Aqueous Two-phase Systems with Ultrasonic Extraction Used for Extracting Phenolic Compounds from *Inonotus obliquus*

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Abstract: **Objective** To optimize the extracting technology of assessing the maximum yield of phenolic compounds (PC) from *Inonotus obliquus* by single factor experiments and orthogonal array design methods through aqueous two-phase systems combined with ultrasonic extraction. **Methods** The range of the independent variables, namely levels of acetone and ammonium sulfate, and ultrasonic time were identified by a first set of single factor experiments. The actual values of the independent variables coded at four levels and three factors were selected based on the results of the single factor experiments. Subsequently, the levels of acetone and ammonium sulfate, and ultrasonic time were optimized using the orthogonal array method. **Results** The optimum conditions for the extraction of PC were found to use 7.0 mL acetone, 5.5 mg ammonium sulfate, with ultrasonic time for 5 min. Under these optimized conditions, the experimental maximum yield of PC was 37.8 mg/g, much higher than that of the traditional ultrasonic extraction (UE, 29.0 mg/g). And the PC obtained by this method had stronger anti-oxidative activities than those by traditional UE method. **Conclusion** These results indicate the suitability of the models developed and the success in optimizing the extraction conditions. This is an economical and efficient method for extracting polyphenols from *I. obliquus*.

Key words: aqueous two-phase systems; free radicals; *Inonotus obliquus*; phenolic compounds; ultrasonic extraction
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Introduction

The medicinal fungus *Inonotus obliquus* (Fr.) Pilát has been used as a folk remedy in Russia and Eastern Europe for more than four centuries, where its powerful effects on several human diseases without any unacceptable toxic side effects have been reported (Saar, 1991). This fungus synthesizes a range of phenolic compounds (PC) which possess remarkable potentials for scavenging free radicals (Babitskaya, Shcherba, and Lkonnikova, 2000; Leitner *et al*, 2009). It is believed that these PC were the principles able to reduce the incidence of oxidative stress-induced diseases including cancer, hypertension, neurodegenerative, and auto-immune diseases (Singh *et al*, 2003; Zheng *et al*, 2010).

The investigation has shown that *I. obliquus* is rich in PC, such as naringin, rutin, narirutin, epicatechin gallate, epigallocatechin gallate, narigenin, and phelligridin G (Zheng *et al*, 2009). So far, there are several methods for the extraction of polyphenols. However, almost all the methods have lower extraction ratios. Hence, there is a need for the optimization of efficient and economical extraction methods, in order to achieve higher extraction ratio. One such extraction method that meets all these criteria is aqueous two-phase extraction (ATPE). ATPE in many cases offers a better alternative to existing methods, especially in the early processing stages, with regard to scale of operation, low processing time, and enrichment of the product. In ATPE, selective partitioning of the desired polyphenols to one phase and contaminant to the other phase not only purify the polyphenols but also concentrate them in one of the phases (Srinivas, Barhate, and Raghavarao, 2002; Raghavarao, Guinn, and Todd, 1998; Lin and Huo, 2002). Ultrasonic extraction (UE) is widely used to broke the biological cells and extraction composition from Chinese herbal medicines (Ma *et al*, 2008; Zhao

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et al, 2009). In view of these it was thought to be prudent to use ATPE associated with UE technology for the extraction of polyphenols from mycelia of *I. obliquus*. The main objective of the study is to develop a simple and more efficient technology for the extraction of polyphenols. More emphasis is given to arrive at the optimal process parameters of ATPE associated with UE by considering a case study of polyphenols.

Materials and methods

Reagents and instruments

Ethanol, isopropanol, acetone, ammonium sulfate, sodium sulfate, and sodium carbonate (Sinopharm Chemical Reagent Co., Ltd., China); Pyrogallol, 1,10-phenanthroline, and 2,2-dipenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich); vitamin C (Beijing Biodee Biotechnology Co., Ltd., China).

FLUKO Homogenizer (Shanghai Frug Fluid Machinery Manufacturing Co., Ltd., China); JY92—IIN Ultrasonic Cell Disrupter (Ningbo Scientz Biotechnology Co., Ltd., China); Spectra Max M2 (Molecular Devices of America, USA).

Organism, inoculums preparation, and culture conditions

Inonotus obliquus (Fr.) Pilát was obtained from the fungal culture collection of Key Laboratory for Biotechnology on Medicinal Plants, Jiangsu Normal University and maintained on potato dextrose agar (PDA). For preparing a standardized inoculum, *I. obliquus* was initially grown on PDA medium for 7 d and then transferred with a sterile self-designed cutter to a 500 mL-conical flask containing 200 mL of medium consisting of glucose (2%), peptone (0.35%), yeast extract (2%), KH_2PO_4 (0.01%), and $MgSO_4 \cdot 7H_2O$ (0.05%), followed by incubation on an orbital platform shaker at 26 ℃ and 140 r/min for 14 d.

Extraction and measurement of polyphenols

The fungal mycelia were filtered by filter paper. Mycelia were washed with distilled water for three times to remove the culture medium components. The mycelia were freeze-dried and the sample was then passed through a miller where the sample size exited at 0.25 mm sieve and stored for the tests.

Accurately milled mycelia (50.0 mg) were weighed. The sample was then transferred into a 50 mL tube which contained 20 mL distilled water, salt, and organic solvent, ultrasonic at low temperature, and centrifugation at 4800 r/min for 10 min. The upper solution was taken and brought to volume by 30% ethanol. Levels of polyphenols were measured with the Folin-Ciocalteu method (Singleton and Rossi, 1965). The extraction of polyphenols was generally evaluated based on the extraction ratio of gallic acid / dry weight (mg/g).

Orthogonal tests for extraction optimization

To examine the interactions among parameters and optimize them for polyphenols extraction, $L_{16}(4^3)$ orthogonal test was selected to examine the effects of four levels and three factors on extraction of polyphenols. All the extractions were carried out for three times.

Free radical scavenging activity

Pyrogallol (3 mmol/L) was used for assaying the capacity of scavenging superoxide anion (Wang *et al*, 2004), 1,10-phenanthroline (5 mmol/L) for hydroxyl radical (Jing *et al*, 1996), and DPPH (0.1 mmol/L) for DPPH radical (Wang *et al*, 2004). The capacity for scavenging free radicals was indicated as 50% effective concentration (EC_{50}), which was calculated as previously described (Mortensen *et al*, 1998). Vitamin C was used as positive control.

Statistical analysis

The data acquired in the test were processed using SPSS 10.0 software. The assumptions of analysis of variance were confirmed to be statistically significant when $P < 0.05$. The results were expressed as $\bar{x} \pm s$.

Results

Effect of organic solvent type

Aqueous methanol, ethanol, isopropanol, and acetone were commonly used to extract PC from samples (Uma *et al*, 2010). There was not any salt could form aqueous two-phase system with methanol, because of the chemical structure of methanol (Liu *et al*, 2010). The PC extraction was done in tubes containing 50.0 mg mycelia, 20 mL distilled water, and 12 mL ethanol, isopropanol, or acetone, diffused for 2 h, then added 6.0 g ammonium sulfate with further UE for 5 min at low temperature and centrifugation at 4800 r/min for 10 min. The upper solution was taken and brought to volume by 30% ethanol. The abilities of

different organic solvents in extracting PC were compared by performing Folic-Ciocalteu assay method.

Fig. 1 showed that ethanol, isopropanol, and acetone were capable of extracting PC but acetone was the most effective solvent for extracting PC from *I. obliquus*. Acetone gave the highest yield of PC (20.65 mg/g), followed by ethanol (11.72 mg/g) and isopropanol (15.14 mg/g). Thus, acetone was chosen for the PC extraction from mycelia of *I. obliquus*.

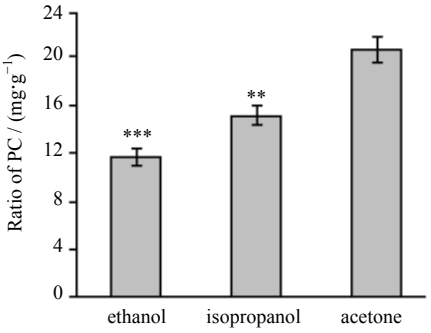

Fig. 1 Effects of organic solvent type on extraction ratio of PC from mycelia of *I. obliquus*

$^{**}P < 0.01$ $^{***}P < 0.001$ *vs* acetone

Effect of acetone level

The usage of acetone was investigated with the range of 4—10 mL. The extraction was done in tubes containing 50.0 mg mycelia, 20 mL distilled water, and acetone, diffused for 2 h, then added 5.0 g ammonium sulfate with further UE for 5 min at low temperature. The extraction procedures were repeated as described. The best level of acetone was chosen according to the highest ratio of PC.

The influence of acetone level on yield of PC is presented in Fig. 2. The PC extraction was at its peak of 7 mL acetone with the ratio of PC showing 24.03 mg/g. After added 8 mL acetone, the yield of PC decreased. Therefore, 6—9 mL acetone was chosen for the optimization of PC extraction from mycelia of *I. obliquus*.

Effect of salt type

Ammonium sulfate, sodium sulfate, sodium carbonate, sodium phosphate, and sodium chloride were used for aqueous two-phase system (Dallora, Klemz, and Filho, 2007; Karakatsanis and Liakopoulou, 2007; Mokhtarani *et al*, 2008). The PC extraction was done in tubes containing 50.0 mg mycelia, 20 mL distilled water, and 8 mL acetone, diffused for 2 h, then added 4.0 g ammonium sulfate, sodium sulfate, and

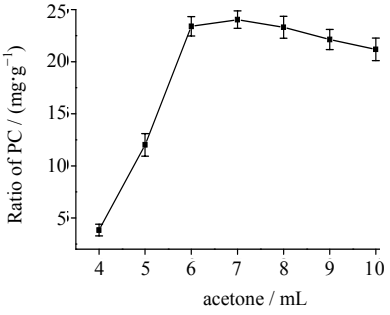

Fig. 2 Effects of acetone level on PC extraction ratio from mycelia of *I. obliquus*

sodium carbonate, respectively, with further UE for 5 min at low temperature. The extraction procedures were repeated as described. The best type of salt was chosen according to the highest yield of PC.

Through adding ammonium sulfate, sodium sulfate, or sodium carbonate into aqueous acetone, aqueous two-phase system could be obtained. But ammonium sulfate was more effective for extracting PC from mycelia of *I. obliquus*. Ammonium sulfate gave the highest ratio of PC (14.25 mg/g) followed by sodium sulfate (12.37 mg/g) and sodium carbonate (10.89 mg/g). We chose ammonium sulfate as the salt to form aqueous two-phase system with acetone, because it is cheap and effective with high solubility at room temperature for the PC extraction from mycelia of *I. obliquus* (Fig. 3).

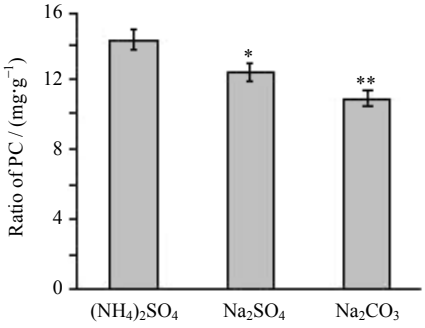

Fig. 3 Effects of salt type on PC extraction ratio from mycelia of *I. obliquus*

$^{*}P < 0.05$ $^{**}P < 0.01$ *vs* $(NH_4)_2SO_4$

Effect of ammonium sulfate level

The PC extraction was done in tubes containing 50.0 mg mycelia, 20 mL distilled water, and 8 mL acetone, diffused for 2 h, then added 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 8.0 g ammonium sulfate, respectively, with further UE for 5 min at low temperature. The extraction procedures were repeated as described. The

best level of ammonium sulfate was chosen according to the highest PC yield.

Fig. 4 showed the effect of ammonium sulfate level on extraction behavior of PC in different phase systems. PC from mycelia of *I. obliquus* extracts increased dramaticly with the ammonium sulfate increasing from 4.0 g (14.25 mg/g) to 5.0 g (23.30 mg/g) after which it began to decrease until it reached a minimum (17.61 mg/g). Thus, the levels of ammonium sulfate were set from 4.5 to 6.0 g for the optimization of PC extraction.

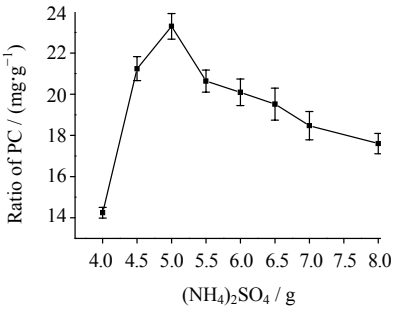

Fig. 4 Effects of ammonium sulfate level on PC extraction ratio from mycelia of *I. obliquus*

Effects of UE time

The impact of UE time on PC was varied from 0, 1, 2, 3, 4, 5, 6, 7, and 8 min. Extraction was accomplished in tubes containing 50.0 mg mycelia, 20 mL distilled water, and 8 mL acetone, diffused for 2 h, then added 5.0 g ammonium sulfate, with further UE at low temperature. The extraction procedures were repeated as described. The best extracting time was chosen according to the highest PC yield.

Fig. 5 presented the PC extraction from *I. obliquus* using various ranges of UE time. The UE time for the highest PC yield in PC extraction was 4 min which was better than at 3 or 5 min. The PC yield at 4 min was 23.38 mg/g. The lowest PC yield at 0 min was 18.58 mg/g. UE time has the significant effect on the PC yield ($P < 0.05$). However, there was no significant difference at the extracting time of 5 to 7 min. Taking from the practical and economical aspects, the extracting time was set from 3 to 6 min.

Orthogonal design

Factor and level assignment including main effects of each factor on the PC extraction are given in Table 1. Each yield for levels was based on the optimized extraction by the single factor test. $L_{16}(4^3)$ orthogonal

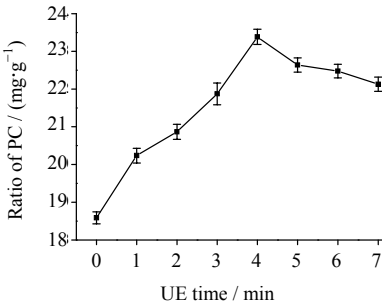

Fig. 5 Effects of UE time on PC yield from mycelia of *I. obliquus*

Table 1 Factors and levels

Levels	Factors		
	Ammonium sulfate / g	Acetone / mL	*t* / min
1	4.5	6.0	3
2	5.0	7.0	4
3	5.5	8.0	5
4	6.0	9.0	6

design was utilized to examine the optimum combination of extraction variables based on the PC yield from mycelia of *I. obliquus*.

Based on $L_{16}(4^3)$ orthogonal design, only 16 tests were carried out in triplicate. The conditions were listed in Table 2, and the PC yield was included in the last column.

The order of affecting factors on PC yield was found to be UE time > acetone > ammonium sulfate. But the maximum PC yield was not recorded with the test parameters of 5.5 mg ammonium sulfate, 7.0 mL acetone, and UE time of 5 min. To confirm the data, a test was carried out using optimal extraction conditions according to Table 2. The measured PC yield (37.8 mg/g) was higher than the extraction yield in Table 2.

The conditions of traditional ultrasonic treatment were 50.0 mg mycelia, 15 mL 80% acetone, ultrasound in ice bath for 5 min, quiet place overnight at 4 ℃, centrifugation at 4800 r/min for 10 min, and repeat for three times (Zhao *et al*, 2009). Extraction yield [(29.0 ± 0.71) mg/g] was lower than the ratio (37.8 mg/g) by aqueous two-phase systems with UE.

Free radical scavenging activity

The PC extraction from mycelia of *I. obliquus* by aqueous two-phase systems combined with UE and traditional UE had the ability of scavenging free radicals. But the scavenging activity against DPPH radical showed the difference ($P < 0.1$), which

presented the striking difference on scavenging capacity against superoxide anion radical ($P < 0.05$). And there was a significant difference between them on scavenging capacity against hydroxyl radical ($P < 0.01$). The PC extraction by aqueous two-phase systems combined with UE had stronger anti-oxidative activities than that by UE method (Fig. 6).

Table 2 $L_{16}(4^3)$ orthogonal design and results

No.	Factors			Extraction yield / ($mg \cdot g^{-1}$)
	Ammonium sulfate / g	Acetone / mL	*t* / min	
1	1	1	1	16.1
2	1	2	2	12.0
3	1	3	3	36.5
4	1	4	4	33.2
5	2	2	3	29.7
6	2	1	4	23.2
7	2	4	1	33.2
8	2	3	2	23.3
9	3	3	4	29.3
10	3	4	3	31.1
11	3	1	2	22.7
12	3	2	1	32.2
13	4	4	2	17.8
14	4	3	1	17.4
15	4	2	4	27.2
16	4	1	3	19.9
T_1	10.77	8.19	10.93	
T_2	11.99	12.85	8.57	
T_3	12.59	11.69	13.83	
T_4	9.97	12.59	11.99	
R	2.62	4.66	5.26	

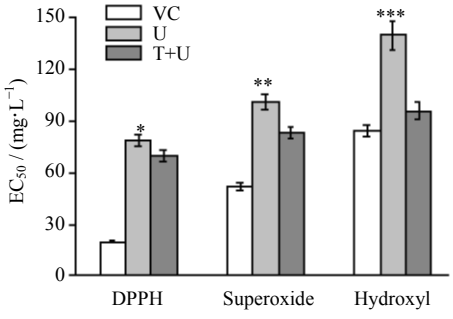

Fig. 6 Scavenging capacities of PC

VC: vitamin C; U: PC by UE;

T+U: PC by aqueous two-phase systems with UE

$^{*}P < 0.05$ $^{**}P < 0.01$ $^{***}P < 0.001$ *vs* scavenging capacities of U (PC by UE) for DPPH, superoxide, and hydroxyl radicals

Discussion

Acetone is an effective solvent for PC extraction from henna leaves or lentil seeds (Uma *et al*, 2010; Alasalvar *et al*, 2006). Through adding ammonium sulfate into aqueous acetone, aqueous two-phase system could be obtained. Polyphenols were allocated to acetone phase and water-soluble ingredients, such as sugar and protein, were distributed in phase of salt in acetone-ammonium sulfate system, which could improve the purity of the products effectively.

Through comparing the contents of PC, the best extraction method has been discussed. The method was successfully employed to optimize the PC extraction conditions. And the results indicated that the acetone level and UE time were the most important affecting factors on the PC yield, followed by the ammonium sulfate level. The optimum operating conditions that maximize the PC extraction were 5.5 mg ammonium sulfate, 7.0 mL acetone, and UE time of 5 min. PC yield under these conditions (37.8 mg/g) was higher than the yield by traditional UE. And PC by aqueous two-phase systems combined with UE had stronger anti-oxidative activities than that by UE method. These indicated the suitability of the models developed and the success in optimizing the extraction conditions. Compared with traditional UE technology, aqueous two-phase system combined with UE could change the chemical equilibrium and extraction process for the active ingredients of dissolving, shorten the extraction time, improve the yield, and obtain the high level of polyphenols. This study also provides a useful reference to the quicker PC extraction. Our further study should be conducted to isolate and purify the extracted PC for the benefit of consumers as medicine.

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Introduction of Cover Photo

Oenothera biennis L. (evening primrose, evening star) is a species of *Oenothera* L. native to eastern and central North America, from Newfoundland west to Alberta, southeast to Florida, and southwest to Texas, and widely naturalized elsewhere in temperate and subtropical regions.

O. biennis has a life span of two years (biennial) growing to 30—150 cm tall. The leaves are lanceolate, 5—20 cm long and 1—2.5 cm broad, produced in a tight rosette the first year, and spirally on a stem the second year.

Blooming lasts from late spring to late summer. The flowers are hermaphrodite, produced on a tall spike and only last until the following noon. They open visibly fast every evening producing an interesting spectacle, hence the name “evening primrose”.

The blooms are yellow, 2.5—5 cm diameter, with four bilobed petals. The flower structure has an invisible to the naked eye bright nectar guide pattern. This pattern is apparent under ultraviolet light and visible to its pollinators, moths, butterflies, and bees.

The fruit is a capsule 2—4 cm long and 4—6 mm broad, containing numerous 1—2 mm long seeds, released when the capsule splits into four sections at maturity.

Provided by ZHOU You

Tonghua Normal University