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Original article

^{1}H NMR based metabolite profiling for optimizing the ethanol extraction of *Wolfiporia cocos*

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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 ^{1}H 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. ^{1}H NMR spectroscopy of *W. cocos* identified 33 chemically diverse metabolites in D_2O, consisting of 13 amino acids, 11 organic acids 2 sugars, 3 sugar alcohols, 1 nucleoside, and 3 others. Among these metabolites, the levels of tyrosine, proline, methionine, sarcosine, choline, acetoacetate, citrate, 4-aminobutyrate, aspartate, maltose, malate, lysine, xylitol, lactate threonine, leucine, valine, isoleucine, uridine, guanidoacetate, arabitol, 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 ^{1}H-NMR spectra were found in $CDCl_3$. 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.

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

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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_2PO_4), 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP), Ethyl alcohol, Deuterated chloroform ($CDCl_3$) and deuterium oxide (D_2O) 99.8%, were purchased from Sigma-AldrichSigma 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_2O 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) usingSimca-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_2O dissolved of *W. cocos* samples at the 5 different ethanol extrac-

Table 1
Metabolite assignments and chemical shifts of distinguishable peaks.

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(d, *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 (GABA)	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, J_1 = 15.36, J_2 = 2.9)
15	Aspartate	2.66(m), 2.82(dd, J_1 = 3.8, J_2 = 3.7)
16	Sarcosine	2.70(s), 3.58(s)
17	Malonate	3.10(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, J_1 = 8.3, J_2 = 1.62), 3.90(m)
22	Xylitol	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*=)
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 guanidoactate, 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).

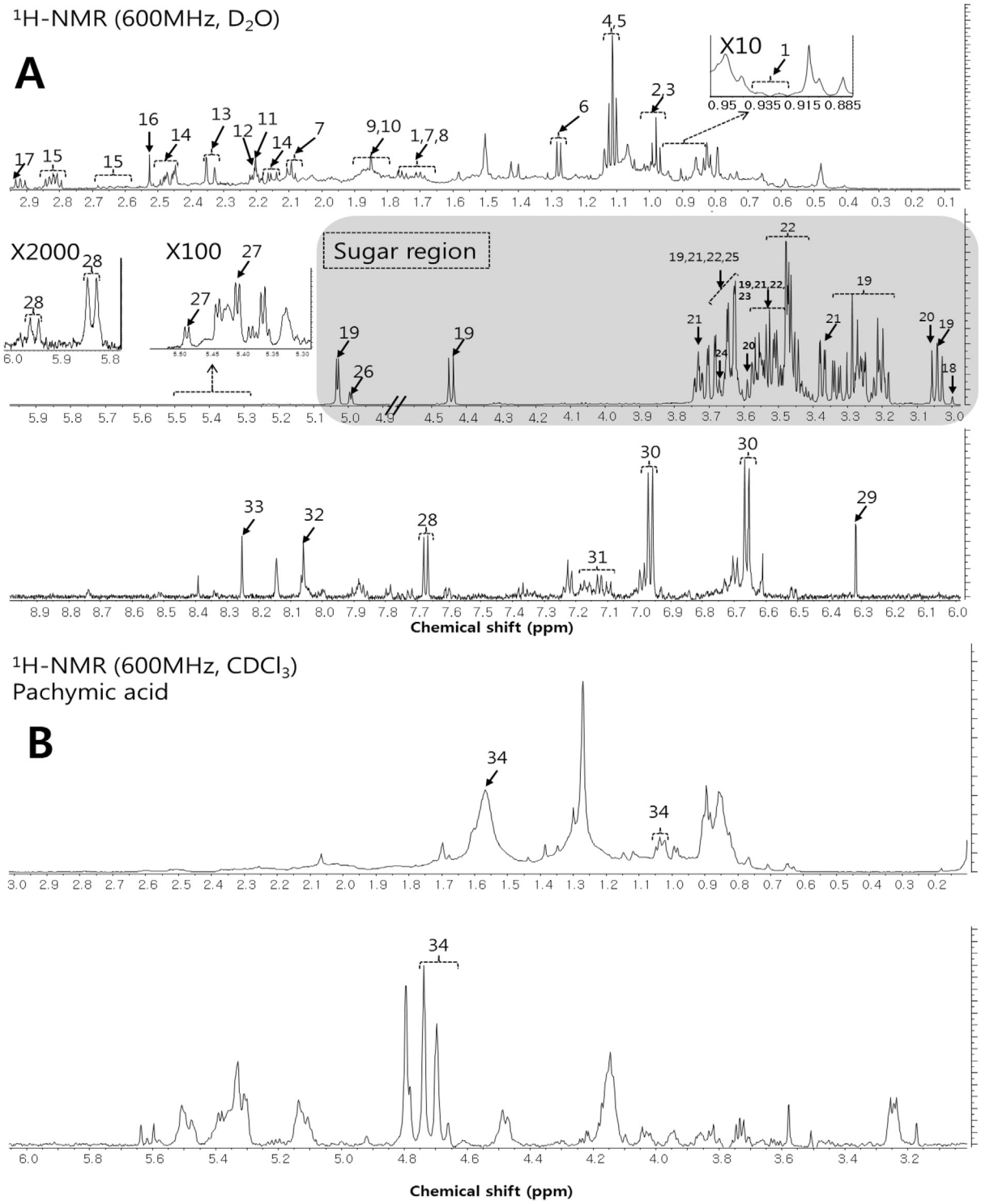

Fig. 1. Representative ^{1}H NMR spectrum (a) NMR spectra of *W. cocos* sample analyzed with D_2O as an NMR dissolution solvent after 95% methanol extraction. (b) pachymic acid analyzed with $CDCl_3$ as an NMR dissolution solvent after 95% ethanol extraction.

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-aminobutlyate, proline, methionine, acetoacetate, citrate, malate, aspartate, sarcosine, malonate, choline, glucose, betaine, arabitol, xylitol, lysine, guanidoacetate, 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 an 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,

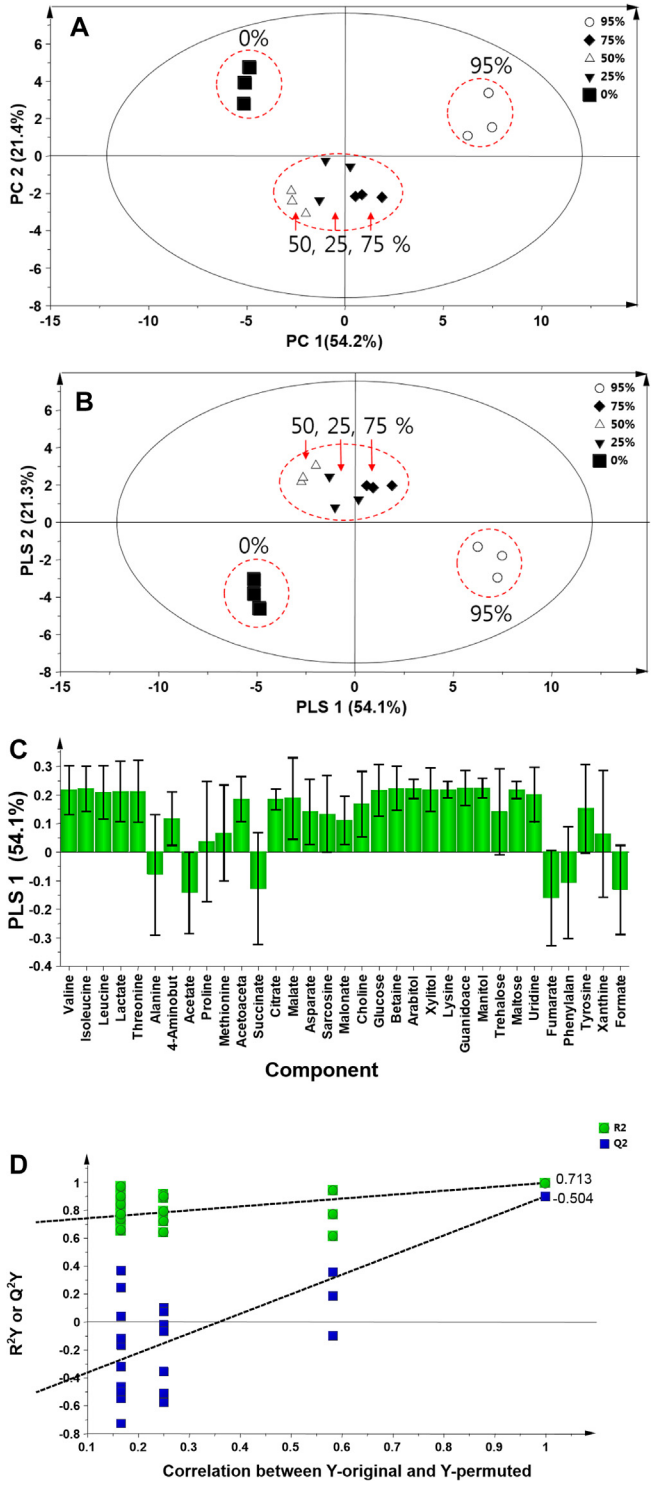

Fig. 2. Validation plots of the PLS-DA model using 5 different ethanol extracts of *W. cocos* samples. PCA model (A), PLS-DA model (B), Metabolites in PLS1 (C), R2 and Q2 intercept values (D).

Table 2
The VIP values of the major metabolites for the separation of *W. cocos* samples in the PLS-DA derived score plots.

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	Alanine	1.145
8	Malate	1.119
9	Choline	1.069
10	Malonate	1.056
11	Uridine	1.052
12	Formate	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

Table 3
Relative levels of 34 metabolites in different ethanol extraction sample of *W. cocos*.

Compounds	*Poria cocos* ethanol extracts				
	0%	25%	50%	75%	95%
Valine	0.148 ± 0.0284^{a}	0.134 ± 0.0037^{a}	0.099 ± 0.0531^{a}	0.352 ± 0.0215^{b}	0.680 ± 0.0321^{c}
Isoleucine	0.120 ± 0.0247^{a}	0.140 ± 0.0166^{a}	0.094 ± 0.0418^{a}	0.335 ± 0.0329^{b}	0.675 ± 0.0396^{c}
Leucine	0.213 ± 0.0326^{a}	0.127 ± 0.0322^{a}	0.125 ± 0.0385^{a}	0.452 ± 0.0281^{b}	0.824 ± 0.0528^{c}
Lactate	0.340 ± 0.0207^{a}	0.285 ± 0.0197^{a}	0.379 ± 0.0679^{a}	0.533 ± 0.0337^{b}	1.174 ± 0.0615^{c}
Threonine	0.279 ± 0.0128^{ab}	0.249 ± 0.0061^{a}	0.275 ± 0.0328^{ab}	0.372 ± 0.0516^{b}	0.900 ± 0.0316^{c}
Alanine	0.479 ± 0.0100^{d}	0.293 ± 0.0106^{bc}	0.237 ± 0.0077^{a}	0.257 ± 0.0191^{ab}	0.324 ± 0.0129^{c}
4-Aminobutyrate	0.471 ± 0.0279^{abc}	0.729 ± 0.0482^{c}	0.415 ± 0.1185^{a}	0.451 ± 0.0356^{ab}	0.697 ± 0.1118^{bc}
Acetate	0.234 ± 0.0061^{b}	0.174 ± 0.0181^{b}	0.083 ± 0.0382^{a}	0.069 ± 0.0045^{a}	0.082 ± 0.0222^{a}
Proline	0.143 ± 0.0303^{a}	0.134 ± 0.0344^{a}	0.114 ± 0.0194^{a}	0.097 ± 0.0023^{a}	0.155 ± 0.0522^{a}
Methionine	0.200 ± 0.0310^{a}	0.236 ± 0.0583^{a}	0.167 ± 0.0261^{a}	0.152 ± 0.0155^{a}	0.239 ± 0.0669^{a}
Acetoacetate	0.053 ± 0.0115^{a}	0.116 ± 0.0368^{a}	0.083 ± 0.0201^{ab}	0.074 ± 0.0061^{a}	0.168 ± 0.0300^{b}
Succinate	0.775 ± 0.0067^{b}	0.200 ± 0.0299^{a}	0.174 ± 0.0330^{a}	0.167 ± 0.0419^{a}	0.232 ± 0.0273^{a}
Citrate	0.032 ± 0.0068^{a}	0.088 ± 0.0367^{ab}	0.054 ± 0.0098^{ab}	0.063 ± 0.0274^{ab}	0.118 ± 0.0130^{b}
Malate	0.108 ± 0.0114^{a}	0.130 ± 0.0134^{a}	0.083 ± 0.0044^{a}	0.087 ± 0.0074^{a}	0.249 ± 0.0277^{b}
Asparate	0.285 ± 0.0130^{a}	0.424 ± 0.0618^{b}	0.248 ± 0.0030^{a}	0.220 ± 0.0155^{a}	0.484 ± 0.0678^{b}
Sarcosine	9.446 ± 0.8679^{bc}	6.527 ± 0.2543^{a}	7.308 ± 0.2788^{ab}	9.019 ± 1.1207^{b}	11.398 ± 0.5247^{c}
Malonate	0.066 ± 0.0079	0.091 ± 0.0191	0.077 ± 0.0420	0.106 ± 0.0357	0.105 ± 0.0074
Choline	1.101 ± 0.1974^{ab}	0.941 ± 0.0400^{b}	0.812 ± 0.1059^{b}	1.258 ± 0.2183^{ab}	1.554 ± 0.0887^{b}
Glucose	41.881 ± 4.2489^{a}	85.977 ± 3.6322^{c}	63.496 ± 1.8225^{b}	88.431 ± 1.6366^{c}	102.242 ± 3.1062^{d}
Betaine	16.182 ± 1.7689^{a}	25.322 ± 1.3454^{b}	22.178 ± 1.0788^{b}	26.971 ± 1.8695^{b}	32.399 ± 1.7217^{c}
Arabitol	20.125 ± 0.5451^{a}	25.829 ± 2.0786^{bc}	24.266 ± 0.3775^{bc}	31.684 ± 4.1390^{bc}	38.088 ± 2.4777^{c}
Xylitol	26.176 ± 0.7212^{ab}	31.144 ± 2.3288^{bc}	21.976 ± 1.7453^{a}	34.731 ± 3.3623^{c}	56.220 ± 2.0529^{d}
Lysine	8.552 ± 1.3995^{a}	13.031 ± 2.5729^{ab}	13.544 ± 2.3630^{ab}	18.874 ± 2.9903^{b}	27.290 ± 2.1021^{c}
Guanidoacetate	3.366 ± 0.2804^{a}	5.768 ± 0.4409^{bc}	5.315 ± 0.7008^{b}	7.104 ± 0.2362^{c}	8.915 ± 0.6533^{d}
Mannitol	10.925 ± 0.3843^{a}	14.576 ± 1.4573^{a}	14.439 ± 0.7339^{a}	18.638 ± 1.3304^{b}	23.531 ± 1.6786^{c}
Trehalose	0.219 ± 0.0131^{a}	2.966 ± 1.1683^{b}	2.497 ± 0.3350^{b}	3.437 ± 0.7295^{b}	3.046 ± 0.0285^{b}
Maltose	0.079 ± 0.0081^{a}	0.206 ± 0.0715^{b}	0.138 ± 0.0194^{ab}	0.208 ± 0.0303^{b}	0.328 ± 0.0101^{c}
Uridine	0.031 ± 0.0056^{a}	0.083 ± 0.0136^{b}	0.060 ± 0.0057^{ab}	0.090 ± 0.0134^{bc}	0.099 ± 0.0051^{c}
Fumarate	0.080 ± 0.0018^{d}	0.037 ± 0.0020^{c}	0.027 ± 0.0008^{b}	0.021 ± 0.0007^{a}	0.024 ± 0.0003^{ab}
Phenylalanine	0.229 ± 0.0569^{b}	0.098 ± 0.0277^{a}	0.056 ± 0.0058^{a}	0.065 ± 0.0081^{a}	0.087 ± 0.0019^{a}
Tyrosine	0.097 ± 0.0482	0.088 ± 0.0065	0.079 ± 0.0107	0.103 ± 0.0041	0.149 ± 0.0073
Xanthine	0.017 ± 0.0015^{a}	0.050 ± 0.0062^{c}	0.041 ± 0.0064^{bc}	0.043 ± 0.0050^{bc}	0.032 ± 0.0019^{b}
Formate	0.037 ± 0.0089^{ab}	0.048 ± 0.0040^{b}	0.057 ± 0.0095^{b}	0.041 ± 0.0041^{ab}	0.024 ± 0.0034^{a}
Pachymic acid	0.383 ± 0.0111^{b}	0.354 ± 0.0070^{b}	0.331 ± 0.0011 ^{a}	0.344 ± 0.0982^{b}	0.426 ± 0.0313^{b}

Tukey HSD a < b<c < d.

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, maltose, malate, lysine, xylitol, lactate threonine, leucine, valine, isoleucine, uridine, guanidoacetate, arabitol, 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 (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 ^{1}H 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.

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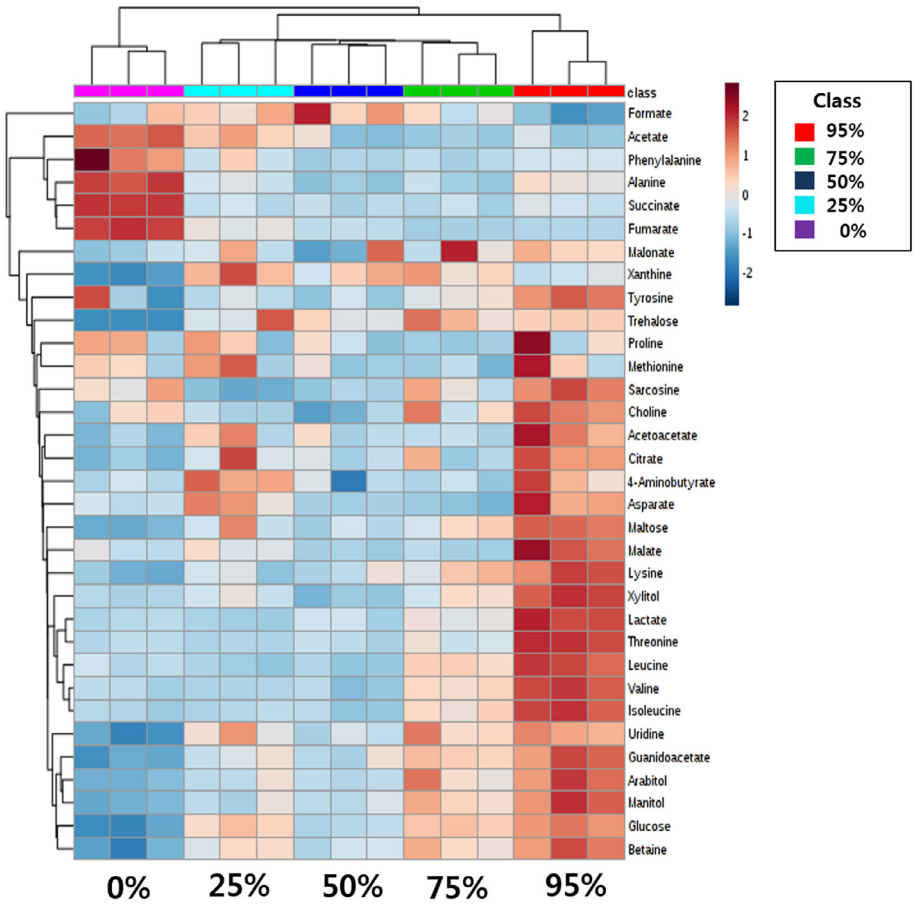

Fig. 3. The heat map constructed based on the differential ethanol extract 34 metabolites of *W. cocos* relative concentration values. A red-blue color scale indicates normalized relative concentration level expression value. The red color indicate a Log2 Fold Change ≥ 2 highest relative concentration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

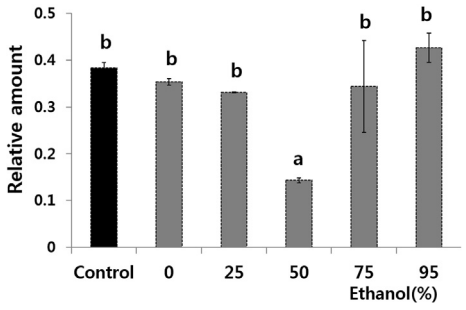

Fig. 4. Relative quantification of pachymic acid in different percentage of ethanol extracts of *W. cocos*.

Conflict of interest statement

There is no conflict of interest.

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