



## Extraction, purification and preliminary characterization of polysaccharides from *Kadsura marmorata* fruits

Hongjun Wang<sup>a,1,2</sup>, Hong Jiang<sup>a,1</sup>, Shude Wang<sup>a</sup>, Xinguo Li<sup>b</sup>, Dan Yao<sup>a</sup>, Jingjing Dong<sup>c</sup>, Tiezhong Zhou<sup>a,\*</sup>, Yingzi Liu<sup>a</sup>, Shenyang Gao<sup>a</sup>, Li Li<sup>a</sup>, Xuming Deng<sup>d,\*\*</sup>

<sup>a</sup> Institute of Animal Husbandry and Veterinary Medicine, Liaoning Medical University, No. 48 People's Street, Jinzhou, Liaoning 121001, PR China

<sup>b</sup> Chinanimal Agricultural Development Co., Ltd., No. 188 West Road of South Fourth Ring, Beijing 100070, PR China

<sup>c</sup> Nanjing Shengyi Pharmacology Technology, LLC, Life Science and Technology Innovation Park Qixia District, Nanjing 210000, PR China

<sup>d</sup> College of Animal Husbandry and Veterinary Medicine, Jilin University, Changchun, Jilin 130062, PR China

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### ABSTRACT

In this study, response surface methodology and central composite design based on single-factor experiments were applied to the optimization of the extraction parameters (extraction time, extraction temperature, and solid-liquid ratio) of polysaccharides from *Kadsura marmorata* fruits. The extraction temperature and solid-liquid ratio affected the extraction yield significantly. The optimal conditions included a solid-liquid ratio of 1:27 (mass/volume), an extraction time of 3 h and an extraction temperature of 98 °C. Under these conditions, the maximal yield of crude *Kadsura* polysaccharide (KPS) was  $2.7735 \pm 0.068\%$ , which agreed with model predictions. Five major fractions, KPS I, KPS II, KPS III, KPS IV and KPS V, were obtained by diethylaminoethyl (DEAE) cellulose-52 column chromatography. The main fraction, KPS III-1, was isolated from KPS III by Sephadex G-100 column chromatography. Preliminary characterization of KPS III-1 by gas chromatography indicated that it was composed mainly of xylose and galacturonic acid.

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## 1. Introduction

*Schisandra chinensis* has been used as a traditional medicine for a long time. *Kadsura marmorata* (E.G. Henderson & A. Henderson) A.C. Smith is one of the better known traditional medicinal *Schisandraceae*. However, there are few reports on this plant. *K. marmorata* is distributed in the Palawan province of the Philippines (Bi, Liu, & Zhao, 2002). Recently, our research group successfully extracted *Kadsura* polysaccharides (KPS) from defatted *K. marmorata* fruits, and proved their immunoregulatory activity in chickens (Wang, Jiang, & Deng, 2011). Nine compounds from *K. marmorata* fruits were isolated for the first time (Wang, Wu, Zhou, Deng, & Wang, 2012). Lignans and triterpenoids from *Schisandra* and *Kadsura*

plants have attracted considerable attention during the past 30 years (Kim, Ha, Kim, & Lee, 2010; Li, 1986; Song, Jin, Feng, & Chen, 2010), but more recently, the polysaccharides from *S. chinensis* have become of interest because of their various biological activities, which include antitumor, anti-aging, and immunoregulatory activities (Chen & Ji, 2007; Huang & Zhang, 2004; Miao, 2002; Yang, Wu, Fan, & Zhao, 2008). However, little attention was devoted to the extraction, purification and characterization of *K. marmorata* polysaccharides. According to a previous study, the extraction yields of KPS were mainly affected by the extraction time, temperature and solid-liquid ratio. To determine the optimal conditions, the procedure consisted of changing one parameter while keeping the others unchanged. However, this single variable optimization did not depict the net effects of various parameters on the reaction rate. Response surface methodology (RSM) has been successfully used for optimizing complex processes, including polysaccharide extraction technology (Chen et al., 2010; Guo, Zou, & Sun, 2010; Li, 2005; Meng et al., 2010; Sun, Liu, & Kennedy, 2010; Wang & Zhang, 2010). The advantage of RSM is that it can reduce the number of experimental trials as well as evaluate the interactions between multiple parameters (Jafari, Nateghi, & Rabbani, 2010); RSM is more effective and precise than many approaches. Therefore, this study aimed to optimize the extraction parameters

\* Corresponding author. Fax: +86 416 2625883.

\*\* Corresponding author. Tel.: +86 431 87836160; fax: +86 431 87836160.

E-mail addresses: [wanghj0312@yahoo.com.cn](mailto:wanghj0312@yahoo.com.cn) (H. Wang), [ztz1818@126.com](mailto:ztz1818@126.com) (T. Zhou), [xumingdeng@jlhu.edu.cn](mailto:xumingdeng@jlhu.edu.cn) (X. Deng).

<sup>1</sup> These authors contributed equally to this work.

<sup>2</sup> Department of Basic Veterinary Medicine, College of Animal Science and Veterinary Medicine, Liaoning Medical University, No. 48 People's Street, Jinzhou, Liaoning 121001, PR China. Tel.: +86 416 4672096; fax: +86 416 2625883.

of a KPS fraction using RSM, and to preliminarily characterize the purified polysaccharide by gas chromatography.

## 2. Materials and methods

### 2.1. Materials

Fruiting bodies of *K. marmorata* were obtained from Guizhou Traditional Chinese Medicine Hospital (Guizhou, China). *K. marmorata* fruits (1000 g) were pulverized in an electric mill to a size able to pass through a 0.425 mm sieve. The flour was packed in a glass jar and stored at room temperature until use. DEAE cellulose-52 and Sephadex G-100 were purchased from Whatman Co. (Maidstone, Kent, UK). Sulfuric acid, anthrone, petroleum ether (60–90 °C), chloroform, 95% ethanol, glucose (Glc) and mannose (Man), which were all analytical grade, and galactose (Gal), galacturonic acid (Gal A), rhamnose (Rha), ribose (Rib), arabinose (Ara) and xylose (Xyl), which were all biological grade, were obtained from Jinzhou City Chemical Reagent Company (Jinzhou, China).

### 2.2. Extraction of crude polysaccharide

500 g of *K. marmorata* fruit powder was defatted by extraction with petroleum ether (60–90 °C), chloroform and 95% ethanol, in that order, using the improved Soxhlet method (Wang et al., 2003). The organic solvent was recycled using a rotary evaporator (Shanghai Broadcom Economic and Trade Co., Ltd., Shanghai, China). The defatted *K. marmorata* fruit powder was diluted and extracted under different conditions of extraction time (1–5 h), extraction temperature (60–120 °C) and solid–liquid ratio (1:15–1:35). Then, the mixtures were centrifuged at 4000 revolutions per minute (rpm) for 15 min. The supernatant was evaporated to a third of the original volume. After cooling, proteins and pigments were removed by the addition of trichloroacetic acid (TCA) and activated carbon. The protein content was estimated by the application of Coomassie brilliant blue (CBB) reagent, using a Sunrise enzyme standard instrument (Tecan, Grödig, Austria). Then, the mixtures were precipitated by the addition of triple volumes of 95% ethanol at 4 °C for 24 h. The precipitates (crude KPS) were washed three times with ether and anhydrous alcohol.

### 2.3. Determination of KPS

The crude KPS was dissolved in distilled water and its content was determined with the anthrone-sulfuric acid method (Wang, Deng, Jiang, Zhou, & Chang, 2011). The yield of KPS was expressed as g KPS/100 g defatted *K. marmorata* fruit powder. All the experiments were conducted in triplicate.

### 2.4. Experimental design and statistical analysis

Single-factor-test was employed to determine the preliminary range of the extraction variables including extraction temperature, extraction time and solid–liquid ratio. A five-level three-factor central composite design (CCD) was used in this study, requiring 20 experiments as shown in Table 1. All trials were performed in triplicate. The extraction yields were treated as responses. Design-Expert Software Version 7.1.6 (Stat-Ease, Inc., Minneapolis, MN, USA) and Minitab Software Version 15 (Minitab Inc., State College, PA, USA) were used to generate the experimental designs, statistical analysis and regression model. The experimental data were fitted to a second-order polynomial model, shown below:

$$Y = k_0 + k_1A + k_2B + k_3C + k_4AB + k_5AC + k_6BC + k_7A^2 + k_8B^2 + k_9C^2$$

where  $Y$  was the predicted response,  $k_0$  was the offset term,  $k_1$ ,  $k_2$  and  $k_3$  were linear effect terms,  $k_4$ ,  $k_5$  and  $k_6$  were interaction

effects and  $k_7$ ,  $k_8$  and  $k_9$  were squared effects. The fitness of the second-order model shown in Table 2 was expressed by the regression coefficient  $R^2$  and its statistical significance was determined by the  $F$ -test (Nakai, Li-Chen, & Dou, 2006).  $t$ -Test was used for evaluating regression significance (Gao, Wang, & Jiang, 2004). The corresponding variables are more significant if the absolute  $F$ -value becomes greater and the  $P$ -value becomes smaller (Atkinson, 1992).

### 2.5. Purification of KPS

The crude polysaccharide was redissolved in 80 mL of distilled water, filtered through 0.45  $\mu$ m filters and applied to a DEAE cellulose-52 column (2.6 cm  $\times$  30 cm) equilibrated with distilled water. The polysaccharide was fractionated and eluted with distilled water and by the stepwise addition of NaCl solutions of different concentrations (0, 0.01, 0.1 and 1.0 M NaCl). The eluate was concentrated to obtain the main fractions, which were centrifuged and dried under vacuum. Then, the main fractions were redissolved in 2.0 mL of distilled water and applied to a Sephadex G-100 gel column (2.6 cm  $\times$  100 cm) equilibrated with distilled water. The main fractions were obtained by elution with distilled water and by the stepwise addition of NaHCO<sub>3</sub> solutions of different concentrations (0, 0.01, 0.02 and 0.05 M NaHCO<sub>3</sub>) at a flow rate of 1.0 mL/min. The obtained fractions were combined according to the total carbohydrate content quantified by the anthrone-sulfuric acid method. The relevant fractions were collected, concentrated, dialyzed and dried in a vacuum dryer (Beijing Zhongjing Keyi Technology Co., Ltd.).

### 2.6. Analysis of monosaccharides

The purified fractions were hydrolyzed with 2 M sulfuric acid at 100 °C for 8 h. The sulfuric acid was removed by neutralization with BaCl<sub>2</sub>. The treated sample and monosaccharides, including Glc, Man, Xyl, Rha, Rib, Ara, Gal and Gal A, were derivatized by the addition of a mixture of hexamethyldisilazane and chlorotrimethylsilane (Liu & Zhang, 2009; Xu, Wang, & Li, 1992). Methylsilane derivatives were separated by a gas chromatograph (GC-2010 Plus, Shimadzu, Kyoto, Japan) fitted with a fused silica capillary column (SE-30, Agilent Technologies, Inc., Santa Clara, CA, USA; 30 m  $\times$  0.25 mm  $\times$  0.25  $\mu$ m) and a flame ionization detector. The injector and detector were set at 220 °C and 230 °C, respectively. The oven was kept at 195 °C. Nitrogen was used as the carrier gas, at a flow rate of 30.0 mL/min.

## 3. Results and discussion

### 3.1. Effect of extraction time on the extraction yield of KPS

Extraction time was one of the factors that influenced the extraction yield. The extraction time was increased from 1 to 5 h, while the solid–liquid ratio was kept at 1:20 and the extraction temperature was kept at 100 °C. The results showed that the extraction yield began to increase from  $1.35 \pm 0.01\%$  to  $2.31 \pm 0.02\%$  (Fig. 1A). A longer extraction time resulted in a slightly increased yield of polysaccharides. Therefore, an extraction time range of 1–3 h was selected as the optimal in the present study, with cost saving taken into consideration.

### 3.2. Effect of solid–liquid ratio on the extraction yield of KPS

The yield of KPS was influenced by the different solid–liquid ratios, from 1:15 to 1:35 (Fig. 1B). The extraction temperature and extraction time were set at 100 °C and 2 h, respectively. When the ratio increased from 1:15 to 1:25, the extraction yields of

**Table 1**

Central composite design matrix (in coded level of three variables) and response values for the KPS yield.

| Run | Coded variables |          |        | Polysaccharide yield (Y) (%) |                     |
|-----|-----------------|----------|--------|------------------------------|---------------------|
|     | A               | B        | C      | Experimental ( $Y_0$ )       | Predicted ( $Y_i$ ) |
| 1   | 1(2.5)          | −1(1:20) | 1(105) | 2.3733                       | 2.2626              |
| 2   | −1(1.5)         | 1(1:30)  | 1(105) | 1.7706                       | 1.6872              |
| 3   | −1(1.5)         | −1(1:20) | −1(95) | 1.703                        | 1.7796              |
| 4   | −1(1.5)         | 1(1:30)  | −1(95) | 2.126                        | 2.3612              |
| 5   | 1(2.5)          | 1(1:30)  | 1(105) | 1.7525                       | 1.947               |
| 6   | −1(1.5)         | −1(1:20) | 1(105) | 1.8136                       | 2.0028              |
| 7   | 1(2.5)          | 1(1:30)  | −1(95) | 2.4731                       | 2.621               |
| 8   | 1(2.5)          | −1(1:20) | −1(95) | 1.7652                       | 2.0394              |
| 9   | 2(3.0)          | 0(1:25)  | 0(100) | 3.0784                       | 2.8344              |
| 10  | −2(1.0)         | 0(1:25)  | 0(100) | 2.5147                       | 2.3148              |
| 11  | 0(2.0)          | 2(1:35)  | 0(100) | 2.0921                       | 1.9604              |
| 12  | 0(2.0)          | −2(1:15) | 0(100) | 1.7933                       | 1.6944              |
| 13  | 0(2.0)          | 0(1:25)  | 2(110) | 1.1276                       | 1.1484              |
| 14  | 0(2.0)          | 0(1:25)  | −2(90) | 1.8503                       | 1.5992              |
| 15  | 0(2.0)          | 0(1:25)  | 0(100) | 2.5223                       | 2.5746              |
| 16  | 0(2.0)          | 0(1:25)  | 0(100) | 2.5514                       | 2.5746              |
| 17  | 0(2.0)          | 0(1:25)  | 0(100) | 2.4501                       | 2.5746              |
| 18  | 0(2.0)          | 0(1:25)  | 0(100) | 2.5069                       | 2.5746              |
| 19  | 0(2.0)          | 0(1:25)  | 0(100) | 2.6821                       | 2.5746              |
| 20  | 0(2.0)          | 0(1:25)  | 0(100) | 2.4085                       | 2.5746              |

**Table 2**

Analysis of variance for the fitted quadratic polynomial model of polysaccharide extraction.

| Variables      | Sum of squares | Df | Mean square          | F value | P-value Prob > F | Significance |
|----------------|----------------|----|----------------------|---------|------------------|--------------|
| Model          | 3.78           | 9  | 0.40                 | 15.91   | <0.0001          | **           |
| Residual       | 0.26           | 10 | 0.042                |         |                  |              |
| Lack of fit    | 0.22           | 5  | 0.044                | 4.89    | 0.0532           |              |
| Pure error     | 0.045          | 5  | 0.009                |         |                  |              |
| Corr. total    | 4.04           | 19 |                      |         |                  |              |
| $R^2 = 0.9347$ |                |    | $R^2_{Adj} = 0.8760$ |         | C.V.% = 7.49     |              |

\*\*  $P < 0.01$ .

the polysaccharides increased from  $1.71 \pm 0.02\%$  to  $2.47 \pm 0.04\%$ . Afterward, it tended to stabilize. The more water was used, the more ethanol was added to precipitate the KPS. Therefore, an extraction ratio of 1:25 was favorable for the extraction of the polysaccharides.

### 3.3. Effect of temperature on the extraction yield of KPS

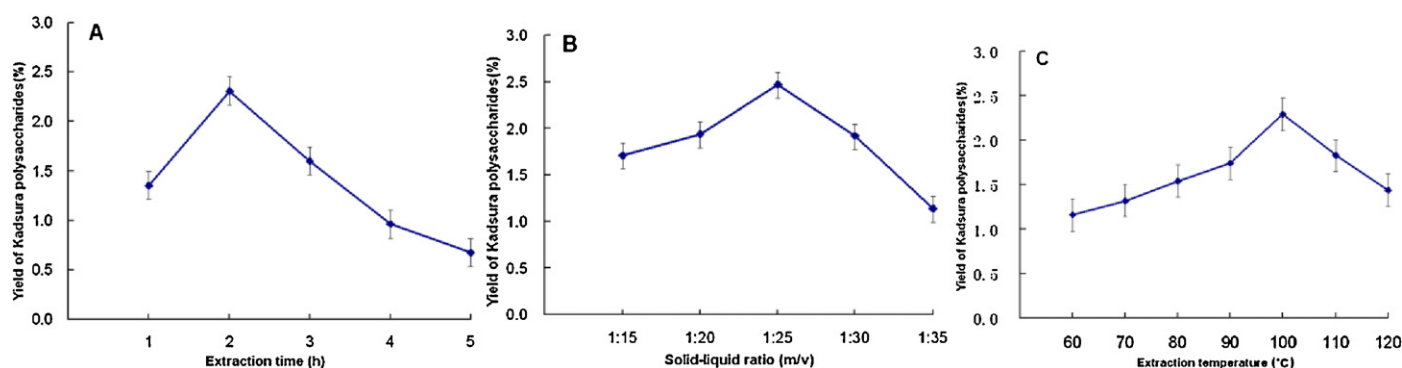
The effect of temperature on the extraction yield was investigated (Fig. 1C). The temperature was increased from  $60^\circ\text{C}$  to  $120^\circ\text{C}$ , while the solid–liquid ratio was kept at 1:20 and the extraction time was 2 h. The yield of KPS increased with an increase in temperature, up to  $100^\circ\text{C}$ , and then began to decrease. The maximum extraction yield was  $2.29 \pm 0.03\%$  at  $100^\circ\text{C}$ . This result indicated that an increase in temperature, up to  $100^\circ\text{C}$ , enhanced

the extraction of polysaccharides from the *K. marmorata* fruits into the water, but higher temperatures resulted in their loss, caused by decomposition.

According to the single-parameter study, the corresponding variables would be more significant if the absolute value becomes larger (Amin & Anggoro, 2004). Thus, we adopted an extraction temperature of  $90\text{--}110^\circ\text{C}$ , a solid–liquid ratio of 1:15–1:35, and an extraction time of 1–3 h for the RSM experiments.

### 3.4. Predicted model and statistical analysis

The design matrix and the corresponding results of the RSM experiments, conducted to determine the effects of the three independent variables (extraction time (A), solid–liquid ratio (B) and extraction temperature (C)), are shown in Table 1. The predicted

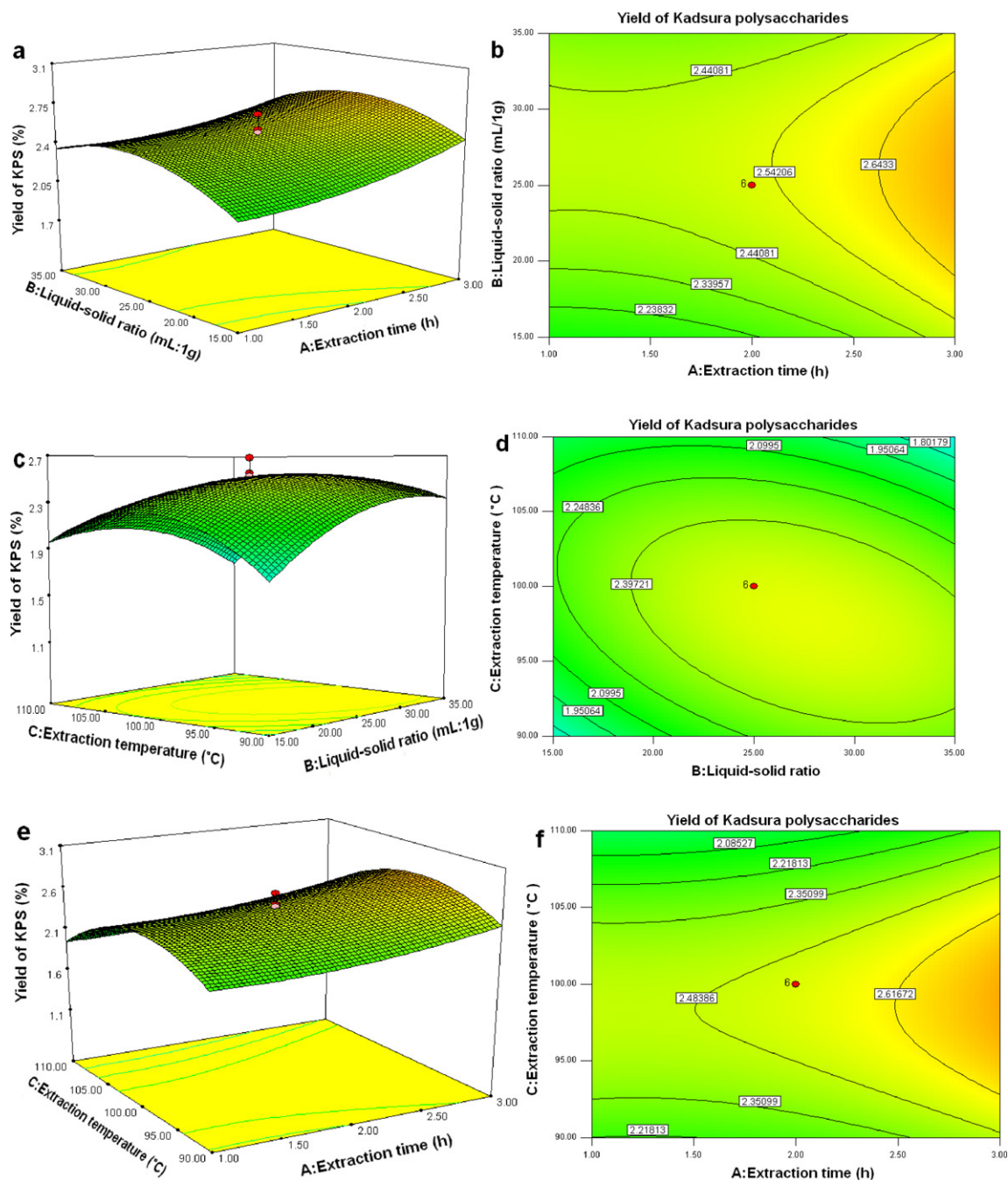
**Fig. 1.** Effects of extraction time (A), solid–liquid ratio (B) and extraction temperature (C) on the KPS yield.

**Table 3**  
Significance of regression coefficient for polysaccharide yield.

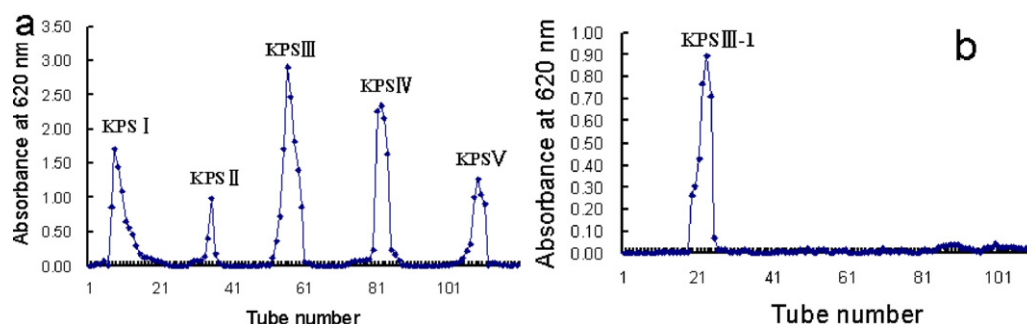
| Variables              | Regression coefficient | Standard error | T value | P-value Prob > F | Significance |
|------------------------|------------------------|----------------|---------|------------------|--------------|
| Intercept              | 2.52                   | 0.066          | 31.7856 | 0.0005           | **           |
| A – extraction time    | 0.14                   | 0.044          | 2.7874  | 0.0101           | *            |
| B – solid–liquid ratio | 0.071                  | 0.044          | 1.428   | 0.1372           |              |
| C – temperature        | –0.12                  | 0.044          | –2.4178 | 0.0256           | *            |
| A <sup>2</sup>         | 0.081                  | 0.043          | 0.9329  | 0.086            |              |
| B <sup>2</sup>         | –0.22                  | 0.043          | –4.6698 | 0.0004           | **           |
| C <sup>2</sup>         | –0.38                  | 0.043          | –7.6472 | <0.0001          | **           |
| AB                     | –0.037                 | 0.057          | –0.5555 | 0.5379           |              |
| AC                     | 0.017                  | 0.057          | 0.2509  | 0.7792           |              |
| BC                     | –0.22                  | 0.057          | –3.4042 | 0.0029           | **           |

\*\*  $P < 0.01$ .

\*  $P < 0.05$ .



**Fig. 2.** Response surface plots (a, c and e) and contour plots (b, d and f) show the effect of extraction time (A), solid–liquid ratio (B) and extraction temperature (C) on the KPS yield.



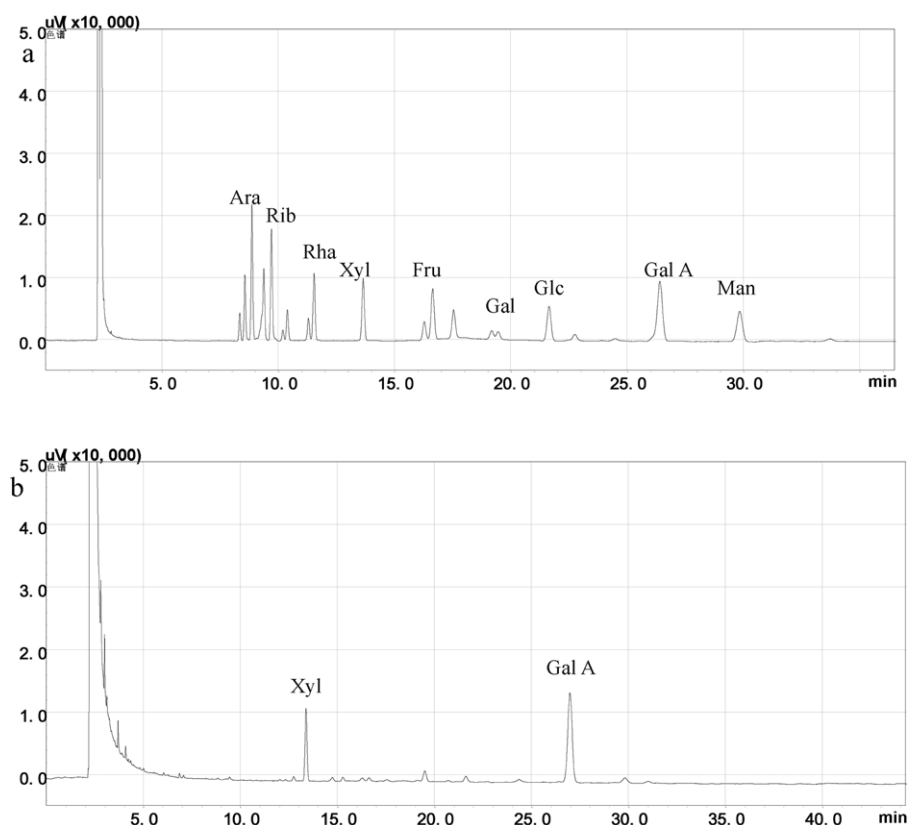
**Fig. 3.** Polysaccharides from crude *K. marmorata* extract were isolated by DEAE cellulose-52 chromatography (a), and named as KPS I, KPS II, KPS III, KPS IV and KPS V. KPS III was fractionated and eluted with distilled water and by the stepwise addition of  $\text{NaHCO}_3$  solutions of different concentrations (0, 0.01, 0.02 and 0.05 M  $\text{NaHCO}_3$ ) at a flow rate of 1.0 mL/min. A single peak was designated as KPS III-1(b).

model was obtained by the following second-order polynomial function:

$$Y = 2.5227 + 0.139A + 0.071B - 0.1152C + 0.0814A^2 - 0.2204B^2 - 0.3808C^2 - 0.0366AB + 0.0165AC - 0.2243BC$$

The fit statistics of extraction yield (Y) for the selected quadratic predictive model are shown in Table 2. The coefficient of the variation (CV) and value of adjusted determination coefficient  $R^2_{\text{Adj}}$  were 7.49 and 0.876, respectively, which indicated a high degree of precision of reliability of the experimental values and a high degree of correlation between the observed and predicted values. The ANOVA analysis is shown in Table 3. The *P*-values were used as a tool to check the significance of each coefficient. The smaller

the *P*-value, the more significant the corresponding coefficient is (Muralidhar, Chirumamila, Marchant, & Nigam, 2001). The variable with the largest effect was the quadratic of extraction temperature ( $C^2$ ), followed by the quadratic of solid–liquid ratio ( $B^2$ ), the interaction effects of solid–liquid ratio and extraction temperature ( $B \times C$ ), and the linear term of extraction time (*A*) and extraction temperature (*C*) (Table 3). The other term coefficients ( $B$ ,  $A^2$ ,  $A \times B$ ,  $A \times C$ ) were not influential ( $P > 0.05$ ). According to previous reports, the effect of linear terms and interaction terms were always different among different species and different material treatments. There are two possible reasons for this. First, polysaccharides from different species have different solubilities. Second, dried materials need a higher extraction temperature, solid–liquid ratio, and a longer extraction time compared with fresh materials (Qiao et al., 2009; Sun et al., 2009). This might be because of the expanded state



**Fig. 4.** GC analysis of trimethylsilyl ether derivatives of KPS III-1 from *K. marmorata* fruits. The chromatogram of the standards is shown in (a). The derivatives were separated, and xylose (Xyl) and galacturonic acid (Gal A) were detected (b).

of the cells of fresh plant material, which makes the polysaccharides easier to extract.

### 3.5. Response surface plot and contour plot

3D response surface and 2D contour plots are graphical representations of the regression function. They show the type of interactions between two tested variables and the relationship between responses and experimental levels of each variable. Different shapes of the contour plots indicate different interactions between variables. Circular contour plots indicate negligible interactions between the corresponding variables, while elliptical contour plots indicate otherwise (Muralidhar et al., 2001). In the present study, the response surface and contour plots (Fig. 2) were obtained using Design-Expert version 7.1.6. As shown in Fig. 2(a) and (b), when the extraction temperature (C) was fixed at level 0, the liquid–solid ratio (B) displayed a quadratic effect on the response yield. When the extraction time was longer, the yield increased at first and then decreased with the increase of the liquid–solid ratio (B). Fig. 2(c) shows that when the extraction time (A) was fixed at level 0, the liquid–solid ratio (B) and extraction temperature (C) demonstrated quadratic effects on the extraction yields. The elliptical contour plot shown in Fig. 2(d) indicates that the mutual interactions between the solid–liquid ratio and the extraction time were significant. Fig. 2(e) and (f) indicate that when the liquid–solid ratio was fixed at level 0, the extraction temperature (C) displayed a quadratic effect on the response yield. When the extraction time was longer, the yield increased at first and then decreased with an increase in temperature (C).

By analyzing the plots and the predicted value of the tested variables for an extraction time of 3 h, a solid–liquid ratio of 1:27 and an extraction temperature of 98 °C was 2.755%. In optimal conditions, the experimental yield of crude KPS was  $2.7735 \pm 0.068\%$ , which agreed with the predicted value. Therefore, the results indicated the suitability of the model and the success of RSM in optimizing the extraction conditions.

### 3.6. Purification of KPS

Crude KPS was extracted from *K. marmorata* fruits and the yield was about 2.77%. As shown in Fig. 3(a), the extracts were fractionated by DEAE-52 cellulose column chromatography to obtain five fractions, which were designated as KPS I, KPS II, KPS III, KPS IV and KPS V. KPS III was the main fraction, representing 52.31% of the extract. The total sugar content of KPS III was  $95.13 \pm 1.12\%$ , as determined by the anthrone-sulfuric acid method. KPS III was subjected to gel filtration on a Sephadex G-100 column. As shown in Fig. 3(b), the purified fraction KPS III-1 yielded a single peak.

### 3.7. Preliminary characterization of KPS fractions

KPS III-1 was mainly composed of the monosaccharides Xyl and Gal A (Fig. 4). However, previous studies showed that *Schisandra chinensis* polysaccharides (SCP) were mainly composed of Rha, Glc, Ara and Gal (Wang, Wu, Liang, & Li, 2009). The polysaccharides SCP-BI and SCP-BII were isolated from *Schisandra chinensis* (Turcz.) Baill (Gao, 2009). SCP-BI was mainly composed of Ara, Gal, Glc and Gal A (Gao, 2009), while SCP-BII was mainly composed of Rha, Ara, Man, Gal, Glc and Gal A (Gao, Meng, & Li, 2008). This suggests that the sugar compositions of *Schisandra* and *Kadsura* differ. The configuration of KPS III-1 and its glycosidic bonds are subjects for further research.

## 4. Conclusion

The single-factor experiments and CCD, along with RSM, were applied for optimizing the extraction parameters of polysaccharides from *K. marmorata* fruits. The optimal conditions for polysaccharide extraction were as follows: extraction time of 3 h, solid–liquid ratio of 1:27 and extraction temperature of 98 °C. In optimal conditions, the experimental yield of KPS was  $2.7735 \pm 0.068\%$ , which agreed with the predicted value. The preliminary characterization of KPS III-1 by GC showed that the polysaccharide was mainly composed of Xyl and Gal A. The experimental conditions allowed for a fast and cost-saving extraction of the polysaccharide. Further studies on the elucidation of the chemical structures and biological functions of the novel polysaccharide are underway.

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