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Original Article

Optimizing the extraction technology of antioxidant substances from *Daldinia concentrica* by orthogonal experiments

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Abstract

This work aimed to optimize the extraction technology of antioxidant substances from *Daldinia concentrica* by orthogonal experiments. Using ultrasonic assisted extraction process, the effects of ethanol concentration, solid-liquid ratio, and extraction time on scavenging rate of DPPH radical were inspected. According to the result of single-factor experiment and the orthogonal design method, a $L_{16}(4^3)$ orthogonal table was used to study the ultrasonic extraction technology of antioxidant active components from the *Daldinia concentrica*. Based on the analysis on the significance of each level and their interactions, the optimum extraction conditions were as follows: extraction time was 30 min, ethanol concentration was 100% and solid-liquid ratio was 3:100 (concentration was 30 $mg \cdot mL^{-1}$). Under these conditions, the DPPH antioxidant activity of the extract was the highest (76.17%). The extraction process could provide theoretical and a practical reference for the development of antioxidant substances in *Daldinia concentrica.*

Keywords: antioxidant activity, *Daldinia concentrica*, extraction technology, orthogonal test

1. Introduction

Daldinia concentrica is a kind of mushroom that belongs to the genus Daldinia and family Xylariaceae. It has been found in Americas, Europe and Asia (Singh, 1994) and mainly lives in the surface of dead and rotting wood (Figure 1), endangers trees and construction timber, especially impacts the cultivation of some economic mushrooms such as *auricularia auricula*-judae, *shiitake* mushroom, and *ganoderma lucidum* (He, 2011). However, in recent years, the intensive research of this mushroom found that it had extensive pharmacological activities. Its volatile oil had anti-fungal effect of plant pathogens (Luo, Tang, Yang, Wang, & Liu, 2007), coumarin and sterols had anti-cancer and anti-bacterial effects (Liarzi, Bar, Lewinsohn, & Ezra, 2016). In particular, Concentricolide, a benzofurolide compound purified from this mushroom also had anti-HIV activity (Qin *et al.*, 2006). These studies indicated this mushroom may have potential medical development value.

Figure 1. Wild ascocarp of *Daldinia concentrica* on the trunk and cross-section of the ascocarp.

Many researchers have found that excessive amounts of reactive oxygen species would damage the body, result in cellular DNA damage and induce heart disease, cancer, and aging (Fan, 2017). Antioxidants are important substances which protect the body from oxidative damage caused by free radicals. Therefore, the discovery of anti-

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oxidants, especially from natural sources, has gained increasing attention in science and industry for the past few years (Mukthapura, Shimogga, K, Shetty, & Rao, 2010). Our research group found that *Daldinia concentrica* had strong antioxidant activity in the series work of a preliminary screen of natural antioxidants. This experiment adopted orthogonal experiment to optimize the extraction process of antioxidant active ingredient in *Daldinia concentrica*, which would provide a theoretical basis for following separation work and application development of this mushroom.

2. Materials and Methods

2.1 Mushroom sample

The fruits of *Daldinia concentrica* were collected from Mianyang in Sichuan province in 2016 (Figure 1). It was authenticated by one author of this article, Xin-sheng He, a professor of mycology. The certificate specimens were kept in the Microbiology Laboratory of Life science and engineering department, Southwest University of Science and Technology. The fruit body was cleaned by pure water, and dried at 50℃ to constant weight. The dried sample was crushed through a 50 mesh screen, protected from light, and stored dry. The entire extraction optimization experiment was completed within one month.

2.2 Chemicals

DPPH (1,1-diphenyl-2-picrylhydrazide) was purchased from TCI (Shanghai, China) Chemical Industry Development Co., Ltd. Ethanol was purchased from Kelong Chemical Reagent Factory (Chengdu, China). The above reagents were analytical grade. Pure water was made in the laboratory (electrical resistivity 18 MΩ·cm).

2.3 Equipment

Visible-light spectrophotometer (7200), Unico (Shanghai) Instrument Co., Ltd.; Micro plant grinding machine, Shanghai Deyuan Jiu Chinese Medicine Machinery Plant; Vortex mixer (XH-C), Jintan Tianchen Experimental Instrument Factory; Benchtop centrifuge (D1008E), US Scilogex Co., Ltd.; Ultrasonic cleaner (KQ-200KDE), Kunshan Ultrasonic Instrument Co., Ltd.; Electronic balance (BSA 124S), Germany Sartorius Co., Ltd.

2.4 Sample extraction

Ultrasound-assisted extraction (power 200 W) was used in this experiment. 500 mg of *Daldinia concentrica* powder and 10 mL ethanol were added in Erlenmeyer flasks. The bottle was sealed and the weight was recorded. After ultrasonic extraction for 10 minutes, the original solvent was replenished according to the recorded weight, the extract was centrifuged at 15,000 g for one minute, and the supernatant was taken for DPPH antioxidant activity detection. Three sets of samples were prepared in parallel.

2.5 Detection of DPPH antioxidant activity

The antioxidant activity of the extract was detected by the DPPH free radical scavenging rate using Pyrzynska's method (Pyrzynska & Pekal, 2013) with a slight modification. 200 μL sample solution was added into 4 mL DPPH solution (30 $mg \cdot mL^{-1}$, dissolved by ethanol and the absorbance at 517 nm of this solution is near to 0.8) in a test tube. After vortex and mix, the tube was centrifuged for 1 min at 15,000 g. The supernatant was transferred to a cuvette and the absorbance at 517 nm was measured at room temperature. This value was accompanied by a decrease in the amount of free radical scavenger (Li & Cui, 2011), and the antioxidant capacity of the sample could be expressed as the Scavenging rate (SR, %). The equation was as follows:

$$\text{Free radical scavenging rate} = (1-(A_i - A_j)/A_0) \times 100\%$$

with A_i: the absorbance value of mixing 0.2mL test solution and 4mL DPPH solution; A_j: the absorbance value of mixing 0.2 mL test solution and 4 mL absolute ethanol; and A_0: the absorbance value of mixing the solvent used in the preparation of the test solution and 4 mL DPPH solution.

2.6 Single factor test

The factors which influence the extraction effect mainly included ethanol concentration, temperature, time, and solid-liquid ratio. According to a preliminary investigation, the extraction temperature had little effect on the antioxidant activity. Hence, the remaining three factors, ethanol concentration (100%, 60%, 50%, 40% and 0%), extraction time (5 min, 30 min, 60 min, 90 min, and 120 min) and the solid-liquid ratio (3:100 (30 mg/mL), 9:100 (90 mg/mL), 15:100 (150 mg/mL) and 30:100(300mg/mL)) were selected for single factor experiment. In order to ensure the accuracy of the solid-liquid ratios determination, the solutions with different ratio were diluted to the same concentration. All above experiments were simultaneously prepared three groups.

2.7 Orthogonal test design

On the basis of the results of single factor test, four levels close to the maximum scavenging rate were selected. The DPPH radical scavenging rate was used as the index, and a three-factor and four-level orthogonal table L_{16} (4^3) (Table 1) was used to optimize the extraction process of antioxidant active constituents from the *Daldinia concentrica*.

Table 1. Factors and levels in the $L_{16}(4^3)$ orthogonal test.

Level	Factor		
	(A) Time (min)	(B) Ethanol concentration (%)	(C) Solid-liquid ratio (mg/mL)
1	30	100	3:100 (30)
2	60	60	6:100 (60)
3	90	50	9:100 (90)
4	120	40	12:100 (120)

3. Results and Discussion

3.1 Effect of ethanol concentration on antioxidant activity

As can be observed from Figure 2, both absolute ethanol and pure water can't obtain the maximum extraction effect of antioxidants in this mushroom. With the increase of ethanol concentration, the DPPH free radical scavenging rate gradually increased. When the ethanol concentration was 60%, scavenging rate reached the maximum, and the antioxidant activity of the extract was the strongest.

The results in Figure 3 showed that the DPPH free radical scavenging rate increased with the extension of extraction time (5 min~90 min). After 90 minutes, the growth of scavenging rate was unobvious and declined slightly over time. This result indicated that the extraction time was of great help in extracting the antioxidant ingredient, but the excessive time did not increase the extraction effect and was adverse to the cost of industrial production.

Figure 4 showed DPPH free radical scavenging rate increased along with the rise of solid-liquid ratio at 3:100~ 9:100 (30 mg/mL~90 mg/mL). When the solid-liquid ratio exceeded 9:100 (90 mg/mL), the scavenging rate turned down. It indicated that it's difficult to extract the antioxidant active ingredient from *Daldinia concentrica* when the solid-liquid ratio is excessively increased. According to Figure 4, the optimum solid-liquid ratio is 9:100(90 mg/mL).

3.2 Orthogonal experiment

The orthogonal test and extreme difference analysis were implemented by statistical software SPSS 20.0. The values of K and R were calculated and the results were listed in Table 2. The R value indicated that the degree of influence of three factors was $R_B > R_C > R_A$, mean B (ethanol concentration) >C (solid-liquid ratio) >A (extraction time). Based on the sort of the K values, the optimal condition was $A_1B_1C_1$, the ultrasonic extraction time was 30 min, the ethanol concentration was 100%, and the solid-liquid ratio was 3:100 (30 mg/mL). The significance of each variable was determined based on a p-value of < 0.05, and the results are summarized in Table 3, along with an analysis of variance (ANOVA) results. Three factors were all significant factors for the extraction of antioxidant substances ($p < 0.05$).

According to the verification experiment with the optimal extraction conditions, the DPPH free radical scavenging rate of the extract from *Daldinia concentrica* was 76.87% which was close to the highest DPPH free radical scavenging rate of Table 2 (76.17%). It had proved that the orthogonal test results had an obvious optimization effect on the extraction of antioxidant active ingredient from *Daldinia concentrica.*

4. Conclusions

Adequate mushroom species around the world are a great source of natural product development (Boa, 1998). Due to the interaction between antioxidant activity and the treatment of many diseases, the discovery of antioxidant active compounds from mushroom should become a hot spot in science and industry.

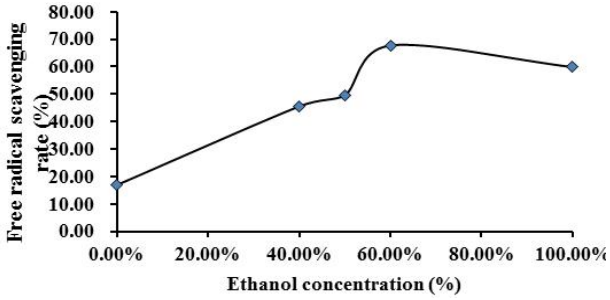

Figure 2. Effect of ethanol concentrations on the antioxidative activity.

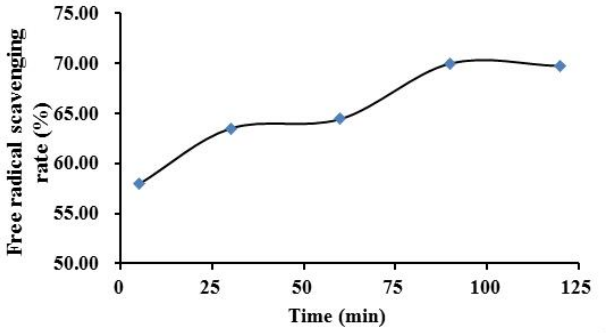

Figure 3. Effect of extraction time on the antioxidative activity.

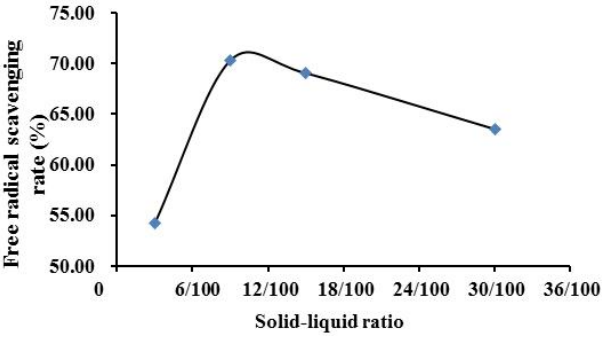

Figure 4. Effect of solid-liquid ratio on the antioxidative activity.

In mushroom, common antioxidant active ingredients are polyphenols, terphenyls, polyketides, alkaloids, terpenes, steroids, and polysaccharides (Li, Wu, Dang, & Li, 2014). Latest literature research shows that the chemical components found in *Daldinia concentrica* include sesquiterpenes, squalene triterpenes and ergosterols (Chang *et al.*, 2014; Liu, 2008; M *et al.*, 2001; Ngoc *et al.*, 2002; & Qin, Shao, Dong), but there are very few reports on the antioxidant activity of these compounds, like isoindolinone derivative Daldinan A, which was separated by Lee from this mushroom in 2015 (Lee *et al.*, 2012). In consequence, what type of compounds are the material basis of the antioxidant activity in this mushroom is unclear.

In this paper, the optimal extraction conditions for extracting the active antioxidant components from *Daldinia concentrica* were obtained by a single factor experiment and L_{16} (4^3) orthogonal experiment: extraction time was 30 min,

Table 2. The result of the orthogonal test and extreme difference analysis.

Number	Time (min)	Ethanol concentration (%)	Solid-liquid ratio (mg/mL)	Free radical scavenging rate (%) ± Standard deviation
1	30	100	3:100(30)	76.17±2.21
2	30	60	6:100(60)	70.44±0.35
3	30	50	9:100(90)	64.47±1.84
4	30	40	12:100(120)	64.69±0.33
5	60	100	6:100(60)	72.27±0.30
6	60	60	3:100(30)	72.64±0.61
7	60	50	12:100(120)	65.52±0.74
8	60	40	9:100(90)	62.31±1.14
9	90	100	9:100(90)	66.60±1.04
10	90	60	12:100(120)	65.35±0.56
11	90	50	3:100(30)	66.89±0.58
12	90	40	6:100(60)	63.54±0.28
13	120	100	12:100(120)	66.27±0.03
14	120	60	9:100(90)	65.23±0.60
15	120	50	6:100(60)	65.27±1.55
16	120	40	3:100(30)	62.48±1.89
∑K1	68.942	70.328	69.545	
∑K2	66.185	68.415	67.880	
∑K3	65.595	65.537	64.653	
∑K4	64.813	63.255	65.457	
R	4.129	7.073	4.892	

Table 3. Results of variance analysis.

Variance factor	Deviation sum of squares	Degrees of freedom	Estimator of variance	F ratio	Signifcant
Time (min)	47.531	3	15.844	11.119	**
Ethanol concentration (%)	116.737	3	38.912	27.308	**
Solid-liquid ratio (mg/mL)	60.350	3	20.117	14.117	**
Error	8.550	6	1.425		

*F ratio >F 0.05 (3,6) =4.76, **F ratio>F 0.01 (3,6) = 9.78

ethanol concentration was 100% and solid-liquid ratio was 3:100 (30 mg/mL). Among them, the concentration of ethanol had the greatest impact on the extraction of antioxidant active ingredients. The above experimental results established a good foundation for the separation process of the antioxidant active ingredient and food and health care product development from this mushroom.

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