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Simultaneous extraction of polysaccharides from *Poria cocos* by ultrasonic technique and its inhibitory activities against oxidative injury in rats with cervical cancer

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ABSTRACT

Orthogonal array design (OAD) was applied for the first time to optimize ultrasonic-assisted extraction (UE) of polysaccharides from *Poria cocos* (PCP). An analysis of variance technique was employed as the data analysis strategy in this study. PCP were successfully extracted from *P. cocos* under the optimum UE conditions: 75 min extraction time, 70 mesh particle size, and 90 °C extraction temperature. An analytical method was developed and validated for the determination of chemical components of PCP by high performance liquid chromatography (HPLC). Wistar rats were fed for 40 days with PCP. The total antioxidant status of organs was assayed by monitoring antioxidant enzyme activities. Results indicated that PCP could increased the level of GSH and activities of antioxidant enzymes in Wistar rats. These results confirm the effectiveness of PCP supplementation in detoxifying free radicals that are produced excessively in rats with cervical cancer.

1. Introduction

The sclerotium of *Poria cocos* Wolf, which grows on the roots of pine trees, has long been used as a sedative and diuretic in traditional Chinese herbal medicine (Lee et al., 2004). *Poria cocos* Wolf is used to treat chronic gastritis, edema, nephrosis, gastric atony, acute gastroenteric catarrh, dizziness, nausea, and emesis (Okui et al., 1996). *P. cocos* is commercially available and is popularly used in the formulation of nutraceuticals, tea supplements, cosmetics, and functional foods in Asia. Chemical compounds found in *P. cocos* include triterpenes (Huang, Jin, Zhang, Cheung, & Kennedy, 2007; Tai et al., 1995) and β-pachyman, a polysaccharide composed of β-pachymarose, pachymic acid, and poricoic acid. Polysaccharides isolated from the mycelia of *P. cocos* showed strong antitumor activity against sarcoma 180 and Ehrlich carcinoma implanted subcutaneously (Chen & Chang, 2004) and antioxidant activity (Wu, Ng, & Lin, 2004; Zhou et al., 2008). Polysaccharide isolated from the sclerotium of *P. cocos* with 1% sodium carbonate (PCSC) induced the proliferation of T lymphocytes measured by mixed lymphocyte responses, the antibody production of B lymphocytes, and the secretion of nitric oxide from macrophage cell line, RAW 264.7 cells (Lee et al., 2006).

The bioactivities of polysaccharides are related to their physical and chemical properties, such as the monosaccharide composition, molecular weight, degree of branching, glycosidic linkages, and substituents. In addition, a number of physicochemical properties, such as solubility, primary structure, molecular weight, extent of branching by side-chain substituents (Bohn & BeMiller, 1995), and the charge on the polymer, may influence the biological activities (Vetvicka & Yvin, 2004).

Ultrasonic-assisted extraction is one of the important techniques for extracting the valuable compounds from the vegetal materials (Vilkhu, Mawson, Simons, & Bates, 2008), and it is quite adaptable on a small or large scale (i.e. on a laboratory or industry scale) (Vinatoru, 2001). Compared with other extraction techniques such as microwave-assisted extraction, the ultrasonic device is cheaper and its operation is much easier (Chen et al., 2008; Wang, Cheng, Mao, Fan, & Wu, 2009).

The goals of this study are to optimize the UE condition for extracting polysaccharides from *P. cocos*, compare UE with boiling extraction, and measure the chemical components of the polysaccharides by HPLC method. Then, antioxidant activities of the polysaccharides were also evaluated in rat with cervical cancer.

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2. Materials and methods

2.1. Materials

Poria cocos was purchased from a local herb market. *P. cocos* was dried and grinded into fine powder.

2.2. Extraction of PCP under ultrasonic irradiation

Poria cocos mycelia were defatted with ethyl acetate for 8 h and then acetone for 8 h. *P. cocos* samples (100 g) were then extracted in a sealed flask (1000 ml) containing a volume of extraction solvent according to the experimental design. The flask was equipped with a device which can sense and control the internal pressure, and the pressure was kept lower than 2×10^5 Pa. The extraction was carried out by means of an integrated temperature controller, which precludes harmful overheating of the sample and guarantees process integrity by terminating the ultrasound when the sample temperature reaches a predetermined limit during the processing cycle. After extraction, the crude extracts were centrifuged, filtered, and then analysed by HPLC.

2.3. HPLC analysis

The extracts were analysed with a Hewlett–Packard 1050 series HPLC pump, a Hewlett–Packard 1050 series variable wavelength UV detector, and a Hewlett–Packard 1050 series autosampler using a 4 μm C8 NOVA-PAK Radial Pak column (8 mm × 100 mm) equipped with a C18 guard column (Waters Corporation, Milford, MA, USA). The mobile phase consisted of 15% methanol and 85% deionized water containing 0.005 M PIC A Reagent (Waters Corporation, Milford, MA, USA). The flow rate was 1.5 ml/min. Nicotinic acid was detected at 254 nm and had a retention time of approximately 9 min. The HPLC run time was set to 1 h to remove late eluting peaks. Chromatograms were monitored with a Millennium software package (Waters Corporation, Milford, MA, USA). Peak areas were used in the calculations.

2.4. Optimization of extraction parameters of PCP

Orthogonal array design (OAD) was used to arrange the experiments and optimise the extraction process for PCP. OAD is a type of fractional factorial design in which orthogonal array is used to assign factors to a series of experimental combinations, the results can be analysed using a common mathematical procedure (Liang, 2008). The effects of extraction time, particle size, and extraction temperature on the extraction yield were investigated. A $L9(_3{}^4)$ orthogonal matrix with three factors, each factor containing three levels was selected to arrange the experiments. Extraction times (A) were 65, 75, and 85 min, and particle size (B) were 50, 60, and 70 mesh, and temperature (C) were 80, 90, and 100 °C (Table 1). All the experiments were repeated in triplicate and the extraction yields were average values.

2.5. Animal experiment

Studies were approved by School of Medicine Animal Care and Use Committee of our University. Female Wistar rats weighing 260–300 g were used. The animals were maintained in a temperature-controlled environment (22 ± 1 °C) in a 12:12 h light–dark cycle with standard laboratory chow and tap-water available ad libitum. Cervical cancer in 24 rats was induced according to our previous work (Chen et al., 2009). Another eight rats served as normal control and received no treatment.

Then, the cervical-cancer rats were divided into three groups of eight animals each; model control and two polysaccharides-treated groups. Group I (normal control) were allowed to free access to food and water and orally received a equal volume of saline for 40 days. Group II (model control) were allowed to free access to food and water and orally received a equal volume of saline for 40 days. Groups III and IV were allowed to free access to food and water and orally received polysaccharides for 40 days (150 and 300 mg/kg/day, respectively). At the end of the experimental period, animals were decapitated. Blood was taken from the heart by midline laparotomy. Glutathione (GSH), superoxide dismutase (SOD), glutathione reductase (GR), catalase (CAT), glutathione peroxidase (GPx), and malondialdehyde (MDA) activities in blood were measured. The cervical was removed, cut open by longitudinal incision, rinsed with saline, and stored at −20 °C for subsequent measurement of tissue enzyme activity. CAT, GSH-Px, GR, MDA, and GSH activities were measured from tissue samples.

Table 1
Factor and level.

Level	A (time, min)	B (particle size, mesh)	C (temperature, °C)
1	65	50	80
2	75	60	90
3	85	70	100

2.6. Biochemical analysis

Catalase (CAT) activity was determined by following the decomposition of H_2O_2 measured as a decrease in absorbance at 240 nm (Aebi, 1987). Glutathione reductase (GR) activity was determined by following the decrease in absorbance due to the oxidation of NADPH utilized in the reduction of oxidized glutathione (Goldberg & Spooner, 1987). The determination of glutathione peroxidase (GPx) activity is based on the oxidation of reduced glutathione by GPx coupled to the disappearance of NADPH by GR (Cao & Ikeda, 2009). Superoxide dismutase (SOD) activity was determined by the Oxis commercial kit Bioxytech SOD-525. Enzyme activities are expressed as units per milliliter of serum or milligram of protein, determined by the Bradford method (Bradford, 1976). Lipid peroxidation (LPO) was assessed by measuring the concentration of malondialdehyde, which can be measured at a wavelength of 532 nm by reacting with thiobarbituric acid (TBA) to form a stable chromophoric production (Buege & Aust, 1978). The level of MDA was expressed as nanomoles per milliliter serum or per milligram protein. GSH was measured by the method of Beutler, Duron, and Kelly (1963).

2.7. Statistical analysis

All the grouped data were statistically evaluated with SPSS/10 software. Hypothesis testing methods included one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test. *P* values of less than 0.05 were considered to indicate statistical significance. All these results were expressed as means ± SD for eight animals in each group.

3. Result and discussion

3.1. Effect of extraction factors on extraction yield of PCP

Effect of extraction time on extraction yield was showed in Fig. 1A. With increasing extraction time from 15 to 85 min, the extraction yield increased from low to high till at 75 min to maximum. Results suggest that the longer the extraction time, the higher the extraction yield obtained for PCP. The yield of extracted PCP no longer increased after 75 min of extraction. Therefore, it seems

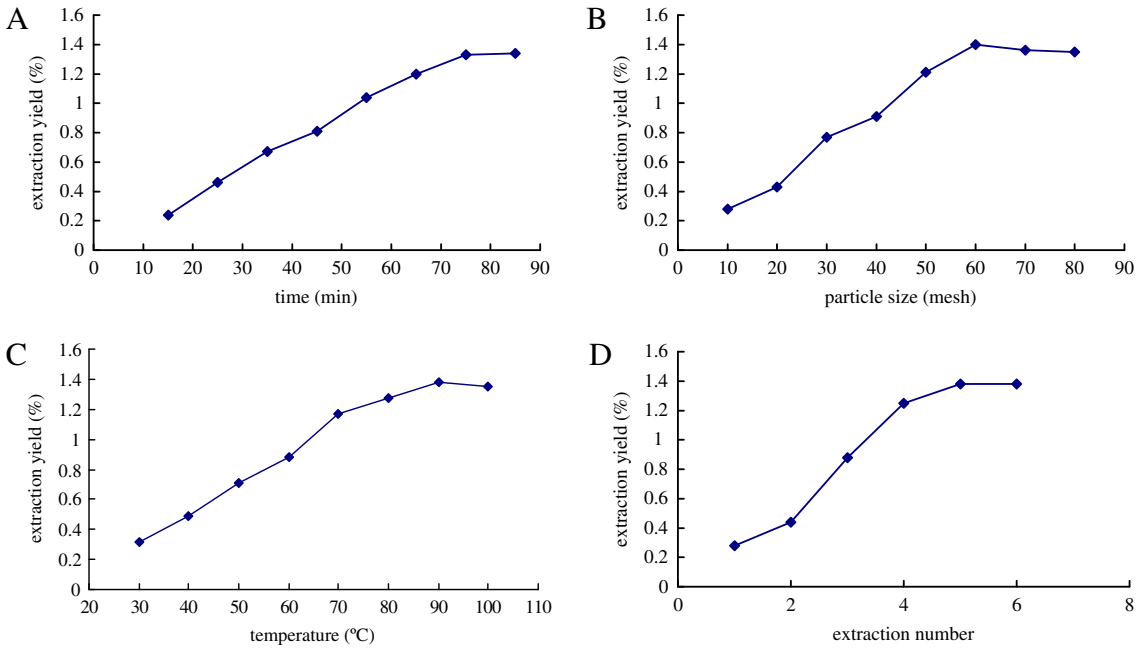

Fig. 1. (A) Effect of extraction time on extraction yield of PCP; (B) effect of particle size on extraction yield of PCP; (C) effect of extraction temperature on extraction yield of PCP; (D) effect of extraction number on extraction yield of PCP.

that the equilibrium was reached after this extraction time. The equilibrium time determines the maximum amount of polysaccharides (Cai, Gu, & Tang, 2008).

Effect of particle size on extraction yield was showed in Fig. 1B. In most cases the effect of particle size was examined in the literature by fractionation of the crushed raw material followed by extraction of the fractions of different size ranges separately (Wang, Luo, & Ena, 2007). As expected, the extraction yield increased with increasing extraction time and decreasing particle size; this means that an increase in the time and in the surface area available for molecular transport contribute to a more extensive mass transfer of solutes between phases – according to the general principles underlying Fick's law. The particle size controls the mass transfer kinetics and the access of water to the soluble components. Accordingly, as shown in Fig. 1B, higher extraction efficiencies can be achieved applying smaller particle sizes, which resulted in an increase in mass transfer surface, and in quantity of soluble fraction on this surface. With increasing particle size from 10 to 60 mesh, the extraction yield increased from low to high till at 60 mesh to maximum. Consequently, extraction yield decreased with the decrease of particle size. A possible explanation is that smaller particle sizes increase diffusion resistance.

Experiment with initial extraction temperature ranged from 30 to 100 °C was conducted to investigate its effect on extraction yield. With increasing extraction temperature from 30 to 90 °C, the extraction yield increased from low to high till at 90 °C to maximum (Fig. 1C). These results indicate that temperature had a significant effect on the extraction of polysaccharides from the plant material. As reported by Ishii (1982) polysaccharides are present in the plant matrix at different sites, some of which can be readily extracted whilst others are tightly bound. Therefore, the increase in temperature may have influenced the release of the tightly bound polysaccharides. However, the decrease in PCP content above 90 °C could be due to slight thermal degradation.

Effect of extraction number on extraction yield was showed in Fig. 1D. The extraction yields increased from 0.28% to 1.38% when the extraction number was increased from 1 to 5. It had no significant influence on the extraction yields of the polysaccharides when extraction number was 6.

3.2. Orthogonal analysis

Orthogonal analysis of results of $L_9(_3{}^4)$ was showed in Table 2. The extreme difference (K) was applied to analyse the data and the results indicated the influence (R) of extraction factors on the extraction yield is $R_B > R_A > R_C$. Namely, particle size > extraction time > extraction temperature. The optimal technique would be the combination $B_3A_2C_2$ (1, 2, 3 are levels). The maximum extraction yield of 1.38% was achieved at a extraction time of 75 min, a particle size of 70 mesh, and a extraction temperature of 90 °C.

3.3. Components analysis of PCP

Separation of major components from PCP by a HPLC can be seen in Table 3. Major components were tentatively identified based on co-chromatography and comparison of their electronic absorption spectra with that of authentic standards. Galactose was the major component followed by glucose, fructose, rhamnose, xylose, galactose, and mannose with a molar ratio of 4.8:21.8:9.4:25.3:5.3:19.57. These results are similar to those previously described for PCP. Other minor monosaccharides components including arabinose were detected but not quantified in

Table 2
Result of orthogonal experiment.

No.	A	B	C	Extraction yield (%)
1	1	1	1	0.52
2	1	2	2	0.79
3	1	3	3	1.37
4	2	1	2	1.26
5	2	2	3	1.01
6	2	3	1	0.89
7	3	1	3	0.69
8	3	2	1	1.25
9	3	3	2	1.03
K1	2.68	2.47	2.66	
K2	3.16	3.05	3.08	
K3	2.97	3.29	3.07	
R	0.48	0.82	0.42	

Table 3
Components of PCP.

Components	RT	Percent (%)
Rhamnose	3.207	9.4
Mannose	5.174	19.57
Galactose	6.053	5.3
Glucose	9.066	4.8
Xylose	15.769	25.3
Fructose	16.949	21.8

Table 4
Effect of PCP on thymus and spleen indices.

Group	Thymus	Spleen
I	1.52 ± 0.13	2.17 ± 0.12
II	1.15 ± 0.09[a]	1.72 ± 0.11[a]
III	1.37 ± 0.13[b]	1.99 ± 0.07[b]
IV	1.53 ± 0.08[b]	2.13 ± 0.14[b]

[a] $P < 0.01$, compared with Group I.
[b] $P < 0.01$, compared with Group II.

our efforts as they represented only a minor portion of the total carbohydrate polymers fraction.

3.4. Comparison of UE with other extraction methods

The primary benefit of the ultrasound action was related to shortening of the extraction time, compared to the classical water extraction (Hromádková, Ebringerová, & Valachovi, 1999; Li et al., 2009). Ultrasound has already been shown to have different effects on the extraction yield and the extraction kinetics for different plant materials (Hromádková & Ebringerová, 2003). To further investigate the advantages of UE method, parallel experiments were carried out with different extraction methods. The extraction yield of total PCP obtained from UE, and hydro-extraction (HE) method is compared. UE were found superior compared to HE as they have yielded higher amount of total PCP. The UE for 90 min gave the highest extract rates than the other extraction methods for several hours, and the extraction efficiency was the highest one. The energy consumption of UE is remarkable less than that of other extraction methods. The ultrasonic enhancement of the extraction is attributed to the cell destruction, capillary effects, better solvent penetration, and mass transfer intensification (Hromádková et al., 1999). Therefore, UE is obviously the best method for extract of polysaccharides from *P. cocos* due to its high efficiency.

3.5. Effect of PCP on thymus and spleen indices

We reported the effect of PCP on thymus and spleen index (Table 4). Compared with normal rats (Group I), a significant decrease ($P < 0.01$) in thymus and spleen indices could be found in rats with cervical cancer (Group II). After 40 days of treatment, PCP exerted a dose-dependent increase ($P < 0.01$) in thymus and spleen indices of rats with cervical cancer (Groups III and IV).

3.6. Effect of PCP on MDA and GSH levels

We reported the effect of PCP on MDA and GSH levels in serum and cervical of experiment rats (Table 5). Compared with normal rats (Group I), a significant increase ($P < 0.01$) in MDA level was found in serum and ovaries of rats with cervical cancer (Group II). In addition, a significant decrease ($P < 0.01$) in GSH level was found in serum and cervical of rats with cervical cancer (Group II). After 40 days of treatment, PCP dose-dependently significantly ($P < 0.01$) decreased MDA and increased GSH levels in serum and cervical of rats with cervical cancer (Groups III and IV).

3.7. Effect of PCP on SOD, CAT, GPx, and GR activities

We reported the effect of PCP on SOD, CAT, GPx, and GR activities in serum and cervical of experiment rats (Table 6). Compared with normal rats (Group I), a significant decrease ($P < 0.01$) in SOD, CAT, GPx, and GR activities was found in serum and cervical of rats with cervical cancer (Group II). After 40 days of treatment, PCP dose-dependently significantly ($P < 0.01$) increased SOD, CAT, GPx, and GR activities in serum and cervical of rats with cervical cancer (Groups III and IV).

4. Conclusion

Using the orthogonal array methodology, the optimum set of the independent variables was obtained in order to obtain the desired levels of crude polysaccharides extraction. It could be found that extraction conditions have significant effects on the yield of crude polysaccharides. Optimum conditions (extraction time, 75 min, particle size, 70 mesh, extraction temperature, 90 °C) for the extraction procedure of crude polysaccharides from *P. cocos* were identified. Further analysis (HPLC) indicated that PCP contained galactose, glucose, fucose, rhamnose, xylose, and mannose. The supplementation of PCP to rats with cervical cancer is effective in decreasing the oxidative stress and oxidative injury, by increasing the activities of antioxidant enzymes like SOD and GPx, limiting lipid peroxidation and superoxide anion production. Nevertheless,

Table 5
Effect of PCP on MDA and GSH levels.

Group	MDA		GSH	
	Serum (nmol/ml)	Cervical (nmol/mg)	Serum (mg/l)	Cervical (mg/g)
I	9.27 ± 0.32	13.17 ± 1.04	383.1 ± 21.6	42.71 ± 3.11
II	15.39 ± 0.22[a]	24.05 ± 0.31[a]	205.7 ± 14.8[a]	21.53 ± 1.64[a]
III	12.11 ± 0.94[b]	18.65 ± 0.41[b]	285.9 ± 12.5[b]	31.52 ± 1.77[b]
IV	9.17 ± 0.76[b]	12.47 ± 0.82[b]	367.3 ± 22.4[b]	43.62 ± 3.66[b]

[a] $P < 0.01$, compared with Group I.
[b] $P < 0.01$, compared with Group II.

Table 6
Effect of PCP on SOD, CAT, GPx, and GR activities.

Group	SOD		CAT		GPx		GR	
	Serum (U/ml)	Cervical (U/mg)	Serum (U/ml)	Cervical (U/mg)	Serum (U/ml)	Cervical (U/mg)	Serum (U/ml)	Cervical (U/mg)
I	139.6 ± 11.8	153.5 ± 12.4	14.61 ± 1.05	17.42 ± 1.14	27.43 ± 2.13	19.47 ± 1.14	20.31 ± 1.41	18.69 ± 1.33
II	68.4 ± 3.7[a]	78.4 ± 6.1[a]	6.83 ± 0.43[a]	9.03 ± 0.57[a]	16.29 ± 1.04[a]	10.41 ± 0.89[a]	11.06 ± 0.89[a]	10.72 ± 1.04[a]
III	94.7 ± 7.4[b]	127.4 ± 11.6[b]	9.53 ± 0.55[b]	15.38 ± 1.32[b]	22.55 ± 1.74[b]	14.84 ± 0.73[b]	17.32 ± 1.17[b]	16.03 ± 1.24[b]
IV	133.5 ± 13.6[b]	151.2 ± 12.8[b]	13.21 ± 1.07[b]	18.27 ± 1.19[b]	26.83 ± 2.07[b]	18.36 ± 1.25[b]	21.77 ± 1.54[b]	18.58 ± 1.72[b]

[a] $P < 0.01$, compared with Group I.
[b] $P < 0.01$, compared with Group II.

PCP contains also abundant vitamins and trace elements that could act synergistically with polysaccharides. Thus, in vivo studies need to be further performed with purified polysaccharides.

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