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Antioxidant responses of *Hericium erinaceus* ingredients based on response surface methodology

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Abstract

Hericium erinaceus, a type of wood-rotting mushroom with many bioactive compounds, is effective to prevent and treat many human diseases. In order to acquire more bioactive materials, evaluation of extraction efficiencies, influencing factors, and available antioxidant activities of water and ethanol extractions were investigated by response surface methodology. The antioxidant activities (reducing power, scavenging activities of DPPH, superoxide anion radical, and hydroxyl radicals) and material contents (protein, polyphenol, and flavonoid) were optimised by a Box-Behnken design. The results showed that extraction temperature and solid-liquid ratio were the dominant factors influencing most responses. In addition, it was difficult to maximise all of the responses simultaneously under any set of condition groups. Flavonoid was the main contributor to the scavenging activity of DPPH and superoxide anion, while protein and polyphenol majorly affected the reducing power and scavenging activity of hydroxyl radicals. The antioxidant activities of the same fruiting bodies were related to extraction conditions, chemical constitutions, and physicochemical properties of the extractions. Following these results, further research on the extracts to identify important bioactive compounds for potential nutraceutical and medicinal use is underway.

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Introduction

Oxidative stress caused by reactive oxygen species (ROS) can induce many diseases such as cognitive and motor disturbances (Grochowski *et al.,* 2018; Guo *et al.,* 2018). Therefore, the improvement of antioxidant levels by the intake of natural antioxidants is urgently necessary to improve the quality of human life. *Hericium erinaceus*, a rare wood-rotting mushroom, possesses edible and medicinal values because it contains a myriad of flavonoids, polyphenols, proteins, and other bioactive constituents. Each of these bioactive constituents plays a role in almost all the physiological systems of organisms including digestive, circulatory and immune system (Jiang *et al.,* 2014b). Antioxidative activity is one of the most remarkable functions of *H. erinaceus*. Besides, *H. erinaceus* is also a good source of exogenous antioxidants. The extracts of *H. erinaceus* by different solvents such as *n*-hexane, xylene, chloroform, anhydrous ether, ethyl acetate, acetone, anhydrous ethanol, and distilled water have antioxidant activities (Jiang *et al.,* 2015; 2016). Novel and natural antioxidant medicines from *H. erinaceus* are important to alleviate the pathological discomforts in oxidative stress related diseases (Abdullah *et al.,* 2012). In crude extracts, flavonoids and polyphenols, which are the representative molecules, can ameliorate various disease symptoms (Yi, 2018). Oral administration of flavonoids can ameliorate adverse effects on the kidney of type II diabetes patients. Moreover, flavonoids exert antitumor activity against renal carcinoma cells with no toxic effects on normal cells (Vargas *et al.,* 2018). Phenolic compounds are considered to maintain human health by antioxidant, antibacterial, anticancer, cardioprotective, and anti-inflammatory activities (Tungmunnithum *et al.,* 2018).

In the extraction of bioactive compounds from plants or herbs, or even mushrooms, extraction conditions are vital since they can noticeably affect the resulting extract's yield and/or constituents. Hence, the evaluation of extraction efficiencies and influencing factors are worth exploring. However, little research has been done on this subject. Previous studies shows that response surface methodology (RSM) can establish models to optimise the complex extraction process based on mathematical and statistical techniques (Wang *et al.,* 2018; Xu *et al.,* 2018; Zhai *et al.,* 2018; Zhang *et al.,* 2018). The most significant feature of RSM is that it can reduce the number of

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experimental trials on the premise that the optimal result can be guaranteed (Nair *et al.,* 2014). Therefore, the objective of the present work was to analyse the effects of extraction conditions on water and ethanol extracts of *H. erinaceus* and their antioxidant properties based on RSM.

Materials and methods

Material preparation

Hericium erinaceus (Houza 19) were cultured in low-pressure polyethylene plastic bags (15 × 55 cm) by using cottonseed hull culture medium that was sterilised and cooled naturally. The mycelia and fruiting bodies were cultured at 26 ± 1 and 18 ± 1°C, respectively. The humidity during the fruiting stage was about 90%. When the thorn was about 0.5 cm, fresh *H. erinaceus* fruiting bodies were gathered, sorted, rinsed in cold running sterilised water, and drained. The mushrooms were lyophilised for 72 h by a vacuum freeze-drier (SCIENTZ-10N, Xinzhi, China), and the dry tissues were grinded into powder by a grinder (DFT300, Dingli, China). Next, the ground tissues were filtered with a 60-mesh sieve to obtain uniform-sized powder.

Preparation of ethanol and water extracts

The powder (2 g) was subjected to extractions under the conditions with prescribed factors and levels. The liquid was collected by centrifugation (8,000 rpm, 15 min) to obtain the crude extracts of water (WE) and ethanol (EE).

Experimental design and optimisation

The single-factor experiments were performed in advance to determine the range of the extraction levels. The RSM was used to determine the effects of the variables on the contents of proteins, polyphenols, and flavonoids, and the antioxidant activities of the crude extracts. The Box-Behnken Design (BBD) in the Design Expert software (version 8.0.6, Stat-Easel, Inc. Minneapolis, USA) was utilised to identify the optimum levels of factors. The factors and levels were solid-liquid ratio, temperature, time, and ethanol concentration (Table 1). The BBD designs comprising of 17 (WE) and 29 (EE) experimental runs were respectively done in a random order to minimise the effects of extraneous factors. The factorial points and centre points were used to estimate the repeatability of the method. The response functions were contents of proteins, polyphenols and flavonoids, and four antioxidant activities of crude extracts. The quadratic model was used to process the data. In BBD, the higher *F*-values and lower *p*-values ($p < 0.05$) represented the significant coefficient. The models were evaluated by ANOVA analysis. The adjusted determination coefficient value ($R^2_{adj} > 0.8$) and the *p*-value for lack-of-fit ($p > 0.05$) also indicated that the model was significant. The calculated data were confirmed by three independent experiments to determine the validity of the quadratic model.

Table 1. Factors and levels of the water and ethanol extractions.

Factor	Mark	Level		
Solid-liquid ratio	A	1:20	1:25	1:30
Temperature (°C)	B	40	50	60
Time (h)	C	1.5	2	2.5
Ethanol concentration (%)	D	40	50	60

Polyphenol, flavonoid and protein content of ingredients in extracts

The total polyphenol content was determined by the Folin-Denis method with gallic acid as standard (Park *et al.,* 2017). In brief, 2 mL of standard gallic acid solution with different concentrations or the samples were mixed with 1 mL of Folin-phenol solution. Then, 1 mL of 7.5% sodium carbonate solution and 6 mL of distilled water were introduced after 5 min. The mixture was shaken well, and incubated in the dark for 90 min at room temperature. The absorbance was measured at 725 nm. Distilled water served as control. The amount of total polyphenol was calculated from the standard curve.

Total flavonoid was determined by aluminium chloride method, and quercetin was used as standard (Shameh *et al.,* 2018). Briefly, 2 mL of standard quercetin acid solution with different concentrations or the samples were mixed with 2 mL of 2% aluminium chloride solution. The mixture was shaken well, and allowed to react for 20 min at room temperature. The absorbance was measured at 415 nm with the distilled water as control. The amount of total flavonoid was calculated from the standard curve.

Protein was measured by Bradford method (Bradford, 1976). The experimental procedures were done according to the kit instructions (Jiancheng, Nanjing, China). In short, 0.05 mL of standard albumin solution or the samples were mixed with 3 mL Coomassie Brilliant Blue G-250 dye solution. After 10 min, the absorbance was measured at 595 nm. In the control, the sample was replaced by water. The concentration of protein was calculated using Eq. 1:

$$\text{Concentration of protein (g/L)} = (A_s - A_0) \times C / (A_c - A_0) \quad \text{(Eq. 1)}$$

where, A_c, A_s, and A_0 = absorbance of standard albumin, sample, and control, respectively; C = concentration of the standard albumin solution.

All experiments were carried out in triplicate and the data were averaged.

Reducing power

Reducing power was determined following the method of Jiang *et al.* (2014a). In brief, reaction mixture containing 2.5 mL of samples was incubated with 2.5 mL of $K_3Fe(CN)_6$ (1%, w/v) at 50°C for 20 min. The reaction was stopped by adding 10% trichloroacetic acid. The mixture was centrifuged at 1,500 *g* for 10 min. The supernatant was gently mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (0.1%, w/v). The absorbance was measured at 700 nm against a blank.

DPPH (1-diphenyl-2-picrylhydrazyl) radical scavenging activity

Radical scavenging activity was measured following the method of Negro *et al.* (2003). A 0.2 mM solution of DPPH in methanol was prepared freshly. The extract solutions (2 mL) were fully mixed with 2 mL of DPPH solution, and incubated for 30 min in the dark. Distilled water served as blank. The absorbance was measured at 517 nm. The scavenging rate was calculated using Eq. 2:

$$\text{Scavenging rate} = (A_c - A_s) \times 100\% / A_c \quad \text{(Eq. 2)}$$

where, Ac and A_s = absorbance of the control and the sample, respectively.

Superoxide anion radical scavenging activity

Superoxide anion radical scavenging activity was determined by Superoxide Anion Free Radical Detection Kit (Spectrophotometry) (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The xanthine oxidase could react on xanthine to produce superoxide anion radicals, which reduce the NBT (nitroblue tetrazolium) to become a colourful substance. It was detected by spectrophotometry at 550 nm. The 0.05 mL extracts were used in reactions according to the kit instructions. The scavenging ability was calculated using Eq. 3:

$$\text{Scavenging ability (U/gprot)} = [(A_{control} - A_{extracts}) / (A_{control} - A_{standard})] \times B \times 1{,}000 / C \quad \text{(Eq. 3)}$$

where, B = concentration of vitamin C (0.15 mg/mL), and C (gprot/L) = protein concentration of the extracts. In this equation, protein concentration was considered as a comparative baseline to adjust different degrees of fragmentation of different samples.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was detected by salicylic acid method with slight modifications (Zhong *et al.,* 2010). The reaction mixture contained 1 mL of $FeSO_4$ (9.1 mM), 1 mL of salicylic acid (9.1 mM), and 1 mL of the sample. Next, 1 mL of H_2O_2 (8.8 mM) was added to the mixture to initiate the reaction. Following incubation at 37°C for 60 min, the reaction mixture was centrifuged at 14,000 *g* for 6 min. The absorbance of the solution was measured at 510 nm. The control solution contained all reagents except water in place of the sample. The scavenging rate was calculated using Eq. 4:

$$\text{Scavenging rate (\%)} = [A_{control} - (A_{sampl} - A_0)] \times 100\% / A_{control}. \quad \text{(Eq. 4)}$$

where, A_0 = absorbance for background (no H_2O_2).

Statistical analysis

The data were analysed by SPSS statistics software (Version 18, IBM Corporation, New York, USA) for data normalisation, correspondence analysis (COA), and correlation analysis. Clustering analysis was carried out by MultiExperiment Viewer software (Howe *et al.,* 2010) by using Euclidean distance method. COA described the relationship between variables in a contingency table in a low-dimensional space. Correlation analysis determined the relationship between extraction conditions and the responses. The Office and Photoshop software (CS2, Adobe Systems Incorporated, San Jose, California, USA) were used to draw the figures.

Results

Based on the preliminary experiments of single-factor design, 17 and 29 BBD experiments were carried out to confirm the optimum extraction conditions by water and ethanol, respectively. The responses were contents of polyphenols, flavonoids, and proteins, reducing power, and scavenging activities of hydroxyl, superoxide anion, and DPPH radicals.

RSM optimums of WE

As shown in Table 2, the reducing power

Table 2. RSM optimum results of WE and antioxidant activities.

Response	Equation	Significant term	R^2_{adj}	Optimum condition			Max (theoretical)	Max (experimental)
				A	B	C		
Reducing power	$2.11-0.227A+0.0219B+1.16C-6.25e^{-6}AB+5.28e^{-3}AC -4.88e^{-3}BC+3.92e^{-3}A^2-1.39e^{-4}B^2-0.293C^2$	A, B, C, BC, A^2, C^2	0.968	20.07	50.38	1.93	0.761	0.801 ± 0.05
Hydroxyl radical	$-97.1+5.74A+4.34B+15.0C+0.0236AB+0.702AC +0.0775BC-0.178A^2-0.0512B^2-8.77C^2$	A, AC, A^2, B^2, C^2	0.930	23.35	49.30	2.01	91.83%	87.56 ± 4.1%
Superoxide anion radical	$7052.375-10.35A-283.663B-414.75C+2.46AB -20.6AC+2.55BC-0.695A^2+2.36B^2+263.5C^2$	A, B, C, AB, AC, BC, A^2, B^2, C^2	0.999	29.91	59.95	2.13	1,310.03 U/gprot	1,500.7 ± 51.2 U/gprot
DPPH radical	$221.55+1.049A-7.141B+15.451C+0.0247AB -0.101AC+0.137BC-0.0392A^2+0.0643B^2-4.702C^2$	B, B^2	0.871	29.46	60.00	2.18	87.24%	85.19 ± 5.96%
Polyphenol	$973.8+560.535A-277.085B+1132.747C-0.0113AB -68.487AC+29.573BC-7.55A^2+1.751B^2-164.67C^2$	A, B, C, AC, BC, A^2, B^2, C^2	0.998	27.17	40.13	1.94	2,586.09 μg/gDW	2,468.03 ± 46.89 μg/gDW
Flavonoid	$56.263+8.516A-7.21B+31.512C-0.0158AB -0.858AC+0.688BC-0.113A^2+0.0734B^2-11.159C^2$	A, B, BC, A^2, B^2, C^2	0.946	24.74	60.00	2.31	65.45 μg/gDW	72.35 ± 2.82 μg/gDW
Protein	$-8.364-0.434A+0.987B-0.558C-7.550e^{-3}AB +0.056AC-0.045BC+0.0136A^2-8.462e^{-3}B^2+0.325C^2$	B, B^2	0.866	29.12	43.86	2.46	7.60 mg/gDW	8.07 ± 0.21 mg/gDW

A = solid-liquid ratio, B = temperature, C = time, RSM = response surface methodology, and WE = water extract.

was significantly influenced by solid-liquid ratio, extraction temperature, and time; 0.761 (theoretical), and 0.801 ± 0.05 (experimental). Hydroxyl radical scavenging activity was significantly influenced by solid-liquid ratio; 87.56 ± 4.1% (theoretical), and 91.83% (experimental). The superoxide anion radical scavenging activity was significantly influenced by almost all factors and their interactions; 1,310.03 U/gprot (theoretical), and 1,500.7 ± 51.2 U/gprot (experimental). DPPH radical scavenging activity was significantly influenced by extraction temperature; 87.24% (theoretical), and 85.19 ± 5.96% (experimental). Polyphenol contents were significantly influenced by almost all single factors and interaction effects; 2,586.09 µg/gDW (theoretical), and 2,468.03 ± 46.89 µg/gDW (experimental). The yield of flavonoid was significantly influenced by solid-liquid ratio and extraction temperature; 65.45 µg/gDW (theoretical) and 72.35 ± 2.82 µg/gDW (experimental). Proteins were significantly influenced by extraction temperature; 7.60 mg/gDW (theoretical), and 8.07 ± 0.21 mg/gDW (experimental).

The optimal extraction condition was also calculated to maximise all responses simultaneously by Design-Expert software. The results showed that under the calculated optimal condition (1:24.76, 40.00°C, and 1.91 h), the reducing power was 0.572, and scavenging activities of hydroxyl, superoxide anion, and DPPH radicals were 86.62%, 614.95 U/gprot, and 82.89%, respectively. The experimental means of yields of polyphenols, flavonoids, and proteins were 2,513.04 µg/gDW, 42.72 µg/gDW, and 6.99 mg/gDW, respectively. The experimental means of reducing power, quenching activities of hydroxyl, superoxide anion, and DPPH radicals were 0.588, 84.24%, 647.12 U/gprot, and 82.98%, respectively. The experimental means of yields of polyphenols, flavonoids, and proteins were 2,596.41 µg/gDW, 47.47 µg/gDW, and 6.27 mg/gDW, respectively.

RSM optimums of EE

As shown in Table 3, the reducing power was significantly influenced by solid-liquid ratio and extraction time; 0.751 (theoretical), and 0.783 ± 0.07 (experimental). Hydroxyl radical scavenging activity was significantly influenced by solid-liquid ratio, temperature, and ethanol concentration; 88.06% (theoretical), and 84.67 ± 2.04% (experimental). Superoxide anion radical scavenging activity was significantly influenced by almost all factors and their interactions; 1,613.22 U/gprot (theoretical), 1,478.09 ± 118.2 U/gprot (experimental). DPPH radical scavenging activity was significantly influenced by solid-liquid ratio, time, and ethanol concentration; 92.15% (theoretical), and 87.35 ± 3.15% (experimental). Polyphenol contents were significantly influenced by almost all single factors and interaction effects; 2,295.06 µg/gDW (theoretical), and 2,364.87 ± 187.9 µg/gDW (experimental). The yield of flavonoids was significantly influenced by the four single factors and most interactions; 94.08 µg/gDW (theoretically), and 106.54 ± 6.18 µg/gDW (experimental). Proteins were significantly influenced by solid-liquid ratio and alcohol concentration; 6.63 mg/gDW (theoretical), and 7.30 ± 0.42 mg/gDW (experimental).

Under the calculated optimal condition (1:30, 60.00°C, 1.50 h, and 58.33%), the reducing power was 0.542, and the scavenging activities of hydroxyl, superoxide anion, and DPPH radicals were 39.45%, 1088.07 U/gprot, and 89.13%, respectively. The contents of polyphenols, flavonoids, and proteins were 2,304.21 µg/gDW, 62.20 µg/gDW, and 6.31 mg/gDW, respectively. The experimental means of reducing power, scavenging activities of hydroxyl, superoxide anion, and DPPH radicals were 0.528, 35.24%, 987.12 U/gprot, and 85.28%, respectively. The experimental means of polyphenol, flavonoid, and protein were 2,366.31 µg/gDW, 64.24 µg/gDW, and 5.99 mg/gDW, respectively, which were very close to the theoretical values.

Clustering analysis

Hierarchical clustering, which is displayed by a heat map, seeks to build a hierarchy dendrogram of cluster data by similarity. Each cluster is distinct from the other cluster, and the objects within each cluster are similar to each other.

As shown in Figure 1A, run 1, 2, 11, 14, and 16 of WE were clustered into one group since they were zero points with same conditions. Run 6, 10, 12, and 17 were gathered since they had the same extraction temperature. As for the responses, they were divided into two groups. The reducing power and scavenging activity of hydroxyl radicals were pairwise coupled, and flavonoid and protein contents were independently clustered. They formed the first group. In the other group, scavenging activities of DPPH, superoxide anion, and polyphenol were gathered together.

In the analyses of EE (Figure 1B), run 1, 17, 23, 27, and 28 were clustered at one branch because they had the same conditions. One group (run 3, 4, 9, 12, 18, and 22) and the zero-point groups made up a big branch. The responses belonged to three subgroups. The first one included reducing power and scavenging activity of hydroxyl radicals. The

Table 3. RSM optimum results of EE and antioxidant activities.

Response	Equation	Significant term	R^2_{adj}	Optimum condition				Max (theoretical)	Max (experimental)
				A	B	C	D		
Reducing power	$2.133-0.117A-0.0325B-0.392C+0.0513D+2.11e^{-3}AB$ $+0.0262AC-1.053e-3AD-2.5e^{-3}BC-3.138e^{-4}BD-4.025e^{-3}CD$	A, C, AB, AC, AD, BD	0.903	20.56	44.37	1.62	52.14	0.751	0.783 ± 0.07
Hydroxyl radical	$805.129-12.398A-7.595B-55.898C-11.271D+0.0838AB$ $+0.419AC+0.0805AD+0.221BC+0.0125BD-1.116CD$ $-6.531e^{-3}A^2+0.0399B^2+23.061C^2+0.0991D^2$	A, B, D, AB, CD, B^2, C^2, D^2	0.941	20.34	41.31	2.27	43.35	88.06%	84.67 ± 2.04%
Superoxide anion radical	$-649.779-478.006A+250.202B-538.005C+36.078D$ $-1.881AB+5.466AC-0.0845AD-60.309BC-0.998BD$ $+11.548CD+12.637A^2-0.388B^2+764.298C^2-0.215D^2$	A, B, C, D, AB, AC, BC, BD, CD, A^2, B^2, C^2, D^2	0.999	29.96	46.70	2.45	40.99	1613.22 U/gprot	1478.09 ± 118.2 U/gprot
DPPH radical	$287.399-4.416A-4.073B-2.009C-1.944D+2.539e^{-3}AB$ $+0.0463AC+0.0232AD-0.174BC-4.780e^{-4}BD+0.189CD$ $+0.0638A^2+0.0436B^2+0.986C^2+9.518e^{-3}D^2$	A, C, D, AD, BC, CD, A^2, B^2, D^2	0.941	21.23	40.01	2.43	40.93	92.15%	87.35 ± 3.15%
Polyphenol	$4340.095-3.285A-5.160B+476.507C-160.621D$ $+0.448AB+25.119AC+1.818AD-3.209BC+0.671BD$ $-9.292CD-2.660A^2-0.194B^2-114.336C^2+1.179D^2$	A, B, C, D, AB, AC, AD, BC, BD, CD, A^2, B^2, C^2, D^2	0.998	26.25	59.06	1.96	59.80	2,295.06 µg/gDW	2,364.87 ± 187.9 µg/gDW
Flavonoid	$-2399.653+49.889A+19.156B+549.514C+30.980D$ $-0.271AB-7.781AC+0.0931AD-0.616BC+0.0608BD$ $-0.0144CD-0.457A^2-0.134B^2-82.240C^2-0.358D^2$	A, B, C, D, AB, AC, AD, BD, A^2, B^2, C^2, D^2	0.978	29.24	50.06	1.82	50.73	94.08 µg/gDW	106.54 ± 6.18 µg/gDW
Protein	$22.015-0.417A-0.0633B+1.943C-0.675D+6.50e^{-4}AB$ $-0.194AC+9.90e^{-3}AD-0.0225BC+3.375e^{-3}BD+0.109CD$ $+6.858e^{-3}A^2-8.229e^{-4}B^2-0.382C^2+1.658e^{-3}D^2$	A, D, AC, AD, CD	0.852	30	60	1.73	59.99	6.63 mg/gDW	7.30 ± 0.42 mg/gDW

A = solid-liquid ratio, B = temperature, C = time, D = ethanol concentration, RSM = response surface methodology, and EE = ethanol extract.

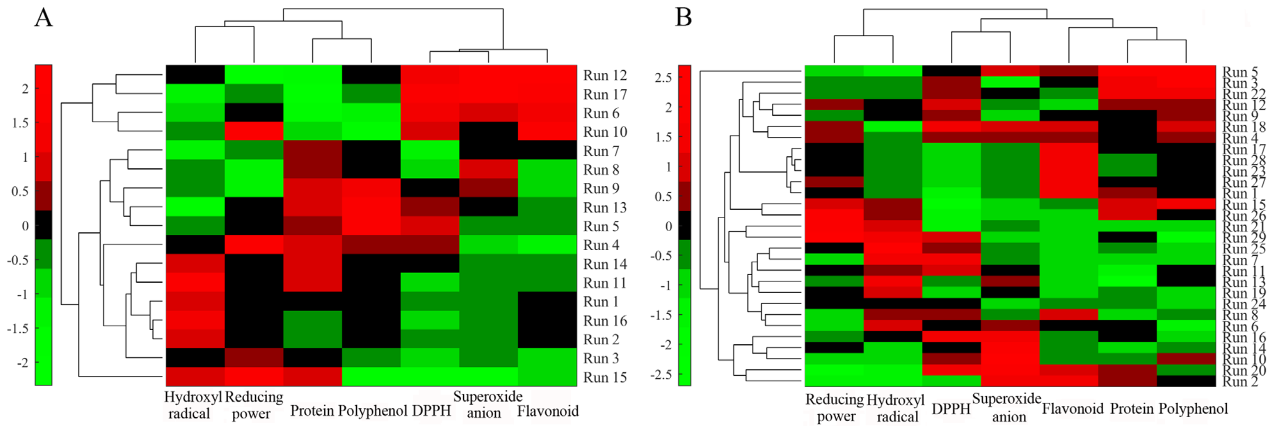

Figure 1. Heatmap and clustering analysis. (A) WE (water extracts) experiments, and (B) EE (ethanol extracts) experiments. Each column represents the results of each response. The maximum values in each column are indicated by red colour, whereas the minimum values are indicated by green colour.

second consisted of scavenging activities of DPPH and superoxide anion. The third group consisted of protein, polyphenol and flavonoid contents.

COA analysis

Correspondence analysis was employed to analyse all the variables in a visualisation plot. Cronbach's Alpha values of WE and EE were 0.964 and 0.927, respectively, which implied that the COA analyses were credible. For each variable, fewer distances between category points indicated closer relationships.

The WE result (Figure 2A) shows that the solid-liquid ratio, extraction time, and scavenging activities of superoxide anion were in the first quadrant. The polyphenol and scavenging activities of hydroxyl radicals were in the second quadrant. The protein and reducing power were in the third quadrant. Temperature, flavonoid, and scavenging activities of DPPH radical were in the fourth quadrant. Moreover, they were in accord with the RSM results. Further vector analyses showed that the impact of extraction temperature on flavonoid was the greatest. The polyphenol was negatively related to temperature.

In COA analysis of EE (Figure 2B), scavenging activity of hydroxyl radical and reducing power were in the first quadrant. Ethanol concentration, temperature, protein, and polyphenol were in the second quadrant. Solid-liquid ratio, extraction time, flavonoid, and scavenging activities of superoxide anion and DPPH radicals were in the third quadrant. Meanwhile the fourth quadrant was vacant.

Canonical correlation analysis

As far as the WE were concerned (Figures 3A, 3B, 3C), all the canonical correlations among extraction conditions, antioxidant activities, and material contents were higher than 0.800 ($p < 0.05$),

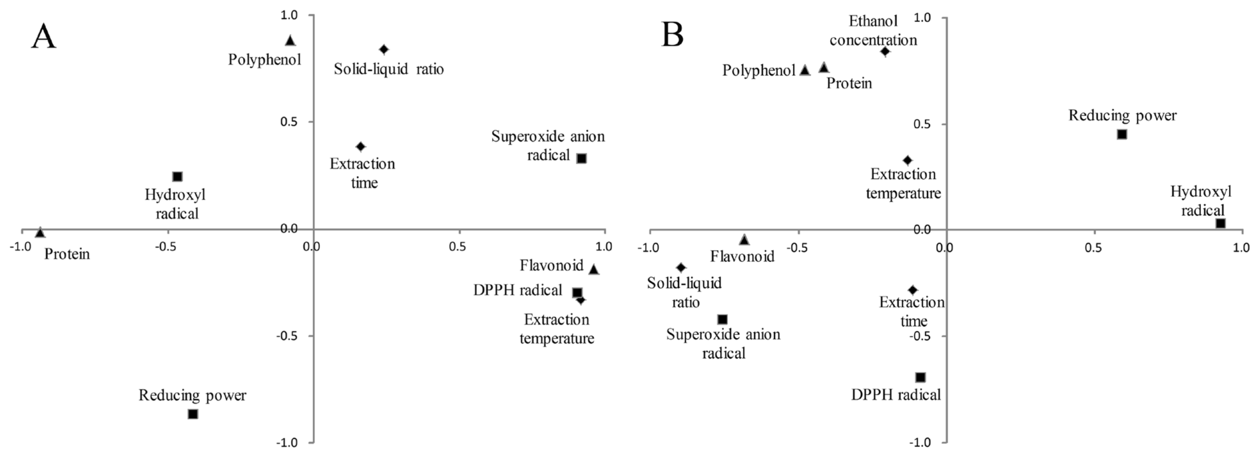

Figure 2. COA analysis. (A) WE (water extracts) experiments, and (B) EE (ethanol extracts) experiments. The extraction conditions are marked by rhombus (♦), substance contents are marked by triangle (▲), and antioxidant activities are marked by square (■).

which implied that they were significantly correlated. The canonical loading of extraction temperature (Figure 3A) was 0.970 (p < 0.05), which was far higher than solid-liquid ratio (-0.195) and extraction time (-0.147). This demonstrated that the contents of the material were positively and strongly dependent on the temperature. The canonical loadings of reducing power and hydroxyl radical scavenging activity were positive, and DPPH and superoxide anion radical scavenging activity were negative (Figure 3B). The absolute values of canonical loadings of reducing power and superoxide anion radical scavenging activity were very high, which indicated that they were important in the antioxidant activities canonical variable. As shown in Figure 3C, the absolute values of canonical loadings of some antioxidant abilities and material contents were bigger than 0.6, which implied that they occupied a greater role proportion in their respective canonical variables. All results were in agreement with the RSM and COA analyses.

The canonical correlations of EE (Figures 3D, 3E, 3F) among extraction conditions, antioxidant activities, and material contents were high (over 0.900, p < 0.05), which meant that they were significantly correlated. Most canonical loadings of the variables in Figure 3D were the minus sign, which indicated that they were negatively related. The higher absolute values of the loadings meant that this variable was the principal contributor to the canonical correlation variable. In short, ethanol concentration and polyphenol contents were the bigger contributors to their corresponding canonical variable. As shown in Figure 3E, the canonical loadings of reducing power and hydroxyl radical scavenging activity were positive values, and those of DPPH and superoxide anion radical scavenging activity were negative values. The absolute value of canonical loading of hydroxyl radical scavenging activity was very high, which demonstrated that it was the dominating influencing factor on the canonical antioxidant activities variable. Solid-liquid ratio was the crucial indicator for canonical extraction conditions because the canonical loading was -0.918. The results (Figure 3F) implied that the absolute values of some canonical loadings such as flavonoid content and hydroxyl radical scavenging activity were bigger than 0.8, which in turn implied that they played important roles in their respective canonical variables.

Discussion

Hericium erinaceus is a mushroom frequently used in the Chinese traditional medicine, and capable of treating clinical diseases especially gastrointestinal disorders, by fortifying the function of the spleen and stomach. Many modern pharmaceutical studies have verified that metabolic compounds in *H. erinaceus* such as flavonoids and polyphenols are the potential bioactive compositions (Jiang *et al.,* 2014b). The RSM method has been implemented to optimise the extraction factors, which could influence the yields and activities of the

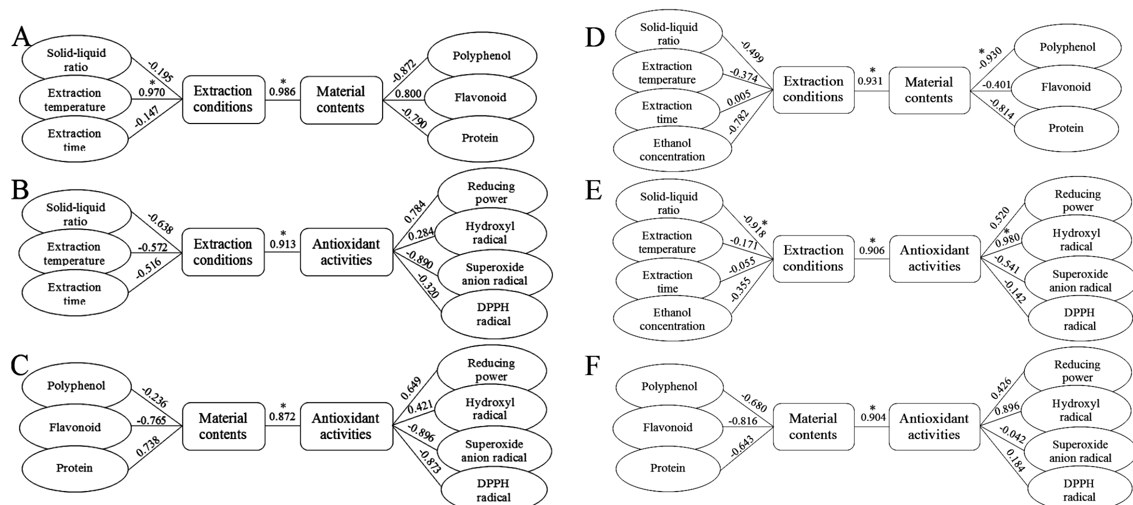

Figure 3. Canonical correlation coefficient chart of WE (water extracts) and EE (ethanol extracts) experiments. (A) and (D) canonical correlation between extraction conditions and material contents of WE and EE. (B) and (E) canonical correlation between extraction conditions and the antioxidant activities of WE and EE. (C) and (F) canonical correlation between materials contents and antioxidant abilities of WE and EE. The first canonical correlation variables are encircled with double elliptical rings. The specific factors are encircled with single oval rings. The numbers above the lines mean the canonical correlation coefficients. The asterisk represents significant correlation (p < 0.05).

natural products in different conditions. In the present work, antioxidant activities of the crude extract of *H. erinaceus* by water and ethanol were determined by RSM. Furthermore, the contents of polyphenols, proteins, and flavonoids in the crude extracts were also determined to investigate their relationships with the observed antioxidant activities.

In the 14 responses, 10 were dramatically influenced by extraction temperatures. The temperature had two effects on the extraction course. Within certain temperature ranges, more components would be extracted with the temperature power-up. However, when the temperature was too high, the structure of some active substances would be damaged, accompanied by the decrease of bioactivity. In RSM combinatorial optimisation, most optimal temperatures were in the range of 40 to 50°C (low- and medium levels). As for the solid-liquid ratio, 10 responses were significantly affected by this factor. Small solid-liquid ratio could lead to insufficient extraction, whereas too large a ratio could exert a dilution effect. Further analysis showed that most responses opted for high solid-liquid ratio (1:25 - 1:30), probably due to the fact that high solid-liquid ratio could efficiently resolve the solubility and contents of the bioactive compounds. In the ethanol extraction trials, six of seven responses were affected by ethanol concentration. When the ethanol concentration was high, polar molecules were still included in the water phase, thus resulting in a decrease of extraction efficiency. Meanwhile, the activities based on the polar molecules would accordingly be influenced. Therefore, the optimal ethanol concentrations were around 40 - 50% in most responses. To obtain set of conditions that could optimise all the responses, theoretical calculations and experimental tests were carried out. The result showed that results obtained from the co-optimisation method were all lower than the individual optimisation, which meant that different response could be influenced by different extraction conditions. In another word, it was hard to maximise all responses simultaneously under any set of conditions.

The reducing power, scavenging activity of hydroxyl radical, polyphenol contents, and protein contents of WE were a little higher than those of EE. The other three responses were mainly affected by ethanol. The observed antioxidant activities were all dependent on the constituents of the extract. Polyphenols and flavonoids, secondary metabolites with aromatic ring, have high structural similarity. Further vector analysis of COA showed that flavonoids might be the main contributor to the scavenging activity of DPPH and superoxide anion; meanwhile proteins and polyphenols might contribute to the reducing power and scavenging of hydroxyl radicals. Mau *et al.* (2002) found that polyphenols were the main ingredient in the methanolic extracts from *H. erinaceus*, and total polyphenols were the major naturally occurring antioxidant components (Mau *et al.,* 2002). This is in agreement with our results. The higher pairwise correlation among extraction conditions, material contents, and antioxidant activities also stated that the extraction conditions determined the chemical compositions and contents of materials, which consequently affected the antioxidant activities. However, the three variables did not strictly coincide with each other because of the complexity of the extracts and different antioxidant mechanisms (Wang *et al.,* 2014). The stimulation with different growth regulators, such as gibberellic acid and chitosan could also improve the production of polyphenol compounds of *H. erinaceus* (Vi *et al.,* 2018). The contents of phenolics and flavonoids in *H. erinaceus* were increased after selenium addition, resulting in superior antioxidant properties of the mushroom (Gąsecka *et al.,* 2016). All results showed that variability of the antioxidant activities might be related to culture conditions, extraction conditions, chemical constitutions, and physicochemical properties of the extracts.

In the present work, four antioxidant activities were selected to study the influences of extraction conditions on the biological functions of WE and EE. In the hierarchical clustering analyses, some responses were often clustered in pairs, such as reducing power and scavenging activity of hydroxyl radicals, scavenging activities of DPPH, and superoxide anion. Similar results were also observed in COA analyses. In COA, reducing power and scavenging activity of hydroxyl radicals had similar abscissa values, whereas scavenging activities of DPPH and superoxide anion exhibited semblable abscissa values. In the canonical analyses, the canonical loadings of the reducing power and scavenging activity of hydroxyl radicals were all positive values, whereas those of the other two activities were always negative values. The similar pattern might be explained by the antioxidant mechanisms. In reducing power, the antioxidants donate electrons, and convert the oxidised form of iron to ferrous. The production of hydroxyl radical also concerned the iron ion in Fenton reaction. As to DPPH radical and superoxide anion, some antioxidants could react directly with the two radicals.

Although *H. erinaceus* has been introduced into various healthcare products and medicines, the exact compounds and accurate mechanisms on diseases amelioration are still unknown. Many people are consuming this mushroom by various means all over the world. However, water and ethanol are the commonly used solutions. The yields and health-promoting functions of the bioactive compounds are dramatically affected by the non-standard household methods. Therefore, the present work would offer them some references. Further in-depth studies on (1) purification and structural identification of the exact bioactive compounds, (2) quantitative structure-activity relationship between bioactivities and chemical structures, and (3) biological mechanisms for ameliorating diseases are warranted.

Conclusion

The effects of each extraction factor have been investigated. Extraction temperature and solid-liquid ratio were the dominant factors. Flavonoid was related to the scavenging of DPPH and superoxide anion radicals. Meanwhile protein and polyphenol acted on reducing power and scavenging of hydroxyl radicals. The antioxidant activities might be influenced by the extraction conditions and chemical constitutions of the extracts. Following these results, further research of the extracts is underway to identify the important bioactive compounds for potential nutraceutical and pharmaceutical use.

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