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Optimization of extraction of *Tremella fuciformis* polysaccharides and its antioxidant and antitumour activities in vitro

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ABSTRACT

Optimization of extraction of *Tremella fuciformis* polysaccharides was investigated using response surface methodology in this paper. *T. fuciformis* polysaccharides were extracted using boiling water at different extraction temperatures (80–100 °C), times (3.5–4.5 h) and ratios of solvent to raw material (4–6). The effect of extraction conditions on the yield of *T. fuciformis* polysaccharides were studied using a three-level three-factor Box–Behnken design. The results showed that the highest yield (3.08%) of *T. fuciformis* polysaccharides was reached at extraction temperature 100 °C, extraction time 4.5 h and ratio of solvent to raw material 5 (v/v). In addition, *T. fuciformis* polysaccharides could scavenge superoxide anion and hydroxyl radicals. At last, it could still be found that antitumour activities of *T. fuciformis* polysaccharides increased from 73.4% to 92.1% with increasing concentration of polysaccharides. Pharmacology experiment indicated that *T. fuciformis* polysaccharides was useful to the therapy of free radical injury and cancer diseases.

1. Introduction

Tremella fuciformis, belonging to the order of the Tremellales and the family of the Tremellaceae, has been appreciated as an edible mushroom. It also has been used for medicinal purposes due to its diverse physiological activities such as improving immunodeficiency and preventing senile degradation of microvessels (Cheung, 1996; Reshetnikov, Wasser, Duckman, & Tsukor, 2000).

Many mushroom polysaccharides, such as (1 → 3)-β-D-glucans, are known to possess immunomodulatory characteristics and may contribute to various therapeutic effects, such as antitumour or anti-inflammatory activities (Adachi, Okazaki, Ohno, & Yadomae, 1994; Borchers, Stern, Hackman, Keen, & Gershwin, 1999; Ma, Lin, Feng, & Putheti, 2009). As compared to other mushroom polysaccharides, the (1 → 3)-β-D-glucans and all of the heteroglycans produced by the *Tremella* species consist of a (1 → 3)-α-mannan backbone with small xylose- and glucuronic acid-containing side chains (Slodki, Wickerham, & Bandoni, 1966). All the fractions and subfractions of the polysaccharide from fruiting body of *T. fuciformis* displayed the ability to induce human monocytes to produce interleukines (IL-1 and IL-6) and tumour necrosis factor in vitro (Gao, Berntzen, Jiang, Killie, & Seljejid, 1998; Gao, Killie, Chen, Jiang, & Seljejid, 1997). Together with this immunomodulatory effect, the acidic heteropolysaccharide lowers the cholesterol level in blood serum (Cheung, 1996; Yui, Ogawa, Kakuta, & Misaki, 1995).

Response surface methodology (RSM) is a collection of statistical and mathematical technique useful for developing, improving and optimizing process (Myers & Montgomery, 2002). The principles of the approach are explained by Henika (1982) and Giovanni (1983). It is a designed regression analysis meant to predict the value of a dependent variable based on the controlled values of the independent variables (Meilgaard, Civille, & Carr, 1991; Resurreccion, 1998; He et al., 2009). The use of RSM in the process optimization stage leads to the need for an experimental design, which can generate a lot of samples for consumer evaluation in a short period of time, and thus laboratory level tests are more efficient (Moskowaitz, 1994).

In the present study, RSM was used to determine the optimum extraction conditions (extraction temperature, time and ratio of solvent to raw material) for maximum polysaccharides yield. Then, in vitro antioxidant and antitumour activities of *T. fuciformis* polysaccharides were investigated.

2. Materials and methods

2.1. Extraction of polysaccharides

Dried *T. fuciformis* were commercially available in Xian, China. It was triturated and boiled in distilled water for 4 h at 100 °C. After filtration to remove debris fragments, the filtrate was concentrated in a rotary evaporator. Protein was removed with the Sevag method (Sun, Zhang, Zhang, & Niu, 2010). Then the solution was precipitated with three volumes of 95% ethanol for 24 h at 4 °C. The precipitate was collected by centrifugation to obtain crude polysaccharides.

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Table 1
Variables in Box–Behnken design.

Factor	Levels used, actual (coded)		
	Low (−1)	Medium (0)	High (+1)
A: extraction temperature (°C)	80	90	100
B: extraction time (h)	3.5	4	4.5
C: ratio of solvent to raw material	4	5	6

2.2. Experimental design

The Box–Behnken design optimizes the number of experiments to be carried out to ascertain the possible interactions between the parameters studied and their effects on extraction yield of polysaccharides. Box–Behnken design is a spherical, revolving design; it consists of a central point and the middle points of the edges of the cube circumscribed on the sphere (Evans, 2003).

Box–Behnken design only has three levels (low, medium, and high, coded as −1, 0, and +1) and need fewer experiments; this design is more efficient and easier to arrange and interpret in comparison with others (Bosque-Sendra et al., 2001). Therefore, this statistical technique was used in this study. A total of 20 runs were used to optimize the process parameters namely temperature, time, and ratio of solvent to raw material. The level and code of variables considered in this study are shown in Table 1. The average from two replicated values of each run was taken as dependent variables or response or yield (extraction yield).

The software Design-Expert (Version 6.0.1 1, State-Ease Inc., Minneapolis, USA) was used for the experimental design, data analysis, quadratic model buildings, and graph (three-dimensional response surface and contour) plotting. The responses function (Y) was partitioned into linear, quadratic and interactive components:

$$Y = \beta_0 + \sum_{i=1}^{k} B_i X_1 + \sum_{i=1}^{k} B_{ii} X^2 + \sum_{i>j}^{k} B_{ij} X_i X_j,$$

where β_0 is defined as the constant, B_i the linear coefficient, B_{ii} the quadratic coefficient and B_{ij} the cross-product coefficient. X_i and X_j are the levels of the independent variables while k equals to the number of the tested factors ($k = 3$). The analysis of variance (ANOVA) tables were generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significances of all terms in the polynomial were judged statistically by computing the F-value at a probability (P) of 0.001, 0.01 or 0.05. The regression coefficients were then used to make statistical calculations to generate contour maps from the regression models.

2.3. Superoxide anion scavenging activity

The phenazine-methosulfate (PMS)-NADH method (Robak & Gryglewski, 1998) was used for the generation of O_2^-. The test tubes contained 12 μM PMS, 100 μM NADH, and 100 μM NBT in 0.1 M phosphate buffer (K_2HPO_4–KH_2PO_4) at pH 7.8. After 2 min of incubation at room temperature, 100 μl of HCl (0.1 M) was added to stop the reaction. The spectrophotometric measurement was recorded at 560 nm against blank samples, in the absence of PMS. Different series of concentrations of polysaccharides were added to the test tubes before adding PMS.

2.4. OH^- scavenging assay

The scavenging capacity of polysaccharides extract on OH^- was evaluated according to the reaction of sodium salicylate and residual hydroxyl radical. OH^- scavenging assay was performed according to a literature procedure (Wang, Gao, Zhou, Cai, & Yao, 2008) with a few modifications. Hydroxyl radicals were generated by Fenton reaction in the system of $FeSO_4$ and H_2O_2. The reaction mixture was consisted of 0.5 ml $FeSO_4$ (8 mM), 0.8 ml H_2O_2 (6 mM), 0.5 ml distilled water, 1.0 ml of various concentrations polysaccharides extract and 0.2 ml sodium salicylate (20 mM). The total mixture (3.0 ml) was incubated at 37 °C for 1 h and then the absorbance of the mixture was recorded at 562 nm. The scavenging activity was calculated using the following equation:

$$\text{scavenging (or inhibition) rate (\%)} = \left[1 - \frac{A_1 - A_2}{A_0}\right] \times 100 \quad (1)$$

where A_0 is the absorbance of the control (without extract), A_1 the absorbance of the extract addition and A_2 the absorbance without sodium salicylate.

2.5. Evaluation of antitumour effect in vitro

The human Hep G22 cells were obtained from School of Medicine, Shanghai JiaoTong University. For the antitumour effect study, Hep G22 cells were seeded for 12 h in 96-well cell culture dishes (Nunclon Surface, Nalge Nunc International, Roskilde, Denmark). After seeding, the cells were exposed to PBS with varying concentration of *T. fuciformis* polysaccharides for 7 days. The cells were then washed twice with PBS. After 72 h in culture, bromodeoxyuridine uptake was measured to determine viable cell counts with a cell proliferation enzyme-linked immunosorbent assay system (Biotrak, Amersham Life Science Ltd, Little Chalfont, UK) (Magaud, Sargent, & Mason, 1988).

3. Result and discussion

3.1. Fitting of data to the model

Twenty trials were performed to locate optimum conditions for extraction of *T. fuciformis* polysaccharides. The experiments were carried out in random order as required in many design procedures. In the experimental design, the optimum conditions sought were the operating conditions for maximizing percent extraction yield of *T. fuciformis* polysaccharides.

Table 2 shows experimental conditions for batch runs and the results (response) in terms of corresponding percent extraction yield of *T. fuciformis* polysaccharides. RSM was applied to build up an empirical model for modeling percent lactose consumption

Table 2
Experimental design runs in Design-Expert 6.0 and corresponding results.

Run	A	B	C	Y
1	0	0	0	3.11
2	0	−1.68179	0	2.59
3	0	1.681793	0	2.64
4	1	1	−1	2.89
5	0	0	0	3.08
6	−1	1	−1	2.48
7	1	1	1	3.03
8	0	0	1.681793	2.76
9	0	0	0	3.09
10	1	−1	−1	2.87
11	0	0	0	3.1
12	−1	−1	1	2.9
13	1	−1	1	2.88
14	0	0	−1.68179	2.79
15	0	0	0	3.1
16	1.681793	0	0	3.01
17	−1	−1	−1	2.73
18	−1.68179	0	0	2.79
19	−1	1	1	2.9
20	0	0	0	3.04

in terms of the operational parameters of extraction temperature, extraction time and ratio of solvent to raw material. Design-Expert 6.0 (trial version) suggested a quadratic equation for percent *T. fuciformis* polysaccharides, as

$$Y = 3.08372 + 0.075420 \times A + 2.99457\text{E}{-}004 \times B + 0.050491 \times C + 0.052500 \times A \times B - 0.055000 \times A \times C + 0.047500 \times B \times C - 0.046734 \times A^2 - 0.14750 \times B^2 - 0.090929 \times C^2 \quad (2)$$

From Table 3, it was also observed that the linear term of extraction temperature (A) and ratio of solvent to raw material (C) has a large effect on the extraction yield significantly due to the high F-value. The quadratic term of extraction time (B^2) and ratio of solvent to raw material (C^2), F-value <45.67 and 17.36 are more significant than the extraction temperature (A^2), F-value 4.59.

ANOVA results of the quadratic model presented in Table 2 indicated that the model equation sufficiently describes the response surface of extraction rate of polysaccharides. Parameters like F-value, probability > F, lack of fit, R^2 are statically obtained values, which are the measures of how the predicted model fits the experimentally monitored data. It should be noted that the effect of each variable on the response is the combination of coefficients and variable values as well as the contribution of joint effect of variables, which cannot be observed by traditional optimization methods.

The optimum conditions for the response were evaluated as extraction temperature 100 °C, extraction time 4.5 h and ratio of solvent to raw material 5 (v/v). The high value of adjusted R^2 (0.8) indicated that the model fits the observed data well (Table 3).

Fig. 1a shows the changes of extraction yield with varying extraction temperature and extraction time. It is observed that, the extraction yield initially increases when there is an increase in extraction temperature and extraction time. The maximum extraction yield of 3.05% was achieved at extraction temperature 90 °C and extraction time 4.5 h.

Fig. 1b shows the changes of extraction yield with varying extraction temperature and ratio of solvent to raw material. It is observed that, the extraction yield increases when there is an increase in extraction temperature and ratio of solvent to raw material. The maximum extraction yield of 2.98% was achieved at extraction temperature 100 °C and ratio of solvent to raw material 6.

Fig. 1c shows the changes of extraction yield with varying extraction time and ratio of solvent to raw material. It is observed that, the extraction yield initially increases when there is an increase in extraction time and ratio of solvent to raw material. The maximum extraction yield of 3.04% was achieved at extraction time 4.2 h and ratio of solvent to raw material 5.2. Due to further increase in extraction time and ratio of solvent to raw material, a significant decrease in extraction yields (%) occurred at any point along the line.

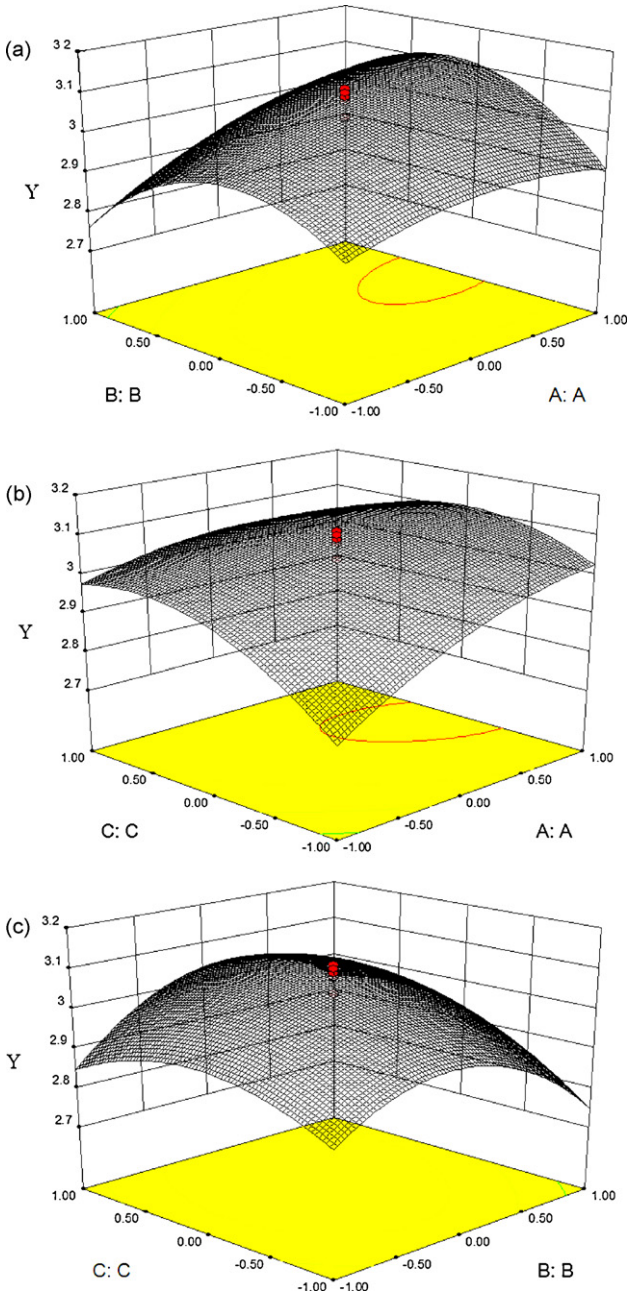

Fig. 1. Response surface plots showing the interaction between variables in the extraction of *Tremella fuciformis* polysaccharides: (a) interaction between extraction temperature and extraction time; (b) interaction between extraction temperature and ratio of solvent to raw material; (c) interaction between extraction time and ratio of solvent to raw material, while keeping other variables at their respective '0' levels.

3.2. O_2^- radical-scavenging activity

Xanthine oxidase catalyzes the oxidation of xanthine to uric acid. During oxidation of xanthine, O_2^- and H_2O_2 are formed (Britigan, Pou, Rosen, Lilleg, & Buettner, 1990; Wang et al., 2009). This enzyme is consequently considered to be an important source of O_2^-. The superoxide anion is a radical which reacts with NO with a high rate constant, generating the oxidant peroxynitrite ($ONOO^-$) (Singh & Evans, 1997; White et al., 1994). At physiological pH, $ONOO^-$ and other reactive nitrogen species have been implicated in the pathophysiology of a variety of diseases including some cardiovascular diseases (Persinger, Poynter, Ckless, & Janssen-Heininger, 2002). *T. fuciformis* polysaccharides exhibited potent scavenging activity for superoxide anion radical. As shown in the figure, O_2^- scavenging activity of the polysaccharides increased from 17.1% to 86.9% with increasing concentration (Fig. 2).

3.3. Hydroxyl radical-scavenging activity

Hydroxyl radical is the most reactive among reactive oxygen species (ROS) and it bears the shortest half-life compared with other ROS. Among the oxygen radicals, hydroxyl radical is the most reactive and induces severe damage to adjacent biomolecules (Sakanaka, Tachibana, & Okada, 2005). The scavenging abilities of polysaccharides extracts on hydroxyl radical inhibition by the 2-

Table 3
ANOVA for the regression model and respective model terms.

Source	Sum of squares	df	Mean square	*F* value	*p*-Value, prob > F
Model	0.584335	9	0.064926	9.458252	0.0008 significant
A–A	0.077682	1	0.077682	11.31646	0.0072
B–B	1.22E-06	1	1.22E−06	0.000178	0.9896
C–C	0.034816	1	0.034816	5.071863	0.0480
AB	0.02205	1	0.02205	3.212181	0.1033
AC	0.0242	1	0.0242	3.525387	0.0899
BC	0.01805	1	0.01805	2.629473	0.1360
A^2	0.031476	1	0.031476	4.585286	0.0579
B^2	0.313523	1	0.313523	45.67315	<0.0001
C^2	0.119152	1	0.119152	17.35779	0.0019
Residual	0.068645	10	0.006864		
Lack of fit	0.065512	5	0.013102	20.90796	0.0023 significant
Pure error	0.003133	5	0.000627		
Cor. total	0.65298	19			

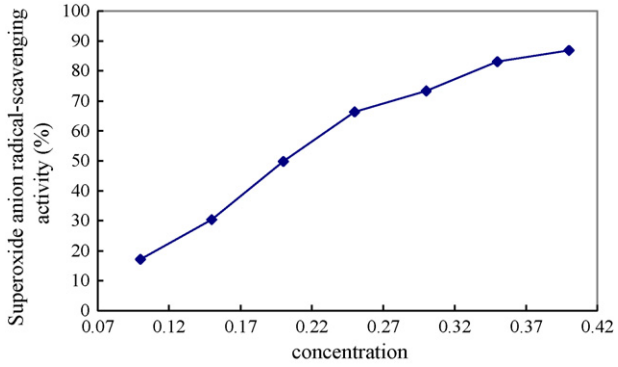

Fig. 2. O_2^- radical-scavenging activity of *Tremella fuciformis* polysaccharides.

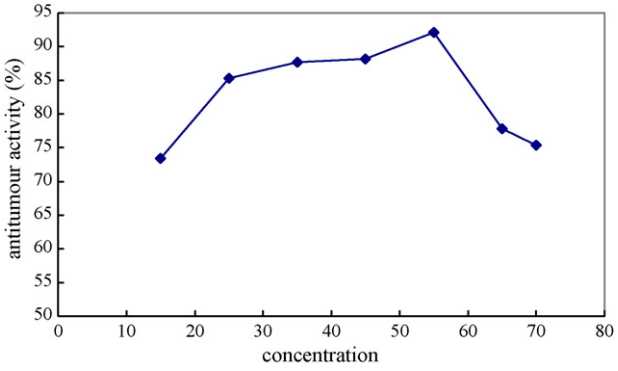

Fig. 4. Antitumour activities of *Tremella fuciformis* polysaccharides.

deoxyribose oxidation method are shown in Fig. 3. The results are indicated as the inhibition rate. *T. fuciformis* polysaccharides showed good hydroxyl radical-scavenging activities (79.8%) at a concentration of 0.2 mg/ml in the reaction mixture. The extract showing hydroxyl radical-scavenging activity was increased with increasing concentration of the extract sample.

3.4. Antitumour activities of T. fuciformis polysaccharides

There have been extensive *in vivo* studies demonstrating the anti-cancer activity of the extracted, purified glucan polysaccharides and polysaccharide peptides in animal models (Wasser & Weis, 1999; Mocanu et al., 2009). There is also increasing experimental evidence that regular incorporation of certain powdered medicinal mushrooms in the diets of animals or topical application of extracts can have a cancer prevention effect and restriction of tumour metastasis (Ikekawa 2001; Vinod et al., 2009). A survey conducted among mushroom workers in the Nagano Prefecture in Japan implied that regular eating of medicinal mushrooms was associated with a much lower death rate from cancer than for other people in the Prefecture (Ikekawa, 2001). Antitumour activities of *T. fuciformis* polysaccharides are shown in Fig. 4. It could be found that antitumour activities of *T. fuciformis* polysaccharides increased from 73.4% to 92.1% with increasing concentration of polysaccharides. However, antitumour activities of *T. fuciformis* polysaccharides no longer increased when concentration of polysaccharides continued to increase.

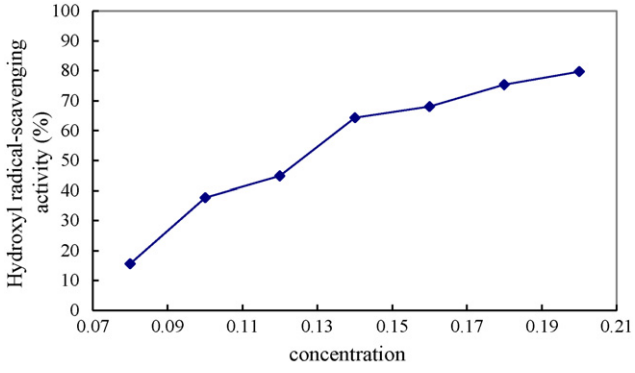

Fig. 3. OH^- radical-scavenging activity of *Tremella fuciformis* polysaccharides.

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