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Article

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

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Phellinus mushrooms have been traditionally used for various medicinal purposes. Protocatechuic acid, which was previously reported to be a component in some *Phellinus* mushrooms, has some pharmacological effects. This study aimed to validate a HPLC method for the quantitative analysis of the protocatechuic acid contents in the extracts from different *Phellinus* mushroom species collected in Thailand. HPLC was carried out using a C18 column and the gradient mobile phases of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. Method validation was performed to assure the linearity, accuracy, precision, limit of detection and limit of quantitation of the analytical method. The linearity range of protocatechuic acid was 1 - 10 µg/ml. The average recovery was 104.16%. The method was shown to be precise with the RSD of repeatability and intermediate precision at less than 3%. The protocatechuic contents in 11 *Phellinus* mushrooms were in the range of less than 0.0099 - 0.4121 %w/w of the extract. The developed HPLC method was reliable and suitable for the quantitative analysis of protocatechuic acid content in *Phellinus* mushrooms.

Keywords: *Phellinus* mushroom. Protocatechuic acid. HPLC. Quantitative analysis. Validation.

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. hippophaëicola* 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

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phenolic compound isolated from *P. linteus* 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 hippophaëicola* 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 *(*RSD*)* was then calculated. The chloroform-ethyl acetate fraction of *Phellinus noxius* was evaluated for the limit of detection (LOD) and the limit of quantitation (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_f), 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 (%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 (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 (Rt = 7.9 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

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

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

System suitability

The system suitability of the optimized HPLC method was performed to simultaneously and quantitatively analyse the protocatechuic acid in the chloroform-ethyl acetate fractions of 11 *Phellinus* mushrooms extracts. 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

System suitability parameters	Acceptant criteria	Result (n=5)
Tailing factor	< 2	0.81 ± 0.02
Theoretical plate	> 2000	28010.40 ± 232.39
Peak area (%RSD)	< 2	0.76
Retention time (RSD)	< 2	0.09

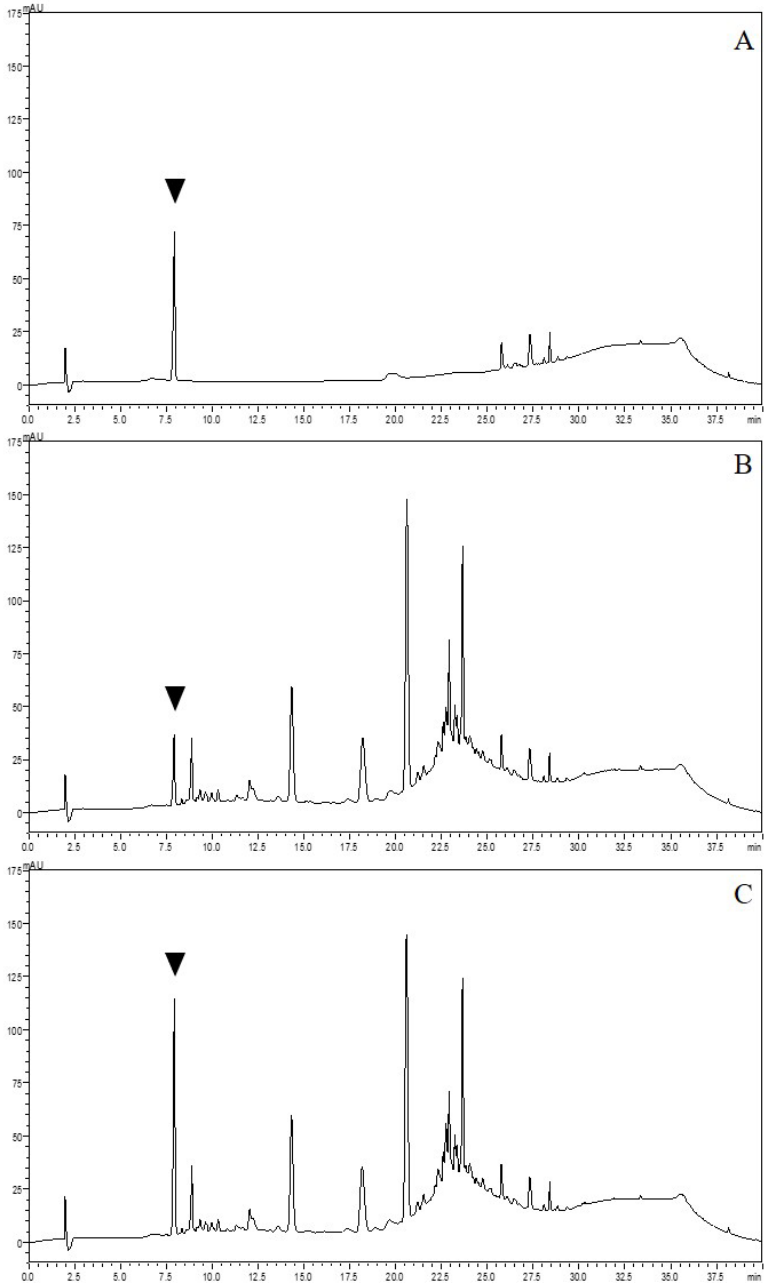

FIGURE 1 - HPLC chromatograms of standard protocatechuic acid (A), chloroform-ethyl acetate fraction of *P. everhartii* (B), and chloroform-ethyl acetate fraction of *P. everhartii* spiked with standard protocatechuic acid (C). ▼ symbol indicates the peaks of protocatechuic acid at the retention time 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. hippophaëicola* (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 (Sunthudlakhar *et al.*, 2019). M4, M8 and M10 samples also showed high antioxidant effects while M1 and M9 samples showed low antioxidant activities (Sunthudlakhar *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 (Sunthudlakhar *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

Sample	Protocatechuic acid content (%w/w of the extract)
M1	< 0.0099*
M2	0.4121 ± 0.03^{a}
M3	0.2337 ± 0.01^{c}
M4	0.3468 ± 0.01^{b}
M5	0.1638 ± 0.01^{d}
M6	0.1540 ± 0.01^{de}
M7	0.1213 ± 0.00^{e}
M8	0.3291 ± 0.01^{b}
M9	< 0.0466*
M10	0.3385 ± 0.01^{b}
M11	0.2314 ± 0.00^{c}

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 gilvus* (Schwein.) Pat., M4 = *Phellinus hippophaëicola* 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. hippophaëicola*, *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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