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Extraction optimisation and lipid-lowering activity of *Auricularia heimuer* polysaccharides

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Abstract: Assessments of molecular weight distribution and activity/efficacy of *Auricularia heimuer* polysaccharides (AAP) are of substantial significance for its extraction process optimisation. In the present study, single-factor orthogonal test and response surface methodology were employed to optimise extraction conditions of AAP. Furthermore, a rat hyperlipidaemia model was established to compare the lipid-lowering activity of polysaccharides obtained by three extraction methods. Conditions for enzymatic hydrolysis were optimised as pH 5.0, 1% cellulase, 2.5% substrate concentration and enzymolysis time of 1.5 h, leading to an up to 31.8% polysaccharide yield and 89.13% of polysaccharides within the molecular weight range of 5 000 Da to 10 000 Da. The results of animal experiments showed that the lipid-lowering activity of enzymolysis-extracted polysaccharides was significantly higher than that of water- and ultrasonic-extracted ones ($P < 0.01$). So the present study revealed that enzymatic hydrolysis-extracted polysaccharides showed the strongest hypolipidaemia activity, providing a basis for the development of *A. heimuer*-based functional foods and drugs.

Keywords: hot water extraction; ultrasonic-assisted; cellulase-assisted; molecular weight; HPLC

Hyperlipidaemia (Jain et al. 2007; Chen et al. 2008) leads to cardiovascular and cerebrovascular conditions, such as cerebral infarction, fatty liver, gallstone disease and atherosclerotic coronary heart disease, which is the leading cause of human death worldwide (Zou et al. 2016). So, lots of research focused on the drugs or foods with significant hypolipidaemic activity but without side effects (Chen et al. 2010).

Auricularia heimuer (Yuan et al. 2017) is a well-known medicinal and edible fungus with hypolipidaemic activity (Zeng et al. 2013; Zhang et al. 2017; Ma et al. 2018). *A. heimuer* polysaccharides (AAP) were

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considered to be the main active component of lipid--lowering activity (Li et al. 2020). Zeng et al. (2013) found that AAP-I significantly decreased the levels of total cholesterol (TC), triglyceride (TG), and low-density lipoprotein cholesterol (LDL-C) in mice in which hyperlipidaemia had been induced by a high-fat diet ($P < 0.05$). Therefore, it is significant to study the activity of AAP, which would contribute to the prevention of cardiovascular and cerebrovascular diseases (Zhou and Yu 2005).

In recent years, the relationship between hypoglycaemic activity and the structure of AAP has become a research hotspot (Zhou and Yu 2005; Xiong et al. 2015). Zhang et al. (1995) studied molecular weights of *A. auricula-judae* polysaccharides. The structure, molecular weight and properties may influence the hypolipidaemic activity of AAP. Thus, conducting comparative studies of different extraction methods of AAP with different molecular weights and lipid-lowering activities is of particular importance. At present, the main extraction method of AAP is hot water extraction (Cai et al. 2015), ultrasound treatment (Wang et al. 2019), ultrasound-assisted extraction (Bian et al. 2020), enzymatic extraction (Yue et al. 2019), etc. However, there are few reports on the hypolipidaemic activity of AAP determined by different extraction methods.

In this study, the processes of three extraction methods of AAP were optimised. The molecular weight distribution of AAP was evaluated by high-performance liquid chromatography (HPLC). The hypolipidaemic effects of AAP were also compared in an Sprague-Dawley (SD) rat model. This provides technical support for *A. heimuer* products in the treatment of hyperlipidaemia.

MATERIAL AND METHODS

Material reagents and animals. No. 29 *A. heimuer* powder (Institute of Microbiology, Heilongjiang Academy of Sciences, China) was crushed and sieved through a 160-mesh sieve (China). Cellulase and pectinase were purchased from Changnuo Biotechnology Co., Ltd. (China) and Sunson Biotechnology Co., Ltd. (China), respectively. Cellulase is a group of enzymes that degrade cellulose to produce glucose. Cellulase represents a synergistic action of endo- and exo-glucanases, and beta--glucosidase. Pectinase is an enzyme that breaks down pectin. Pectinase (pectic enzymes) includes pectolyase and polygalacturonase. Positive drugs were simvastatin, Biomol (ShangHai Haoran Biological Technology Co., Ltd., China). The following kits were purchased from Roche (China): TC Kit, TG Kit, High-Density Lipoprotein Cholesterol (HDL-C) Kit, LDL-C Kit.

Male SD rats were provided by Shanghai Slack Laboratory Animal Co., Ltd., Production License No. SCXK (Shanghai) 2012-0002 [Animal Certificate No. 0214188, specific-pathogen free (SPF) grade]. The quality of sterilised ultra-pure water (second level) used for drinking met the requirements of the National Standard GB5749-2006. The license number of the laboratory animal room was SYXK (Zhejiang) 2015-0008. The temperature of 20 °C to 25 °C and relative humidity of 40% to 70% were applied as feeding environment conditions. The animals were allowed to adapt to the environment in the laboratory animal room for six days before conducting the experiment.

The model feed consisted of 20% sucrose, 15% lard, 1.2% cholesterol, 0.2% sodium cholate, 2% casein, 1% calcium phosphate, 0.6% limestone and maintenance feed. The maintenance feed was provided by the Zhejiang Institute for Food and Drug Control (China) in accordance with the implementation standard GB14924.3-2010.

Hot water extraction of AAP. *A. heimuer* powder was soaked in 40 times the volume of distilled water for 2 h. Then, the mixture was subjected to hot water extraction at 80 °C for 2 h (water bath; Shanghai Yiheng Scientific Instrument Co., Ltd, China), centrifuged at 5 000 rpm for 30 min (Micro 17; Thermo Fisher, US), and the supernatant was collected. A volume of the sediment that was diluted in 40 times its volume of distilled water underwent secondary extraction under the same conditions. Furthermore, the supernatants were combined, concentrated and precipitated with alcohol (at three times the volume of 95% ethanol, 4 °C overnight), and finally dried under reduced pressure at 60 °C (vacuum drying oven; Shanghai Yiheng Scientific Instrument Co., Ltd, China) to obtain crude polysaccharides.

Ultrasonic-assisted hot water extraction of AAP. On the basis of the results of the single-factor experiments, ultrasonic power, extraction time and extraction temperature were selected as independent variables, and the polysaccharide yield was used as the dependent variable to optimise the variable combination by the response surface method.

Cellulase-assisted hot water extraction of AAP. Polysaccharides were extracted from *A. heimuer* powder by pectinase hydrolysis (solid-liquid ratio of 1 : 40, pectinase enzyme of 3%, pH of 5.0, temperature of 52 °C, enzymolysis time of 1.5 h), followed by extraction at 80 °C for 2 h and centrifugation. The sediment obtained was dried at 60 °C and crushed to produce the sediment powder at 100-mesh size. The concentrated

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supernatant was hydrolysed (250 mL beaker; Sichuan Shubo Co., Ltd, China) by 1% cellulase (pH of 5.0, enzymolysis time of 1.5 h), concentrated and precipitated by three volumes of 95% ethanol at 4°C for 24 h, and centrifuged. Dried AAP E1 was obtained under reduced pressure (from the concentrated supernatant).

The sediment powder after E1 extraction was subjected to further treatment with cellulase hydrolysis. Enzyme reaction pH, amount of enzyme, substrate concentration and enzymolysis time were selected as experimental factors. In addition, an orthogonal design of L_9 $(3)^4$ was used to determine the optimal conditions for cellulase hydrolysis, by which AAP E2 (from the sediment powder after E1 extraction) was obtained. The total amount of polysaccharide (E), which was prepared by enzymolysis-assisted hot water extraction, was equal to E1 plus E2.

Molecular weight distribution of AAP by HPLC. The filtrate was analysed by HPLC using the following conditions: chromatographic column TSK-Gel G3000--SWXL, analytical column (5 μm, 7.8 mm × 30 cm) and TSKgel G 3000 SWXL pre-column (7 μm, 6.0 mm × 4 cm) (Tosoh, Japan). The mobile phase was 0.1 mol mL^{-1} sodium sulphate of 10 mmol mL^{-1} phosphate buffer (pH 6.8), the velocity of flow was 1 mL min^{-1}. Both the temperature of the column and detector were maintained at 25 °C. The refractive index detector (RID-10A; Waters, US) was used to detect, and GPC software was used for data analysis.

Standard products T10, T40, T70, T500, and T2000 (5 mg mL^{-1}) represent the molecular weight of 10, 40, 70, 500, and 2 000 kDa. According to the retention time (t_R) of the five standards, the linear regression of the standard curve was calculated (R = 0.998). Based on the t_R of a 1 mg mL^{-1} sample, the molecular weight distribution of each sample (the percentage of the total sample) was calculated.

Lipid-lowering activity of AAP by animal experiments. Male SD rats weighing 200 g to 250 g [Production License No. SCXK (Shanghai) 2012-0002] were purchased from Shanghai Slack Laboratory Animal Co., Ltd. (China). These rats were randomly divided (n = 10 rats per group) into the following groups: normal group, model group, positive drug group and three groups in which different extraction methods were used. Except for the animals in the normal group, where only drinking water was given, rats in the other groups were fed a high-fat diet (Nantong Telofi feed, China) three times a day for three weeks. The serum levels of TC, TG, HDL-C and LDL-C in the blood samples collected from rat eyes were determined.

In compliance with the requirements of Attachment 6 of Food and Drug Supervision (2012) No. 107 issued by the State Food and Drug Administration, the model rats were subjected to tests for the lipid-lowering effect of AAP derived by different extraction methods. Except for the normal group, rats in the other groups were continuously fed a high-fat diet daily. The positive drug group (simvastatin) was orally administered at 10 mg kg^{-1} day^{-1} (saltwater dilution and intragastric administration). The polysaccharides obtained by water extraction, enzymolysis and ultrasonic extraction were orally administered [intragastric administration at 250 mg kg^{-1} day^{-1} (AAP dosage) and 20 mL kg^{-1} day^{-1} (dosing volume)] for eight weeks. Rats in the normal group were given purified water. Then, blood samples were collected, and serum TC, TG, LDL-C and HDL-C levels were determined using a C501 automatic biochemical analyser (Roche, Switzerland).

Data processing. The data were expressed as mean ± ± standard deviation ($\overline{x}$ ± SD). Analysis of variance was performed using DAS statistical software 2.0. $P < 0.05$ at the 0.05 level and $P < 0.01$ at the 0.01 level, were considered statistically significant.

RESULTS AND DISCUSSION

AAP by hot water extraction. The yield rate of AAP obtained by hot water extraction was 9.68%. Bao et al. (2020) also successfully isolated *A. auricula* polysaccharide by hot water extraction, which showed that this method was an important method for the extraction of AAP.

AAP by ultrasonic-assisted method. In this study, the response surface methodology design and polysaccharide yield are presented in Table 1. Extraction time (X_1), extraction temperature (X_2) and ultrasonic power (X_3) were selected as independent variables, whereas polysaccharide yield was chosen as the dependent variable for the response surface analysis performed to determine optimum extraction conditions.

By using the Design-Expert software, the second--order polynomial model describing the correlation between polysaccharide yield and the three variables in this study was obtained. The results of analysis of variance (ANOVA) of the regression models are shown in Table 2. The results of the ANOVA revealed that X_1, X_2, X_3, X_1X_3, X_1^2, X_2^2, and X_3^2 had extremely significant impacts ($P < 0.01$) on the yield of AAP. The influencing factors can be arranged in a descending order as follows: extraction temperature (X_2) > extraction time (X_1) > ultrasonic power (X_3). Therefore, extraction

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Table 1. Design and results of response surface method

Test No.	X_1 (h)	X_2 (°C)	X_3 (W)	Yield (%)
1	0 (2)	1 (90)	1 (600)	13.87
2	0	0 (80)	0 (500)	13.93
3	1 (2.5)	0	1	13.88
4	1	−1 (70)	0	11.23
5	0	0	0	14.01
6	0	0	0	14.05
7	1	1	0	14.13
8	0	−1	1	11.25
9	0	1	−1 (400)	13.24
10	0	−1	−1	10.46
11	−1 (1.5)	1	0	12.95
12	1	0	−1	12.14
13	−1	−1	0	10.72
14	0	0	0	13.99
15	−1	0	1	12.27
16	−1	0	−1	12.70
17	0	0	0	13.93

X_1 – extraction time; X_2 – extraction temperature; X_3 – ultrasonic power; X_1, X_2, and X_3 were chosen as independent variables; the yield of polysaccharide was used as dependent variable to do response surface optimisation combination; the extraction time was 1.5, 2.0, and 2.5 h; the extraction temperature was 70, 80, and 90 °C; the ultrasonic power was 400, 500, and 600 W

temperature had the most significant effect on the extraction of polysaccharides.

The model *F*-value of 283.90 indicated that the model was significant ($P < 0.01$). There is only a 0.01% chance that this large model *F*-value could occur due to noise. The R^2 value was 0.9983, showing that 99.83% of changes in the response value came from the selected variables, indicating that the model of the equation was extremely significant.

The response surface maps were shown in Figure 1. The effect of extraction time and extraction temperature is shown by response surface map and contour map, when ultrasonic power was set at 500 W. When extraction time was set, the polysaccharide yield increased when extraction temperature reached approximately 86 °C, and then it declined. When extraction temperature was set, the polysaccharide yield increased when extraction time reached about 2.3 h, and then it declined. The effect of extraction time and ultrasonic power is shown by response surface map and contour map, when extraction temperature was set at 80 °C. When extraction time was set, the polysaccharide yield increased when ultrasonic power reached approximately 560 W, and then it declined. When ultrasonic power was set, the polysaccharide yield increased when extraction time reached about 2.3 h, and then it declined. The effect of extraction temperature and ultrasonic power is shown by response surface map and contour map, when extraction time was

Table 2. ANOVA of the regression models

Sources of variation	Sum of squares	Degree of freedom	Mean square	*F*-value	*P*-value
Model	26.54	9	2.95	283.90	< 0.0001
X_1	0.94	1	0.94	90.35	< 0.0001
X_2	13.86	1	13.86	1 334.45	< 0.0001
X_3	0.93	1	0.93	89.70	< 0.0001
X_1X_2	0.11	1	0.11	10.81	0.0134
X_1X_3	1.18	1	1.18	113.34	< 0.0001
X_2X_3	0.01	1	0.01	0.62	0.4582
X_1^2	1.47	1	1.47	141.59	< 0.0001
X_2^2	5.41	1	5.41	520.85	< 0.0001
X_3^2	1.74	1	1.74	167.87	< 0.0001
Residual	0.07	7	0.01	–	–
Lack of fit	0.06	3	0.02	7.58	0.0398
Pure error	0.01	4	0.0027	–	–
Sum	26.61	16	–	–	–

ANOVA – analysis of variance; X_1 – extraction time; X_2 – extraction temperature; X_3 – ultrasonic power; the results of ANOVA of the regression models were showed; the statistical significance of equation was checked by *F*-test $P < 0.05$ means significant difference

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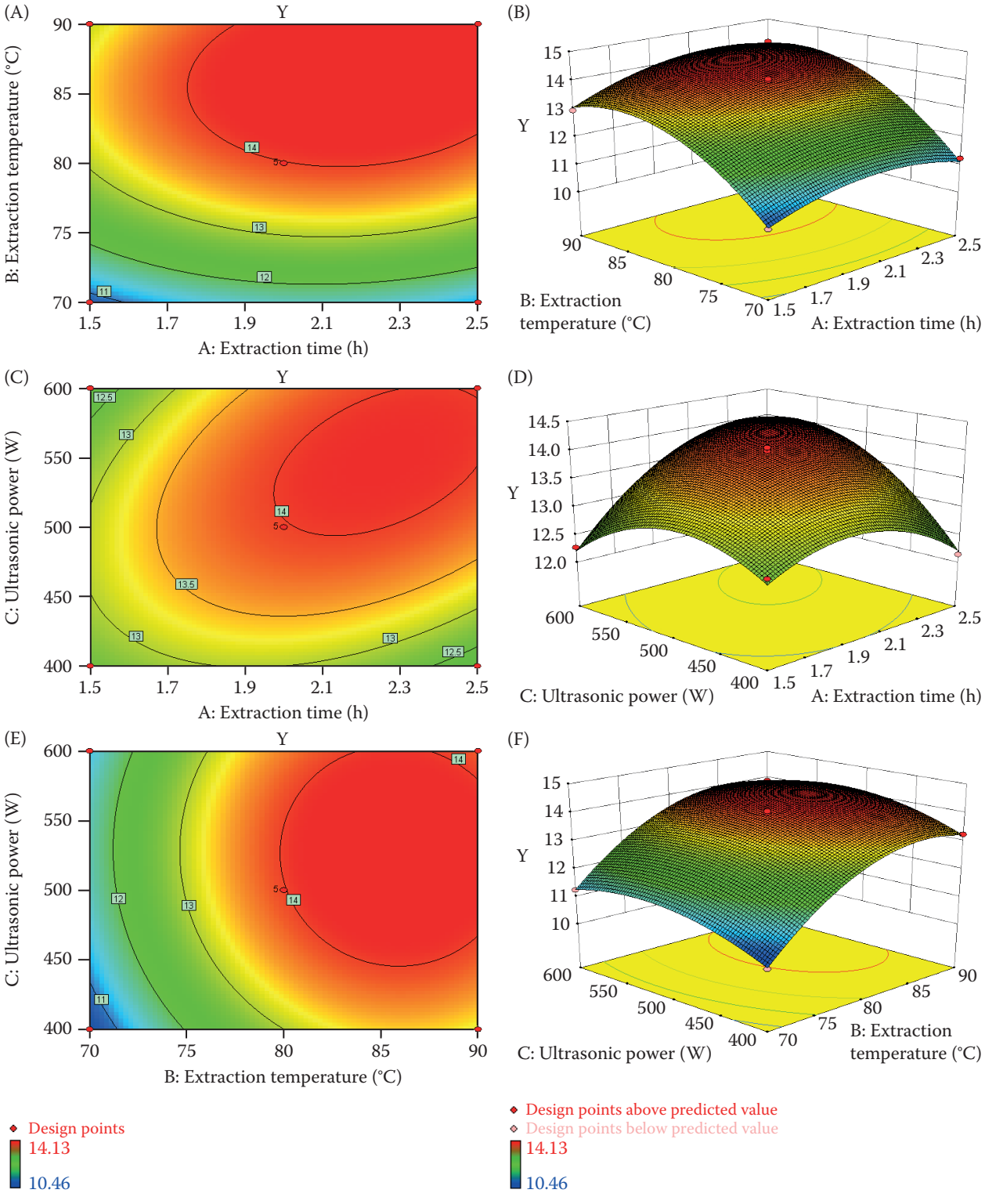

Figure 1. Response surface analysis of AAP prepared by ultrasonic-assisted hot water extraction: (A) contour map of X_1X_2, (B) 3D map of X_1X_2 (X_1 = A: extraction time; X_2 = B: extraction temperature; X_3 = C: ultrasonic power = 500.00), (C) contour map of X_1X_3, (D) 3D map of X_1X_3 (X_1 = A: extraction time; X_3 = C: ultrasonic power; X_2 = B: extraction temperature = 80.00), (E) contour map of X_2X_3, and (F) 3D map of X_2X_3 (X_2 = B: extraction temperature; X_3 = C: ultrasonic power; X_1 = A: extraction time = 2.00)

AAP – *Auricularia heimuer* polysaccharides

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set as 2 h. When extraction temperature was set, the polysaccharide yield increased when ultrasonic power reached approximately 560 W, and then it declined. When ultrasonic power was set, the polysaccharide yield increased when extraction temperature reached about 85 °C, and then it declined.

The optimal combination of factors was as follows: X_1 = 2.3 h, X_2 = 86.17 °C, and X_3 = 550.25 W. These denoted extraction time of 2.3 h, extraction temperature of 86 °C, and ultrasonic power of 550 W. The maximum value was 14.58%. Under these conditions, three parallel experiments were conducted, which resulted in an average polysaccharide yield of 14.55%. This indicated that the regression model had good fitting properties.

It is important that the extraction process should be optimised to maintain the pharmacological activities of AAP (Cai et al. 2015; Bian et al. 2020). Gu et al. (2021) found that when ultrasonic power was 130 W, there was a significant difference from the other four groups in the yield of AAP ($P < 0.05$). The optimal microwave power in this study is different from that. Jambrak et al. (2007) found that ultrasound had an effect on the tissue surface of button mushrooms. So ultrasound may result in the dissolution of polysaccharides because of the cavitation effect and thermal effect of ultrasound.

Table 3. Design and results of orthogonal experiment (primary and secondary factors order by influence: A > D > C > B; optimal scheme: $A_3D_2C_3B_1$)

Test No.	A	B (%)	C (%)	D (h)	Yield (%)
1	1(3)	1(1.0)	1(1.0)	1(1.0)	7.0
2	1	2(1.5)	2(1.5)	2(1.5)	10.8
3	1	3(2.0)	3(2.5)	3(2.0)	9.8
4	2(4)	1	2	3	12.4
5	2	2	3	1	12.0
6	2	3	1	2	12.6
7	3(5)	1	3	2	19.0
8	3	2	1	3	15.4
9	3	3	2	1	15.8
*K*1	27.6	38.8	35.0	34.8	–
*K*2	37.4	38.2	39.4	42.4	–
*K*3	50.2	38.2	40.8	38.0	–
*k*1	9.2	13.0	11.6	11.6	–
*k*2	12.4	12.8	13.2	14.2	–
*k*3	16.8	12.8	13.6	12.6	–
R	41.0	26.0	29.2	30.8	–

A – pH; B – cellulase content; C – substrate concentration; D – enzymolysis time; A, B, C, and D were chosen as independent variables, the yield of polysaccharide was used as dependent variable to obtain the optimal process conditions; pH was 3, 4, and 5; the cellulase content was 1.0, 1.5, and 2.0%; the substrate concentration was 1.0, 1.5, and 2.5%; the enzymolysis time was 1.0, 1.5, and 2.0 h

AAP by cellulase-assisted method. The yield of E1 was 12.80% by pectinase-assisted hot water extraction. The optimum conditions for cellulase hydrolysis of *A. auricular* residues after pectinase extraction were determined by an orthogonal design of Table 3, and the results are shown in Table 3. The effect level on the yield

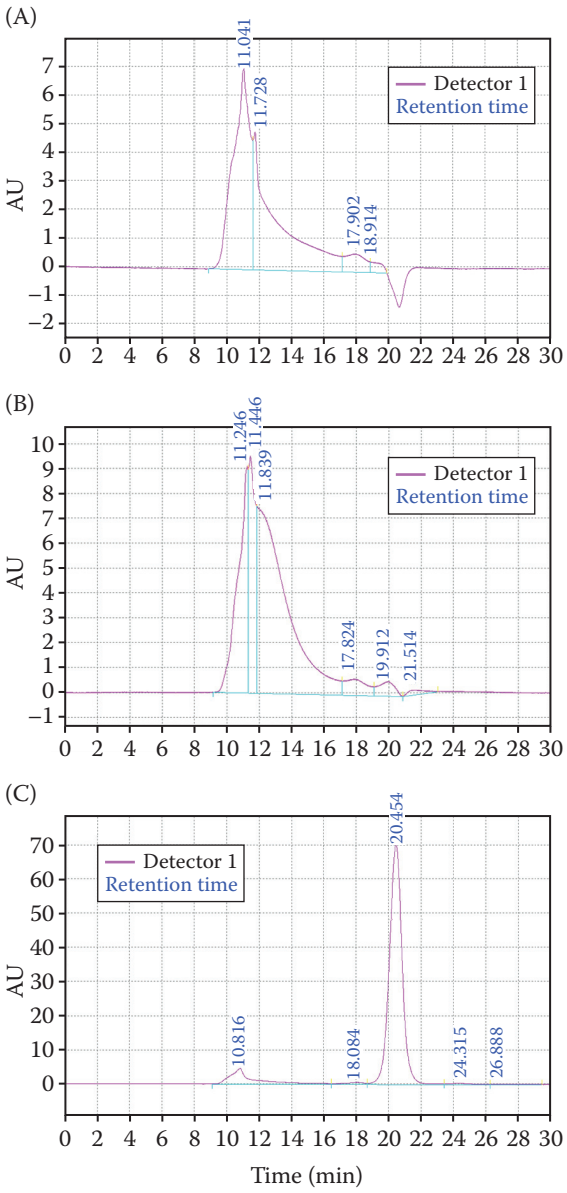

Figure 2. HPLC chromatography of polysaccharides extracted by (A) hot water, (B) ultrasonic-assisted and (C) cellulase-assisted method

HPLC – high-performance liquid chromatography

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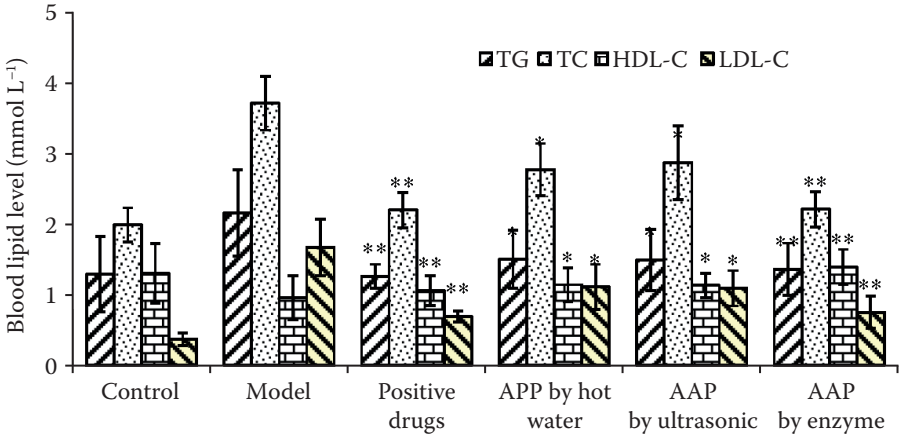

Figure 3. Hypolipidaemia activity of AAP extracted by three methods on hyperlipidaemic rats (mean ± SD; n = 10, mmol L^{-1})

AAP – *Auricularia heimuer* polysaccharides; TG – triglyceride; TC – total cholesterol; HDL-C – high-density lipoprotein cholesterol; LDL-C – low-density lipoprotein cholesterol; SD – standard deviation

of *A. heimuer* polysaccharide was A > D > C > B. The optimal scheme was $A_3D_2C_3B_1$, which had pH of 5.0, cellulase of 1%, enzymolysis time of 1.5 h and substrate concentration of 2.5%, resulting in an E2 yield reaching 19.0%. The sequential enzymatic extraction resulted in a total polysaccharide yield of 31.80%. Yue et al. (2019) obtained the crude polysaccharides from *Armillaria mellea* with an ultrasound-assisted enzymatic extraction. Wu et al. (2020) developed and optimised an efficient enzymatic hydrolysis method for the degradation of *A. auricula* polysaccharide. The degradation product of *A. auricula* polysaccharide had better antioxidant activity than AAP. Therefore, enzymatic hydrolysis could significantly increase the yield of AAP.

HPLC analysis of the molecular weight distribution of AAP. The polysaccharide yields by ultrasonic method (14.55%) and sequential enzymatic method (31.80%) were both higher than that by hot water extraction method (9.68%).

The standard curve regression equation used was log (y) = $-0.004945X + 9.436487R^2 = 0.99$. The differences in molecular weight distribution between ultrasonic (Figure 2B) or enzymatic hydrolysis-extracted polysaccharides (Figure 2C) and hot water-extracted polysaccharides (Figure 2A) by HPLC. There was no significant difference in the molecular weight distribution of polysaccharides between ultrasonic and hot water extraction. The percentage of high-molecular-weight polysaccharides accounted for 94.05% and 92.76%, respectively, whereas low-molecular-weight fractions accounted for only 7.23% and 5.95%, respectively, for these two methods. However, only 10% of the polysaccharides obtained by enzymatic hydrolysis had a large molecular weight, while 89.13% of the polysaccharides obtained were low-molecular-weight polysaccharides with molecular weights ranging from 5 000 Da to 10 000 Da (Figure 2).

Lipid-lowering activity of AAP. The purpose of extraction and separation/purification of AAP is the medical or edible application (Zeng et al. 2013), so it is of crucial significance to elucidate the extraction methods and lipid-lowering activity of AAP.

Compared with the normal group, TC, TG and LDL-C levels in the model group significantly increased ($P < 0.01$), indicating that the model was successfully established. The serum levels of TC, TG and LDL-C in the enzymolysis group were significantly lower ($P < 0.01$), whereas the serum level of HDL-C was significantly higher than the respective values obtained in the control group. Similarly, the serum levels of TC, TG and LDL-C in the hot water and ultrasonic groups also significantly decreased ($P < 0.05$), while only the content of HDL-C exhibited a non-significant increasing trend. These results indicate that the polysaccharides extracted by sequential enzymolysis had significantly more pronounced effects than hot water and ultrasonic extraction (Figure 3).

CONCLUSION

In this study, three extraction methods have been established based on AAP yield. The yields of AAP extracted through ultrasonic-assisted, enzymatic-assisted and hot water extraction were 14.55, 31.80, and 9.68%, respectively.

The molecular weight distribution of AAP extracted by hot water and ultrasonic-assisted method is almost the same, except for the enzymolysis method. Only 10% of AAP obtained by the enzymatic method had a large molecular weight, while 89.13% of AAP obtained were low-molecular-weight polysaccharides. It was found that AAP from enzymatic extraction significantly reduced the serum lipid levels of hyperlipidaemic rats.

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