Original article

1H NMR based metabolite profiling for optimizing the ethanol extraction of Wolfiporia cocos

Junsang Oh a,b, Deok-Hyo Yoon a, Jae-Gu Han c, Hyung-Kyoon Choi b,*, Gi-Ho Sung a,d,*

*a Institute for Healthcare and Life Science, International St. Mary’s Hospital and College of Medicine, Catholic Kwandong University, Incheon 22711, Republic of Korea
b College of Pharmacy, Chung-Ang University, Republic of Korea
Mushroom Research Division, National Institute of Horticultural and Herbal Science, Rural Development Administration, Republic of Korea
4 Department of Microbiology, College of Medicine, Catholic Kwandong University, Republic of Korea

Article history:
Received 22 February 2018
Revised 2 April 2018
Accepted 4 April 2018
Available online 24 April 2018

Keywords:
Wolfiporia cocos
Ethanol extraction
1H NMR
Metabolites profiling
Pachymic acid

Abstract

Metabolite profiling of Wolfiporia cocos (family: Polyporaceae) had been much advancement in recent days, and its analysis by nuclear magnetic resonance (NMR) spectroscopy has become well established. However, the highly important trait of W. cocos still needs advanced protocols despite some standardization. Partial least squares discriminant analysis (PLS-DA) was used as the multivariate statistical analysis of the 1H NMR data set. The PLS-DA model was validated, and the key metabolites contributing to the separation in the score plots of different ethanol W. cocos extract. 1H NMR spectroscopy of W. cocos identified 33 chemically diverse metabolites in D2O, consisting of 13 amino acids, 11 organic acids 2 sugars, 3 sugar alcohols, 1 nucleotide, and 3 others. Among these metabolites, the levels of tyrosine, proline, methionine, sarcosine, choline, acetoacetate, citrate, 4-amino butyrate, aspartate, malate, lactate, lysine, xylitol, lactate threonine, leucine, valine, isoleucine, uridine, guanidoacetate, arbutin, mannitol, glucose, and betaine were increased in the 95% ethanol extraction sample compared with the levels in other samples, whereas level of acetate, phenylalanine, alanine, succinate, and fumarate were significantly increased in the 0% ethanol extraction sample. A biological triterpenoid, namely pachymic acid, was detected from different ethanol P. cocos extract using 1H-NMR spectra were found in CDCl3. This is the first report to perform the metabolomics profiling of different ethanol W. cocos extract. These researches suggest that W. cocos can be used to obtain substantial amounts of bioactive ingredients for use as potential pharmacological and nutraceuticals agents.

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1. Introduction

Wolfiporia cocos is a fungus in the family Polyporaceae. It is a wood-decay fungus but has a subterranean growth habit. It is notable in the development of a large, long-lasting underground sclerotium that resembles a small coconut. This sclerotium called “(Chinese) Tuckahoe” or fu-ling, is not the same as the true tuckahoe used as Indian bread by Native Americans, which is the arrow arum, Peltandra virginica, a flowering tuberous plant in the arum family. W. cocos is also used extensively as a medicinal mushroom in Chinese medicine (Esteban, 2009; Wu et al., 2018; Liu et al., 2018). Indications for use in the traditional Chinese medicine include promoting urination, invigorating the spleen function (i.e., digestive function) and calming the mind (Shah et al., 2014). Alcoholic extracts of W. cocos have been reported to contain various lanostane-type triterpenoids (Akihisa et al., 2007; Wang et al., 2018; Zhu et al., 2018; Chen et al., 2017). W. cocos also possesses abundant medicinal compounds including polysaccharides and triterpenoids (Feng et al., 2013). These compounds have been used to treat many diseases such as gastritis, nephrosis, edema, dizziness, nausea, and emesis. In addition, the surface layer of W. cocos has known to be functional in significant diuretic effects (Zhao et al., 2012; Shi et al., 2017; Hu et al., 2017; Lee et al., 2017) and...
famous for its biological efficacy such as anti-tumor effect (Kanayama et al., 1983; Jin et al., 2003; Li et al., 2017).

Until now, the metabolomic profiling using $^1$H NMR and multivariate statistical analysis of W. cocos has not been reported. The W. cocos extract according to different ethanol extraction is mainly performed by visual inspection. Therefore, such different ethanol extraction has been rather subjective and relies on a few experts in the experiment. Nowadays, metabolomics techniques combining spectrometric methods and multivariate statistical analysis such as principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and hierarchical cluster analysis (HCA) (Eriksson et al., 2006). Additionally, the use of PLS makes it possible to estimate the important activities from multivariate data sets. These techniques are the rapid and reliable identification of different ethanol extract and will require all the traditional approaches of natural products chemistry and metabolomics as well as improved analytical methods and statistical tools. The multivariate statistical analysis techniques coupled with $^1$H NMR analysis using various selection protocols were used for metabolic profiling and trait of various kinds of plants, plant-derived preparations, foods, and tissues (Kim et al., 2010; Sekiyama et al., 2010; Wishart, 2008). We report the first identification and quantification of pachymic acid by $^1$H NMR and our hypothesis was that the metabolic profiles of compounds of W. cocos might change during different ethanol extracts. In this study, we first described $^1$H NMR spectroscopy followed by PLS-DA in metabolomic analysis of different ethanol W. cocos extracts.

2. Materials and methods

2.1. Solvents and chemicals

The following chemicals were obtained commercially: Monopotassium phosphate (KH$_2$PO$_4$), 3-(trimethylisilyl)-propionic-2,2,3,3-d$_4$ acid sodium salt (TSP), Ethyl alcohol, Deuterated chloroform (CDCl$_3$) and deuterium oxide (D$_2$O) 99.8%, were purchased from Sigma-Aldrich (St. Louis, MO, USA). NMR tubes were obtained from Optima (Tokyo, Japan).

2.2. P. cocos microwave-assisted extraction

The microwave-assisted extraction method used for W. cocos samples had as follows: Powdered W. cocos (2 g) were placed into a 250 mL in an extraction vessel with 40 mL each solvent (0, 25, 50, 75, and 95% ethanol). Each extraction vessel was inserted to the microwave oven for 50 min at 85 °C (960 W) (Transform 800. AR0800-MW-1800, Aurora instruments Ltd, Vancouver, B.C., Canada). First extraction was transferred to new flask and then the residue was re-extracted twice for 50 min at 85 °C (960 W). The extracts were evaporated, freeze-dried and then stored at -70 °C until analysis. The extraction samples were used for analysis of $^1$H NMR.

2.3. $^1$H NMR analysis of W. cocos metabolomic profiling

To analyzing the hydrophilic substance, 100 mg of W. cocos extracts were dissolved in 1 mL phosphate buffer (90 mM, pH 7.0–7.4) in D$_2$O containing 0.01% sodium salt (TSP) as an internal standard using an ultra-sonication for 60 min (Lab companion, Daejeon, Korea) to extract intracellular metabolites. Analyzing for pachymic acid, 100 mg of W. cocos extracts were dissolved in 1 mL CDCl$_3$. After metabolite extraction, supernatants were clarified by centrifuging at 12,000 rpm for 20 min at room temperature (Labogene, Seoul, Korea), filtered using an Amicon Ultra 0.5 mL centrifugal filters (Millipore, Darmstadt, Germany), and collected into 1.5 mL tube (Eppendorf, Hamburg, Germany). 700 μL of each filtered extracts were loaded into 5 mm NMR tubes ($n = 3$). $^1$H NMR spectra were acquired at 300 K on a 600.13-MHz Bruker Advance spectrometer (Rheinstetten, Germany) using the ZGPR pulse sequence with water pre-saturation. In total, 128 transients were gathered into 32 K data points with a relaxation delay of 2 s with an acquisition time per scan of 1.70 s and a spectra width of 10.0 ppm. The NMR spectra were analyzed using Chenomx NMR suite software (version 8.2, Chenomx Inc., Edmonton, Alberta).

2.4. Data preprocessing and multivariate statistical analysis of $^1$H NMR data

$^1$H NMR data processing and assigned were performed through Chenomx NMR suite software (version 8.2, Chenomx Inc., Edmonton, Alberta). Multivariate statistical analyses were performed by one-way ANOVA using PASW Statistics 22 software (IBM, Somers, NY, USA) following which a Tukey’s significant difference test. Significance was determined with a P-value threshold (<0.05). Metabolites levels were normalized using log2 function and then, mean centering and UV scaling was applied for all principal component analysis (PCA), and partial least squares discriminant analysis (PLS-DA) using Simca-P+11.0 (Umetrics, Umeå, Sweden).

3. Results and discussion

3.1. Metabolic assignments of W. cocos different ethanol extractions

Table 1 and Fig. 1 indicate that 33 compounds were identified in D$_2$O dissolved of W. cocos samples at the 5 different ethanol extractions.

| Assigned No | Metabolite compound | Chemical shift (ppm) |
|-------------|---------------------|----------------------|
| 1           | Leucine             | 0.94(t, J = 6.5), 1.70(m) |
| 2           | Valine              | 1.02(d, J = 6.0), 0.98(8, J = 6.87) |
| 3           | Isoleucine          | 1.02(t, J = 6.5), 1.06(d, J = 6.9) |
| 4           | Lactate             | 1.30(d, J = 6.91) |
| 5           | Threonine           | 1.34(d, J = 6.58) |
| 6           | Alanine             | 1.46(d, J = 8.31) |
| 7           | 4-Aminobutyrate     | 1.90(m), 2.30(t, J = 7.36), 3.02(t, J = 7.58) |
| 8           | Acetate             | 1.90(s) |
| 9           | Proline             | 1.98(m), 2.02(m) |
| 10          | Methionine          | 2.14(s), 2.22(m) |
| 11          | Acetoacetate        | 2.30(s) |
| 12          | Succinate           | 2.38(s) |
| 13          | Citrate             | 2.50(d, J = 15.92) |
| 14          | Malate              | 2.66(dd, J1 = 15.36, J2 = 2.9) |
| 15          | Aspartate           | 2.66(m), 2.82(dd, J1 = 3.8, J2 = 3.7) |
| 16          | Sarcosine           | 2.70(s), 3.58(s) |
| 17          | Malonate            | 3.18(s) |
| 18          | Choline             | 3.18(s), 4.06(m) |
| 19          | Glucose             | 3.22(t, J = 8.9), 3.46(m), 4.62(m), 5.22(d, J = 4.07) |
| 20          | Betaine             | 3.22(s), 3.86(s) |
| 21          | Arabitol            | 3.54(dd, J1 = 8.3, J2 = 1.62), 3.90(m) |
| 22          | Xyitol              | 3.66(m) |
| 23          | Lysine              | 3.74(t, J = 6.09) |
| 24          | Guanidoacetate      | 3.78(s) |
| 25          | Mannitol            | 3.79(m), 3.90(m) |
| 26          | Trehalose           | 5.18(d, J = 3.8) |
| 27          | Maltose             | 5.34(d, J = 3.8), 5.42(d, J = 3.89) |
| 28          | Uridine             | 5.9(d, J = 7.86(d, J = 3.8)) |
| 29          | Fumarate            | 6.5(s) |
| 30          | Tyrosine            | 6.88(d, J = 8.45), 7.18(d, J = 8.41) |
| 31          | Phenylalanine       | 7.34(m), 7.42(m) |
| 32          | Xanthine            | 8.26(s) |
| 33          | Formate             | 8.46(s) |
| 34          | Pachymic acid       | 0.97–1.01(m), 1.54(s), 4.54-4.78(m) |
tions. 11 amino acids, including leucine, valine, isoleucine, threonine, alanine, proline, methionine, aspartate, lysine, tyrosine, and phenylalanine, 2 amino acid derivatives, including sarcosine, and guanidoacetate, 5 organic acid, including lactate, acetoacetate, acetate, citrate, and malate, 6 organic acid derivative, including malonate, fumarate, formate, 4-aminobutyrate (GABA), succinate, betaine, 2 sugars glucose and maltose, 3 sugar alcohols, including arabitol, xylitol, and mannitol, 1 nucleoside, including uridine, and 3 others, including trehalose, xanthine, choline were detected (Fig. 1A). In addition, pachymic acid was identified CDCl$_3$ dissolved of *W. cocos* samples at the 5 different ethanol extractions. Fig. 1B shows a presentive NMR specrum of the CDCl$_3$ extracts of *W. cocos* samples. The peak of pachymic acid were assigned by comparisons with the previously study (Wang et al., 2012).

Fig. 1. Representative $^1$H NMR spectrum (a) NMR spectra of *W. cocos* sample analyzed with D$_2$O as an NMR dissolution solvent after 95% methanol extraction. (b) pachymic acid analyzed with CDCl$_3$ as an NMR dissolution solvent after 95% ethanol extraction.
3.2. Multivariate data analysis and metabolic characterization of W. cocos

The NMR data sets that included 33 aqueous metabolite data from the W. cocos of different ethanol extraction either the 0, 25, 50, 75, and 95 % were assessed using PCA and PLS-DA (Fig. 2A and B) score plotting. As an unsupervised method, PCA enables recognition of any inherent sample clustering without any bias because it does not require any information on the data sets (Lu, et al., 2008; Woo et al., 2009; Carraro et al., 2009). Therefore, it was applied to confirm the clustering prior to analysis by supervised methods such as PLS-DA. A supervised PLS-DA is one of the classification methods where the response variable is a ‘dummy’ Y matrix expressing an orthogonal unit vector of each class (Barker and Rayens, 2003).

Our data show that significant differences were observed among the five groups. The 0% and 95% extraction samples were clearly separated from the 25%, 50% and 75% extraction samples (PCA and PLS-DA score plots). PLS component 1 versus PLS component 2 explained separation among W. cocos samples of 3 groups. Combining PLS component 1 and PLS component 2 explained 75.4% of total variance (54.1% and 21.3%, respectively) (Fig. 2 B). When internal cross-validation on PLS-DA was performed, component 7 and parameters, such as R2X = 0.95, R2Y = 0.99, and Q2Y = 0.83, which showed its predictive power and degree of fit to the data, were obtained. The major metabolites involved in the separation along PLS1 (positive: 95% ethanol extraction sample) were valine, isoleucine, leucine, lactate threonine, 4-aminobutyrate, proline, methionine, acetate, citrate, malate, aspartate, sarcosine, malonate, choline, glucose, betaine, arabitol, xyitol, lysine, guanidinoacetate, mannitol, trehalose, maltose, uridine, tyrosine, and xanthine (Fig. 2C), while alanine, acetate, succinate, fumarate, phenylalanine, and formate were the major compounds contributing to the separation along PLS1 (negative: 0% ethanol extraction sample) (Fig. 2C). A permutation test was processing in order to further validate the PLS-DA models (Bijlsma et al., 2006). The R2 and Q2 intercept values were 0.713 and –0.504 after 20 permutations (Fig. 2D). Table 2 shows the PLS-DA-derived VIP values of the major compounds contributing to the separation of each W. cocos extraction sample in the PLS-DA model. Variable importance in the projection (VIP) value is a weighted sum of squares of the PLS-DA weight, both with respect to Y as the correlation to all the responses and X as its projection, picking components that play important roles in the separation. It has been indicated that cutting-off for VIP around 1.0 worked well for variable selection even though the variables with larger than 1.0 were most influential for model (Lu et al., 2008; Woo et al., 2009). As shown in Table 2. The VIP values of the major contributing compounds for separation in the score plots derived from PLS-DA. The metabolites with VIP values over 1.0, such as proline, aspartate, xanthine, and citrate, were significantly different between groups.

![Fig. 2. Validation plots of the PLS-DA model using 5 different ethanol extracts of W. cocos samples. PCA model (A), PLS-DA model (B), Metabolites in PLS1 (C), R2 and Q2 intercept values (D).](image_url)

Table 2

| No. | Compounds          | VIP  |
|-----|--------------------|------|
| 1   | Proline            | 1.333|
| 2   | Asparate           | 1.276|
| 3   | 4-Aminobutyrate    | 1.255|
| 4   | Acetate            | 1.204|
| 5   | Xanthine           | 1.168|
| 6   | Methionine         | 1.163|
| 7   | Alaicine           | 1.145|
| 8   | Malate             | 1.119|
| 9   | Choline            | 1.069|
| 10  | Malonate           | 1.056|
| 11  | Uridine            | 1.052|
| 12  | Fumarate           | 1.050|
| 13  | Acetoacetate       | 1.047|
| 14  | Sarcosine          | 1.044|
| 15  | Phenylalanine      | 1.043|
| 16  | Fumarate           | 1.031|
| 17  | Glucose            | 1.020|
| 18  | Succinate          | 1.017|
| 19  | Threonine          | 1.007|
aspartate, 4-aminobutyrate, acetate, xanthine, methionine, alanine, malate, choline, malonate, uridine, formate, acetoacetate, sarcosine, phenylalanine, fumarate, glucose, succinate, and threonine were selected for further comparison by one-way analysis of variance (ANOVA). The relative changes in these metabolites and significant differences between the five groups were determined by ANOVA test. Among these metabolites, the levels of tyrosine, proline, methionine, sarcosine, choline, acetoacetate, citrate, 4-aminobutyrate, aspartate, malate, alanine, xylitol, lactate, threonine, leucine, valine, isoleucine, uridine, guanosine, arabinose, mannitol, glucose, and betaine were increased in the 95% ethanol extraction sample with the levels in other samples, whereas levels of acetate, phenylalanine, alanine, succinate, and fumarate were significantly increased in the 0% ethanol extraction sample (Table 3). Main variations are summarized in the form of a heatmap shown in Fig. 3. In this figure the red color indicates increased relative concentration of the metabolites within the different ethanol W. cocos, while green color indicates a decreased relative concentration (or absence) of metabolites.

In addition, pachymic acid contents of five different ethanol concentration W. cocos were analyzed and the results were summarized in Fig. 4. The pachymic acid content of 95% ethanol was significantly high value (0.426 ± 0.0313). Pachymic acid is a natural triterpenoid known to inhibit the phospholipase A2 family of arachidonic acid – producing enzymes. Pachymic acid has antioxidant activity, anti-inflammatory and anticancer properties, it can inhibit cell growth and modulate arachidonic acid metabolism in lung cancer and impair breast cancer cell invasion by suppressing nuclear factor kappa B-dependent metalloproteinase-9 expression (Gapter et al., 2005; Ling et al., 2010; Ling et al., 2011). W. cocos is used widely as traditional medicines and food in Korea, China, and Japan. W. cocos consumption is likely to increase in recently, due to its important bioactivities.

4. Conclusion

In this study, we used a metabolomics approach based on 1H NMR spectroscopic analysis to show that the characteristic metabolic profiles of W. cocos. The results revealed that the metabolomics profiles different ethanol W. cocos extraction, making further investigations of the bioactivities of appropriated ethanol W. cocos extractions. This study can be offered that the combined use of NMR and PLS-DA is an efficient technique for the different ethanol W. cocos extractions and would be suitable for discriminating samples in industrial application.

Acknowledgment

This research was supported by the Convergence of Conventional Medicine and Traditional Korean Medicine R&D project funded by the Ministry of Health & Welfare through the Korea Health Industry Development Institute (HI15C0094) and Cooperative Research Program for National Genome Project (Project No. PJ01337601) from Rural Development Administration in Korea.
Conflict of interest statement

There is no conflict of interest.

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Fig. 3. The heat map constructed based on the differential ethanol extract 34 metabolites of W. cocos relative concentration values. A red-blue color scale indicates normalized relative concentration level expression value. The red color indicate a Log2 Fold Change ≥ 2 highest relative concentration value while the blue color means Log2 FC ≤ −2 lowest relative concentration value respectively.

Fig. 4. Relative quantification of pachymic acid in different percentage of ethanol extracts of W. cocos.
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