate hormonal and cell-mediated immune functions enhancing the effects of some conventional chemotherapeutic drugs (Zhu, Kim, Chen, 2008). From our previous study, an ethanol extract from many species of Phellinus mushrooms exhibited moderate antioxidant effects; especially, extracts from P. everhartii, P. hippophaëcicola and P. nigricans var. resupinatus. P. igniarius var. cinereus ethanol extract exhibited inhibitory effects on Helicobacter pylori - both normal and resistant strains - and also showed it had the strongest inhibitory effect against Staphylococcus aureus (Sunthudlakhar et al., 2019).

Phellinus mushrooms have been reported to contain triterpenoids, phenolics, lignins, ergosterol and styrylpyrone compounds, which can be related to their biological activities (Lee, Yun, 2011; Soković et al., 2018; Zhang, Reddy, Koyyalamudi, 2014). Hispolon is a...
phenolic compound isolated from *P. lineus* that possesses strong antioxidant, anti-inflammatory, anticancer and antidiabetic properties (Chen et al., 2008; Chang et al., 2011; De Silva et al., 2012; Chen et al., 2013). Further, lanostane- triterpenoids from *P. gilvus* exhibit hypoglycemic activity (Liu et al., 2009). Moreover, we have discovered that most of the *Phellinus* mushroom extracts exhibited similar TLC and HPLC fingerprints with the chromatographic bands corresponding to phenolics, flavonoids and terpenoids. Protocatechuic acid was identified in most *Phellinus* mushroom extracts (Sunthudlakhar et al., 2019).

Protocatechuic acid has previously reported as exhibiting various pharmacological effects (Kakkar, Bais, 2014) including as an *in vitro* and *in vivo* antioxidant (Herrmann, 1989; Kayano et al., 2002; Sang et al., 2002; Pacheco-Palencia, Mertens-Talcott, Talcott, 2008; Li et al., 2011; Semaming et al., 2015), a cancer chemo-preventative (Hudson et al., 2000), an antifungal agent (Link, Angell, Walker, 1929), as well as having antibacterial (Chao, Yin, 2009), antispasmodic (Hassan et al., 2009), anti-inflammatory (Liu et al., 2002; Jaijoy et al., 2010), hepatoprotective (Liu et al., 2002), antiviral (Zhou, Zuo, Chow, 2005), antiatherosclerotic (Zhou, Zuo, Chow, 2005), cardioprotective (Zhou et al., 2012), analgesic (Jaijoy et al., 2010) and antiaging (Guan et al., 2006) effects. Moreover, protocatechuic acid exhibited a strong antioxidant effect using the DPPH scavenging antioxidant method (Sunthudlakhar et al., 2019).

Thus, protocatechuic acid could be regarded as a chemical marker compound for the quality assessment of the raw materials and extracts from *Phellinus* mushrooms. The qualitative and quantitative analysis of herbal medicinal products is an important process for quality control. The chromatographic fingerprint and methods for determination of the active ingredients using the HPLC technique are considered as key strategies in quality control (Liang, Xie, Chan, 2004).

Therefore, the aim of this study was to validate a HPLC method for the quantification of the protocatechuic acid content in *Phellinus* mushroom extracts for the quality control of the raw materials and phytopharmaceutical products.

**MATERIAL AND METHODS**

**Chemicals and reagents**

Standard protocatechuic acid was purchased from Chromadex (USA). Acetonitrile, methanol (HPLC grade) and formic acid were purchased from Merck (Germany). Deionized water was purchased from GHP (Thailand). An analytical grade of chloroform and ethyl acetate were purchased from Labscan (Thailand). 95% ethanol was purchased from Liquor distillery organization (Thailand).

**Mushrooms preparation**

The 11 *Phellinus* mushrooms: *Phellinus conchatus f. alni* Bondartsev (M1), *Phellinus everhartii* (Ellis & Galloway) A. Ames (M2), *Phellinus gilvus* (Schwein.) Pat. (M3), *Phellinus hippophæicola* H. Jahn (M4), *Phellinus igniarius* (L.) Quél. (M5), *Phellinus igniarius* var. *cinereus* Niemelä (M6), *Phellinus nigricans* (Fr.) P. Karst. (M7), *Phellinus nigricans* var. *resupinatus* Bourdot & Galzin (M8), *Phellinus noxius* (Corner) G. Cunn. (M9), *Phellinus pini* var. *cancriformans* M.J. Larsen Lombard & Aho (M10) and *Phellinus pini* var. *microporus* Pilát (M11) were collected from Phu-Pha-Kood, Mukdahan, Thailand in 2013. The mushrooms were morphologically identified by specialists from the Natural medicinal mushroom museum, Mahasarakham University, Mahasarakham, Thailand. All of the mushrooms were dried in a hot air oven at 60 °C for 24 h, and ground using an electric grinder. The powder was kept in the refrigerator at 4 °C before use.

**Ethanol extract preparation**

According to the extraction method previously described (Sunthudlakhar et al., 2019), each *Phellinus* mushroom powder was macerated with 95% ethanol (1:10 w/v) using a water bath at 80 °C for 2 h. Then the sample solution was filtered through Whatman filter paper No. 1 and the supernatant was kept in a separate glass bottle. The residue was re-extracted using the same procedure, then the supernatants were combined in a separate glass bottle. The collected supernatant was evaporated using a
rotary evaporator under a pressure of 176 mbar at 40 °C to remove the solvent. The concentrated ethanol extract was stored at 5 °C until use.

**HPLC method for quantitative analysis of protocatechuic acid in Phellinus mushrooms**

**Chloroform-ethyl acetate fraction preparation**

Each *Phellinus* ethanol extract was partitioned with aqueous phase of one part of distilled water and one part of non-polar phase composed of chloroform and ethyl acetate (1:1 v/v). The chloroform-ethyl acetate part was collected and kept in a separate glass bottle. The residue was re-partitioned using the same procedure then the chloroform-ethyl acetate parts were combined. The collected chloroform-ethyl acetate part was evaporated using a rotary evaporator under a pressure 176 mbar at 40 °C to remove the solvent. The percentage of the fractional yield (%yield) was then calculated. The dried chloroform-ethyl acetate fraction was then stored at 5 °C until use.

**HPLC instrument and analytical condition**

The chromatographic analysis was performed using HPLC Shimadzu LC-10ADVP with a column oven and a Shimadzu diode array SPD-M10AVP (Shimadzu, Japan). A Hypersil™ BDS C18 column (4.6 x 150 mm, 5µm) with a C18 guard column (Thermofisher scientific, USA) was used for the separation and quantitation of the protocatechuic acid content. The conditions used in the analysis were an injection volume of 20 µl, a flow rate of 1.0 ml/min and a column temperature of 40 °C. The gradient elution composed of 0.1% formic acid in water (pH 2.6) as mobile phase A and the 1% formic acid in acetonitrile as mobile phase B. The UV-Vis absorption spectra were recorded from 200 - 400 nm during the HPLC analysis. The UV detection was conducted at 254 nm. The data were processed using Labsolution software.

**Standard preparation**

The primary and secondary stock solution of standard protocatechuic acid was prepared at concentrations of 1000 and 100 µg/mL in methanol, respectively. The working solutions were prepared by diluting the secondary stock solution to a concentration of 1 to 10 µg/ml. The solutions were filtered using a 0.45 µm, PTFE syringe filter.

**Sample preparation**

The primary stock solution of chloroform-ethyl acetate fraction of the 11 *Phellinus* mushrooms was prepared at a concentration of 5 mg/ml in methanol. The working solution was prepared by diluting the stock solution to a concentration of 500 µg/ml. The solutions were filtered using a 0.45 µm, PTFE syringe filter.

**Method optimization**

The effect of the composition of the mobile phase was examined to separate the protocatechuic acid from the other compounds. The specificity of the method was obtained by a comparison of the HPLC chromatograms of the chloroform-ethyl acetate fractions of *Phellinus* mushroom and standard protocatechuic acid. UV spectra in the range of 200 - 400 nm of the peak at retention time corresponding to protocatechuic acid in the sample were evaluated.

**Method validation**

The linearity of the method was assessed by analysing three linearity curves obtained from linear regressions analysed by statistical method of protocatechuic acid composed of seven concentration levels of 1, 2, 3, 4, 6, 8 and 10 µg/ml. The calibration curves were obtained by plotting the peak area and the concentration. Data were evaluated for slope, intercept values and correlation coefficient ($r$). The accuracy of the method was examined by the recovery of the known amounts of protocatechuic acid that was added to each *Phellinus* chloroform-ethyl acetate fraction solution. The three concentration levels of protocatechuic acid were spiked in the working solution (500 µg/ml) of the chloroform-ethyl acetate fraction of *Phellinus everhartii*. The final concentrations of protocatechuic...
acid were 2, 4, 8 µg/ml. The percentage of recovery of each protocatechuic acid at different concentrations was then calculated. Repeatability and intermediate precision were obtained by analyzing the chloroform-ethyl acetate fraction of the Phellinus everhartii at a concentration of 500 µg/ml on a same day \((n = 6)\) and over three different days \((n = 18)\), respectively. The relative standard deviation \((\text{RSD})\) was then calculated. The chloroform-ethyl acetate fraction of Phellinus noxius was evaluated for the limit of detection \((\text{LOD})\) and the limit of quantitation \((\text{LOQ})\). LOD was examined by the RSD using the known amounts of protocatechuic acid added to the sample solution. The 10 µg/ml solution of protocatechuic acid was separately added into the 500 µg/mL chloroform-ethyl acetate fraction of the Phellinus noxius solution. The final concentration of protocatechuic acid were 0.5 µg/ml. LOD were obtained by the analysis of the sample added standard solution \((n = 6)\). The RSD was then calculated. LOQ was examined by the recovery and RSD of the known amounts of protocatechuic acid added to the sample solution. The 10 µg/ml solution of protocatechuic acid was separately added into the 500 µg/mL chloroform-ethyl acetate fraction of the Phellinus noxius solution. The final concentration of protocatechuic acid was 1 µg/ml. The percentage of recovery of protocatechuic acid and RSD were then calculated.

**System suitability**

The system suitability was evaluated in term of number of theoretical pate \((N)\), tailing factor \((T_t)\), and RSD values of the peak area and retention time.

**Quantitative analysis of protocatechuic acid in Phellinus mushrooms**

Each solution of chloroform-ethyl acetate fraction of Phellinus mushroom was prepared as specified in the sample preparation. The sample solutions were analysed using the HPLC validated method. The protocatechuic acid contents were calculated from the linear equation obtained from the standard curve of protocatechuic acid, as grams per 100 gram of ethanol extract \((\% \text{w/w of extract})\). The experiments were carried out in triplicate and the average protocatechuic acid contents were calculated and expressed as the mean ± SD.

**Statistical analysis**

All experiments were carried out in triplicate and the results were expressed as mean ± standard deviation \((\text{SD})\). Linear correlations were calculated using the correlation coefficient statistical option in Microsoft Excel software (version 2016).

**RESULTS AND DISCUSSION**

**Method optimization**

HPLC chromatogram of chloroform-ethyl acetate fraction of each Phellinus mushroom extract exhibited the good separation of protocatechuic acid from other peaks in the extract. Moreover, the UV spectra of the peak in the chloroform-ethyl acetate fraction of each Phellinus mushroom extract was similar to the peak at the same retention time of standard protocatechuic acid \((R_t = 7.9 \text{ min})\). From the results, the analytical method was confirmed to have a good specificity.

**Method validation**

HPLC fingerprinting has been widely accepted for performing quality control on raw materials and herbal medicinal products (AOAC International, 2002). The optimization of HPLC conditions are an important step for good sensitivity, feasibility, and reproducibility. A HPLC method was therefore developed for the analysis of protocatechuic acid, the active antioxidant effect component in Phellinus mushrooms. This method was modified from our previous report (Sunthudlakhar et al., 2019) by adjusting the mobile phase and gradient system for a quantitative analysis of the protocatechuic acid contents in the chloroform-ethyl acetate fraction of the Phellinus mushroom extracts. The HPLC fingerprints of the chloroform-ethyl acetate fraction of Phellinus everhartii and standard protocatechuic acid are shown in Figure 1.

The optimized HPLC method for the quantitative analysis of protocatechuic acid in chloroform-ethyl
acetate fraction of Phellinus mushrooms was validated in terms of linearity, accuracy, precision, limit of detection (LOD) and limit of quantitation (LOQ) according to the AOAC guideline (AOAC International, 2002; AOAC International, 2013). The calibration curve of protocatechuic acid was linear in the concentration range of 1 - 10 µg/ml. The correlation coefficients (r) of the equations were higher than 0.99 indicating good linearity. The recoveries of the protocatechuic acid contents from the chloroform-ethyl acetate fraction of Phellinus mushrooms were within the range of 102.00 - 106.68% and the average recovery was 104.16%. Therefore, the analytical method is accurate. For repeatability, the percentages of the RSD of protocatechuic acid contents in the chloroform-ethyl acetate fraction of Phellinus mushrooms were less than 3% (ranged from 0.76 - 1.48%). For intermediate precision, the percentage of the RSD of the protocatechuic acid content was 2.75%. Thus, the analytical method is precise. The chloroform-ethyl acetate fraction of Phellinus noxius mushroom extract, which did not contain protocatechuic acid, was used for the evaluation of the limit of detection (LOD) and the limit of quantitation (LOQ). LOD and LOQ values for the HPLC analysis of protocatechuic acid content in the chloroform-ethyl acetate fraction of Phellinus mushrooms were 0.5 and 1.0 µg/ml, respectively, with acceptable RSD values (0.86 and 0.72%, respectively). According to the AOAC guideline, all validation parameters are within the acceptance criteria ranges (Table I).

**TABLE I - Validation parameter from HPLC analysis of protocatechuic acid contents in Phellinus mushrooms**

<table>
<thead>
<tr>
<th>Validation parameters</th>
<th>Acceptant criteria*</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear equation*</td>
<td>Y = 64767.9X – 12378.03</td>
<td></td>
</tr>
<tr>
<td>Linearity</td>
<td>r ≥ 0.99</td>
<td>r = 0.9997</td>
</tr>
<tr>
<td>Range (µg/ml)</td>
<td>1- 10</td>
<td></td>
</tr>
<tr>
<td>Accuracy (%recovery)</td>
<td>90 – 108</td>
<td>102.00 – 106.68</td>
</tr>
<tr>
<td>Repeatability (RSD, n = 6)</td>
<td>3</td>
<td>0.76 – 1.48</td>
</tr>
<tr>
<td>Intermediate precision (RSD, n = 18)</td>
<td>3</td>
<td>2.75</td>
</tr>
<tr>
<td>LOD (µg/ml)</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>(%RSD, n = 6)</td>
<td>3</td>
<td>0.86</td>
</tr>
<tr>
<td>LOQ (µg/ml)</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>(%RSD, n = 6)</td>
<td>3</td>
<td>0.72</td>
</tr>
</tbody>
</table>

* AOAC International, *X is the concentration of protocatechuic acid (µg/ml), Y is the peak area at 254 nm.

**System suitability**

The system suitability was evaluated in terms of the tailing factor, number of theoretical plates, the RSD of the peak area and the retention time. All system suitability parameters were within the limits of the acceptance criteria as shown in Table II. The results suggest that the HPLC conditions are suitable for quantitative analysis.
**TABLE II** - System suitability of HPLC analysis of protocatechuic acid contents in *Phellinus* mushrooms

<table>
<thead>
<tr>
<th>System suitability parameters</th>
<th>Acceptant criteria</th>
<th>Result (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tailing factor</td>
<td>&lt; 2</td>
<td>0.81 ± 0.02</td>
</tr>
<tr>
<td>Theoretical plate</td>
<td>&gt; 2000</td>
<td>28010.40 ± 232.39</td>
</tr>
<tr>
<td>Peak area (%RSD)</td>
<td>&lt; 2</td>
<td>0.76</td>
</tr>
<tr>
<td>Retention time (RSD)</td>
<td>&lt; 2</td>
<td>0.09</td>
</tr>
</tbody>
</table>

**FIGURE 1** - HPLC chromatograms of standard protocatechuic acid (A), chloroform-ethyl acetate fraction of *P. everhartii* (B), and chloroform-ethyl acetate fraction of *P. everhartii* spiked with standard protocatechuic acid (C). ▼ symbol indicates the peaks of protocatechuic acid at the retention time of 7.90 min.
Quantitative analysis of the protocatechuic acid contents in Phellinus mushrooms

Quantitative analysis of protocatechuic acid in the chloroform-ethyl acetate fraction of 11 Phellinus mushrooms was performed using the optimized and validated HPLC method. The HPLC fingerprint of 11 Phellinus mushroom fractions exhibited specific chromatographic characteristics. Each fraction from Phellinus mushroom species showed quite similar chromatographic patterns except the chromatograms from the fractions of Phellinus conchatus f. alni (M1) and P. noxius (M9). The chloroform-ethyl acetate fractions of M1 and M9 did not show the peak corresponding to protocatechuic acid at the retention time of 7.9 min suggesting that there was no protocatechuic acid in these two Phellinus mushrooms or the amounts of this compound in these two Phellinus mushrooms are lower than the LOD. The protocatechuic acid contents in 11 Phellinus mushroom extracts are shown in Table III. Phellinus everhartii (M2) contained the highest protocatechuic acid content of 0.4121 ± 0.03 %w/w of the extract, followed by P. hippophaëcola (M4), P. pini var. cancriformans (M10) and P. nigricans var. resupinatus (M8), which contained protocatechuic contents of 0.3468 ± 0.01, 0.3385 ± 0.01 and 0.3291 ± 0.01 %w/w of the extract, respectively. From our previous work, which screened the in vitro antioxidant activities of these 11 Phellinus mushrooms, it was found that among the tested samples, M2 ethanol and water extracts promoted strong antioxidant activities determined by a DPPL scavenging assay and the FRAP method (Sonthudlakhar et al., 2019). M4, M8 and M10 samples also showed high antioxidant effects while M1 and M9 samples showed low antioxidant activities (Sonthudlakhar et al., 2019). Moreover, ethanol extracts from M2 and M4 also promoted stronger in vitro antibacterial effects on some bacteria including Escherichia coli, Salmonella Typhimurium, Salmonella Enteritidis, Staphylococcus aureus, Helicobacter pylori strain T96 and Helicobacter pylori strain 2R suggesting that protocatechuic acid could be responsible for the inhibitory effects of these extracts (Sonthudlakhar et al., 2019). There are some previous reports that demonstrated the antioxidant activities (Herrmann, 1989; Kayano et al., 2002; Sang et al., 2002; Pacheco-Palencia, Mertens-Talcott, Talcott, 2008; Li et al., 2011; Semaming et al., 2015) and antibacterial effects (Chao, Yin, 2009) of protocatechuic acid. The results confirm that protocatechuic acid plays an important role in the antioxidant and antibacterial activities of Phellinus mushrooms and could be used as a chemical marker for quality control of Phellinus mushroom raw materials and products in the future.

TABLE III - Protocatechuic acid contents in Phellinus mushroom extracts analysed by HPLC

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protocatechuic acid content (%w/w of the extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>&lt; 0.0099*</td>
</tr>
<tr>
<td>M2</td>
<td>0.4121 ± 0.03a</td>
</tr>
<tr>
<td>M3</td>
<td>0.2337 ± 0.01c</td>
</tr>
<tr>
<td>M4</td>
<td>0.3468 ± 0.01b</td>
</tr>
<tr>
<td>M5</td>
<td>0.1638 ± 0.01d</td>
</tr>
<tr>
<td>M6</td>
<td>0.1540 ± 0.01c</td>
</tr>
<tr>
<td>M7</td>
<td>0.1213 ± 0.00c</td>
</tr>
<tr>
<td>M8</td>
<td>0.3291 ± 0.01b</td>
</tr>
<tr>
<td>M9</td>
<td>&lt; 0.0466*</td>
</tr>
<tr>
<td>M10</td>
<td>0.3385 ± 0.01b</td>
</tr>
<tr>
<td>M11</td>
<td>0.2314 ± 0.00c</td>
</tr>
</tbody>
</table>

Different letters in the same column are significantly different (P < 0.05), *protocatechuic acid contents less than the LOD value analyzed by the HPLC condition in this study; M1 = Phellinus conchatus f. alni Bondartsev, M2 = Phellinus everhartii (Ellis & Galloway) A. Ames, M3 = Phellinus gigivus (Schwein.) Pat., M4 = Phellinus hippophaëcola H. Jahn, M5 = Phellinus igniarius (L.) Quél., M6 = Phellinus igniarius var. cinereus Niemelä, M7 = Phellinus nigricans (Fr.) P. Karst., M8 = Phellinus nigricans var. resupinatus Bourdot & Galzin, M9 = Phellinus noxius (Corner) G. Cunn., M10 = Phellinus pini var. cancriformans M.J. Larsen Lombard & Aho, M11 = Phellinus pini var. microporus Pilát.
CONCLUSION

Phellinus is a mushroom species available in Thailand, especially in the North-eastern part of the country. This mushroom species has been used for medicinal purposes for a long time. However, there is not much research about its biological activities and phytochemicals. This study developed a HPLC analytical method for the quantitative analysis of the contents of protocatechuic acid, an active compound in 11 Phellinus mushroom species collected in Thailand. The analytical method was precise, accurate and suitable for quantitative analysis of protocatechuic acid content in Phellinus mushroom raw materials and products. Phellinus everhartii contained the highest protocatechuic acid content along with P. hippophaécola, P. pini var. cancriformans and P. nigricans var. resupinatus suggesting they are good sources for development of pharmaceutical or health products in the future.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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REFERENCES


Chao CY, Yin MC. Antibacterial effects of roselle calyx extracts and protocatechuic acid in ground beef and apple juice. Foodborne Pathog Dis. 2009;6(2):201-206.


HPLC quantitative analysis of protocatechuic acid contents in 11 *Phellinus* mushroom species collected in Thailand


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