

Article

Comparison and Optimization of Three Extraction Methods for *Epimedium polysaccharides*

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Abstract: *Epimedium* is used in traditional Chinese medicine. *Epimedium polysaccharides* have a variety of physiological properties. This study compared three different processes for the extraction of polysaccharides from *Epimedium* spp., including ultrasonic, aqueous enzymatic, and microwave extraction, to optimize the extraction conditions and determine the optimal extraction method. The optimal parameters for each method were analyzed. The results showed that the optimal process for ultrasonic extraction was an ultrasonic power of 250 W, an extraction time of 60 min, a temperature of 50 °C, and a solid–liquid ratio of 1:35. The optimal conditions for the aqueous enzymatic method were a papain concentration of 70 U/mL, extraction time of 70 min, a temperature of 50 °C, and a material-to-liquid ratio of 1:30, while those for microwave extraction were a microwave power of 650 W, an extraction time of 50 min, a temperature of 40 °C, and a material-to-liquid ratio of 1:25. The polysaccharide yields were 4.85%, 4.72%, and 3.98% for the three methods, respectively, indicating that ultrasonic extraction resulted in the highest yield of polysaccharides from *Epimedium brevicornum*. After purification with DEAE-cellulose, the polysaccharide yields were 4.13%, 3.67%, and 3.12%, respectively. A comprehensive comparison demonstrated the superiority of the ultrasonic extraction method in terms of both extraction yield and purification efficiency. Characterization of the extracted *Epimedium polysaccharides* showed the presence of five monosaccharides, i.e., glucose, galactose, mannose, galacturonic acid, and rhamnose, and a number average molecular weight M_n of 1.65×10^5 Da and weight average molecular weight M_w of 6.61×10^5 Da. These results provide a scientific basis for the in-depth study and application of *Epimedium polysaccharides*.

Keywords: *Epimedium polysaccharides*; ultrasonic extraction; aqueous enzymatic extraction; microwave extraction; optimization



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1. Introduction

Epimedium is a perennial herb belonging to the genus *Epimedium* in the family Berberidaceae. Originating from regions in central-northern, central-southern, and southeastern China, it has been used as a traditional medicine in China for over 2000 years [1]. *Epimedium* spp. have a wide distribution range and are largely cultivated in southwestern, central-southern, and northeastern China, covering provinces such as Sichuan, Guizhou, Yunnan, Hubei, Shaanxi, Shanxi, Gansu, Qinghai, and Jilin [2]. There are approximately 68 *Epimedium* species worldwide, of which 58 are found in China [3]. The 2020 edition of the Pharmacopoeia of the People's Republic of China includes 15 major medicinal varieties of *Epimedium*, with *Epimedium pubescens*, *Epimedium sagittatum*, and *Epimedium koreanum* being the primary species [4]. Besides medicinal uses, *Epimedium* spp. are also frequently

used in foods, such as teas, noodles, bread, and alcoholic beverages [5,6]. The main bioactive components of *Epimedium* are flavonoids and polysaccharides [7,8]. In recent years, polysaccharides have attracted increasing attention due to their remarkable biological activities and good safety profiles. *Epimedium polysaccharides* (EPS) have been found to possess various physiological properties, such as immunomodulation, antiviral, anti-aging, antioxidant, and anti-radiation activities [9–13], and have been used clinically in enhancing vitality, promoting wound healing, alleviating the symptoms of bronchial asthma, improving infertility caused by cold deficiency, and assisting in the treatment of coronary heart disease [14–16]. Furthermore, given their lack of toxicity, EPS are also regarded as natural antibacterial and antioxidant agents that can be added to functional foods [17]. Optimal methods for the extraction and purification of plant polysaccharides are, thus, crucial for their utilization.

The polysaccharide content of *Epimedium* is about 5.8%. The extraction rate of most extraction methods is about 1–4% [18]. Studies have shown that the effective extraction methods for polysaccharide extraction from plants include the ultrasonic extraction wave method [19], microwave extraction method [20], and enzyme extraction method [21]. This study aimed to determine the optimal extraction process for EPS by comparing the efficiency and selectivity of different methods for the extraction and purification of EPS to ensure a high-purity product. The results of this study will provide a reference for further research and commercial production of EPS.

2. Materials and Methods

2.1. Experimental Materials and Reagents

(1) Materials: *Epimedium* spp. (specifically *Epimedium sagittatum*) was collected from forest land in Gaoguan Town, Chengkou County, Chongqing Municipality, China. The leaves were air-dried naturally, followed by further drying in an oven to constant weight at 60 °C, crushing in a plant mill, and sieving through a 40–60 mesh sieve for later use. The *epimedium* used in each method in the experiment was randomly assigned.

(2) Reagents: Sulfuric acid, glucose, phenol, 95% ethanol, petroleum ether, trifluoroacetic acid, 1-phenyl-3-methyl-5-pyrazolone (PMP), and other reagents of analytical grade were purchased from Chongqing Titanium New Chemical Co., Ltd., Chongqing, China. Papain was purchased from Guangxi Nanning Dongheng Huadao Biotechnology Co., Ltd., Guangxi, China.

2.2. Main Instruments

The main instruments used were: a thermostatic oscillator (Changzhou Huapu Instrument Manufacturing Co., Ltd., Changzhou, China); high-speed universal crusher (Guangzhou Leader Instrument Equipment Co., Ltd., Changzhou, China); rotary evaporator (Tianjin Jinteng Experimental Equipment Co., Ltd., Tianjin, China); freeze dryer (Shanghai Boxun Scientific Instruments Factory, Shanghai, China); ultraviolet spectrophotometer (Beijing Puxi General Instrument Co., Ltd., Beijing, China); Nicolet 6700 infrared spectrometer (Thermo Fisher, Waltham, MA, USA); Waters 1515 gel permeation chromatography (Waters Corporation, Milford, MA, USA); Shimadzu LC 2030 high-performance liquid chromatography (Shimadzu Corporation, Kyoto, Japan); MYR-2 Microwave extraction instrument (Nanjing Yanzheng experimental Equipment Co., Ltd., Nanjing, China); Ultrasonic machine (XM-400VDE, Xiaomei Ultrasonic Instrument Co., Ltd., Suzhou, China).

2.3. Experimental Methods

2.3.1. Preparation of the Glucose Standard Curve

The content of polysaccharide was determined by the phenol-sulfuric acid method. The principle is that the polysaccharide is hydrolyzed into monosaccharides under sulfuric acid, and then dehydrated to produce furfural or hydroxymethylfurfural derivatives, which can be condensed with phenol into an orange-yellow compound. The color depth is proportional to the polysaccharide content in a certain concentration range, and the

maximum absorption peak is at 490 nm wavelength. The polysaccharide content can be determined by comparing it with the standard solution of glucose. The content of polysaccharide is directly expressed by the content of glucose. Two grams of oven-dried analytical-grade glucose were dissolved in 1 L of distilled water to yield a 2.0 mg/mL standard solution. Volumes between 1 and 8 mL of the standard solution were pipetted out and diluted to 100 mL. After dilution, glucose concentrations were 20, 40, 60, 80, 100, 120, 140, and 160 µg/mL. This was followed by the addition of 1.2 mL of 5% phenol solution to each glucose solution, followed by 6.0 mL of concentrated sulfuric acid to promote the color reaction. The operation was repeated and absorbances at 490 nm were measured. By measuring the absorbance value of the sample to be measured, the concentration of glucose in the sample to be measured can be obtained through the standard curve. The glucose standard curve and following formula were used to calculate the extraction rate of polysaccharide.

$$Y = \frac{C \times V \times n}{m} \times 100\%$$

where C is the mass concentration of epimedium polysaccharide calculated according to the absorption value, %; V is the total amount of epimedium polysaccharide extract Volume, mL; m is the weight of epimedium powder, mg; and n is the dilution ratio.

2.3.2. Ultrasonic Extraction Method

Ten grams of epimedium powder were used for ultrasonic extraction of the organic components with 100 mL 95% ethanol. After drying, an appropriate amount of distilled water was added and the solution was concentrated to 10–20 mL in a rotary evaporator operating at 100 rpm. Two volumes of ethanol were then added for alcohol precipitation, after which the precipitate was dissolved in 10 mL of distilled water. The proteins were removed using the Sevag method (Chloroform and n-butanol were mixed at a ratio of 4:1, added to the sample solution, and centrifuged at 8000 r/min for 10 min after oscillation) and the material was freeze-dried to yield the epimedium polysaccharides. We obtained the glucose concentration using the regression equation of the glucose standard curve, and then calculated the polysaccharide yield [22]. Orthogonal experiments and single factor experiments were conducted to optimize the parameters such as ultrasonic power, extraction time, extraction temperature, and solid-to-liquid ratio. We repeated the measurement three times.

2.3.3. Aqueous Enzymatic Extraction Method

Papain can improve the extraction rate of plant polysaccharides [23,24]. Two grams of epimedium powder was added to 50 mL of distilled water. We adjusted the pH of the solution to 5.8 with citric acid and sodium bicarbonate, after which 0.08 g papain was added to facilitate the release of the polysaccharides. The extraction process was carried out at room temperature for 45 min, followed by incubation in a water bath at 60 °C for 3 h and subsequent boiling for 5 min to inactivate the enzyme. The solution was cooled to room temperature and the residue was removed, after which the crude polysaccharide content was determined as described [25]. Orthogonal experiments and single-factor experiments were conducted to optimize the parameters such as papain concentration, extraction time, extraction temperature, and solid-to-liquid ratio. We repeated the measurement three times.

2.3.4. Microwave Extraction Method

Five grams of epimedium powder was soaked in distilled 100 mL water for 30 min at room temperature, before microwave treatment with a microwave extraction instrument with a power setting of 500 W for 60 min. After treatment, the precipitate was collected, dried, and the crude polysaccharide content was calculated [26]. Orthogonal experiments and single factor experiments were conducted to optimize the parameters of microwave power, extraction time, extraction temperature, and solid-to-liquid ratio. We repeated the measurement three times.

2.3.5. Purification of *Epimedium polysaccharides*

The crude epimedium polysaccharide solutions obtained by the three methods described above contain impurities such as proteins. For further purification, the 20 mL crude polysaccharides were dissolved in 1 L water and 60 mL diluent passed through a DEAE-cellulose column (Inner diameter 1.0 cm × height 40 cm). The column was first eluted with distilled water (Mobile phase A) at a flow rate of 1.5 mL/min, collecting 4 mL fractions into small tubes for a total of 20 fractions (10 mL/19 × 24, Aoqi Chemical Medical Supply chain Management Service Co., Ltd., Tianjin, China). This was followed by elution with the gradient of 0.04, 0.05, and 0.06 mol/L NaOH (Mobile phase B) at a flow rate of 1.0 mL/min, where the ratio of mobile phase A to mobile phase B is 2:3, collecting 20 fractions for each concentration. The eluted material was analyzed using the phenol-sulfuric acid method [27].

2.3.6. Infrared Spectroscopy Analysis

A background scan was first performed on a Nicolet 6700 infrared spectrometer (processing control blank). A small amount of sample was then placed on the ATR crystal for testing within the range of 400–4000 cm^{−1}, with a scan speed of 400 mm^{−1}.

2.3.7. Determination of Molecular Weight

A Waters 1515 gel permeation chromatograph equipped with a PLMIXED 7.5 × 50 mm guard column (G3000PWXL, Guangzhou Green Baicao scientific Instrument Co., Ltd., Guangzhou, China) and two PLMIXED-C 7.5 × 300 mm analytical columns (GMPW, Guangzhou Green Baicao scientific Instrument Co., Ltd., Guangzhou, China) connected in series was used. The extracted epimedium polysaccharide sample was dissolved in distilled water to prepare a uniform sample solution. The instrument was calibrated with polysaccharide standards (wkq-08909, Sichuan Vicchi Biotechnology Co., Ltd., Chengdu, China). The chromatographic analysis was performed at a constant temperature of 35 °C with a constant flow rate of 1 mL·min^{−1} through the column. The chromatogram was processed using Cirrus GPC software 2.0.

2.3.8. Liquid Chromatography Analysis

The *Epimedium polysaccharides* were hydrolyzed and derivatized, firstly, by dissolving 10.0 mg of epimedium polysaccharide powder in 4 mL of distilled water, with heating until completely dissolved. One milliliter of 0.2 mol/L trifluoroacetic acid was then added and allowed to hydrolyze at 110 °C for 8 h, after which the solution was allowed to cool to room temperature. The pH was adjusted to neutral (pH 7.0) with an appropriate amount of 0.2 mol/L NaOH, and diluted to 10 mL with distilled water to yield the sample for analysis.

One mL of the sample solution were placed in test tubes, after which 100 µL of prepared 0.02 mol/L monosaccharide standard solution (glucose, galactose, mannose, galacturonic acid, rhamnose) was added to each test tube, followed by 50 µL of 0.5 mol/L PMP solution and 50 µL of 0.3 mol/L NaOH solution. The test tubes were then placed in a water bath at 70 °C for 30 min for derivatization. The sample was then cooled to room temperature and 50 µL of 0.3 mol/L HCl solution for neutralization. Moreover, 200 mL of chloroform for extraction to remove non-sugar impurities in the derivatization reaction. The material was then centrifuged and the supernatant was analyzed to assess the types and proportions of monosaccharides by using Liquid chromatography. C18 column (518905-902 Agilent HC-C18, 250 × 4.6 mm 5 µm, Agilent Technologies, Santa Clara, CA, USA) was used as the chromatographic column. The mobile phase was acetonitrile and water, and the ratio of acetonitrile to water was 4:1, the mobile phase velocity was 0.3 mL/min, the sample size was 20 µL, the column temperature was set to 30 °C, and the UV detection wavelength was 190 nm.

3. Results

3.1. Results of the Single-Factor Test of the Ultrasonic Method

3.1.1. The Effect of Ultrasonic Power on the Extraction of *Epimedium polysaccharides*

Ultrasonic power is one of the key parameters affecting the extraction efficiency of plant polysaccharides. For the effect of ultrasonic power on the extraction of *Epimedium polysaccharides*, the ultrasound power ranged from 100–300 W at 50 W intervals, a solid–liquid ratio of 1:30 was used, and ultrasonic extraction was performed at 60 °C for 60 min. The magnitude of ultrasonic power directly affects the cavitation effect of ultrasound during the extraction process, which in turn affects the extraction efficiency [26]. As shown in Figure 1, as the power increased, the rate of polysaccharide extraction first increased and then decreased. The minimum polysaccharide yield was 2.13% at 100 W, and the maximum was 4.44% at 250 W.

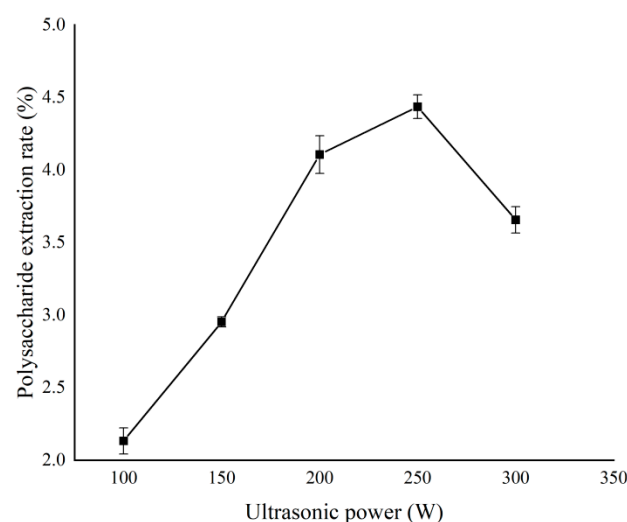


Figure 1. Effect of ultrasound power on *Epimedium polysaccharide* extraction.

3.1.2. Effect of Time on the Extraction of *Epimedium polysaccharides* by Ultrasonic Extraction

For the effect of extraction time on the extraction of *Epimedium polysaccharides*, the extraction time ranged from 30–70 min at 10 min intervals, a solid–liquid ratio of 1:30 was used, and ultrasonic power was performed at 250 W of 60 °C.

As shown in Figure 2, the effect of the extraction time on yield first increased and then decreased. This was due to the slow penetration of the ethanol slowly into the cells, dissolving the polysaccharides. The minimum polysaccharide yield was 2.47% at 30 min, and the maximum was 4.33% at 60 min. While prolongation of ultrasonication can improve the extraction efficiency by increasing the destruction of cell walls resulting in the release of polysaccharides into the solvent, there is an optimal time range for ultrasonication beyond which degradation of the polysaccharides may occur, reducing their molecular weight and potentially affecting their biological activity [28].

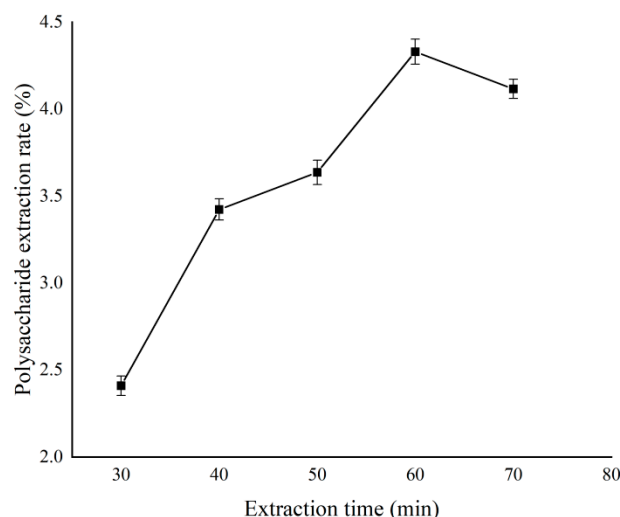


Figure 2. Effect of extraction time on *Epimedium polysaccharide* yield by ultrasonic extraction.

3.1.3. Effect of Extraction Temperature on the Yield of *Epimedium polysaccharides* by Ultrasonic Extraction

For the effect of extraction temperature on the extraction of *Epimedium polysaccharides*, the extraction temperature ranged from 30–70 °C at 10 °C intervals, a solid–liquid ratio of 1:30 was used, and ultrasonic power was performed at 250 W of 60 min.

As shown in Figure 3, as the extraction temperature increased, the polysaccharide yield first increased to a maximum and then decreased. Specifically, when the extraction temperature was set at 60 °C, the polysaccharide yield reached a peak of 4.27%. When the temperature was raised to 70 °C, the polysaccharide yield decreased, which may be related to the acoustic hole effect during ultrasonic extraction. During ultrasonication, the mechanical action of the sound waves can promote solvent penetration and material release. However, when the temperature is too high, the internal pressure of cavitation bubbles generated by the sound waves may not be sufficient to maintain their stability, weakening the cavitation effect of the ultrasound [26]. This weakened cavitation effect may be insufficient to maintain the efficient release of material, thereby affecting the polysaccharide extraction efficiency. In addition, high temperatures may also alter and even degrade the polysaccharide structure, which may also adversely affect the yield [29].

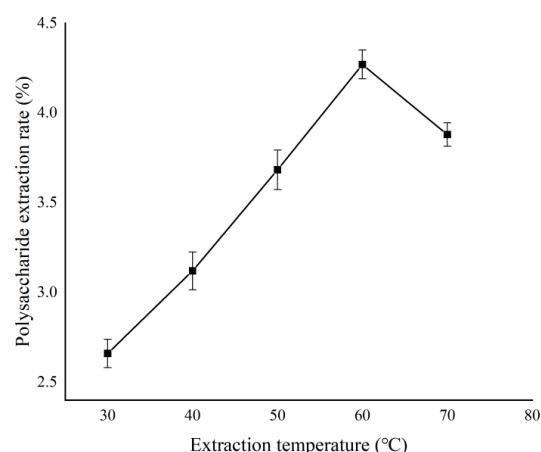


Figure 3. Effect of extraction temperature on glycan yield in *Epimedium* by ultrasonic extraction.

3.1.4. Effect of the Solid–Liquid Ratio on the Yield of *Epimedium polysaccharides* by Ultrasonic Extraction

For the effect of solid–liquid ratio on the extraction of *Epimedium polysaccharides*, the solid–liquid ratio ranged from 1:20–1:40, ultrasonic power was performed at 250 W, and ultrasonic extraction was performed at 60 °C for 60 min.

As shown in Figure 4, the material-to-liquid ratio led to an initial increase and subsequent decrease in the polysaccharide yield. The polysaccharide yield showed a maximum value of 4.11% at a material-to-liquid ratio of 1:30. Increasing the material-to-liquid ratio can enhance the permeability of the material to the solvent, increasing the extraction rate, but can also lead to a marked elevation in energy consumption [30]. In addition, too high a material-to-liquid ratio may promote the dissolution of non-target components in the raw material, not only diluting the concentration of the target polysaccharides, but also introducing impurities, thereby reducing both the purity and yield of the polysaccharides [31].

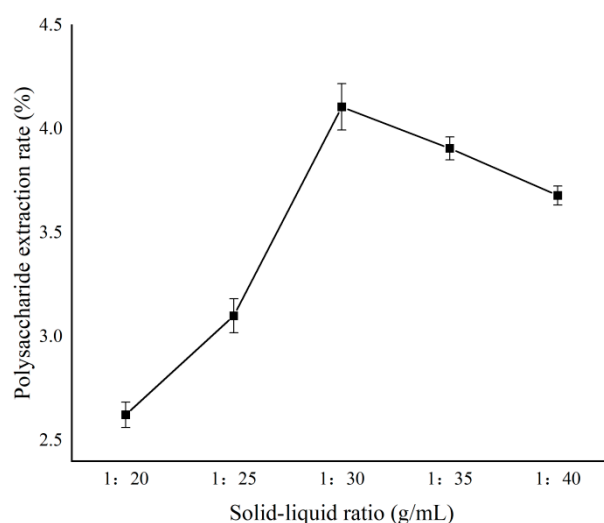


Figure 4. Effect of the liquid–material ratio on polysaccharide extraction of *Epimedium* by ultrasonic extraction.

3.1.5. Optimization of *Epimedium polysaccharide* Ultrasonic Extraction Using Orthogonal Experiments

Single-factor analysis of ultrasonic power, extraction time and temperature, and solid–liquid ratio was performed by using an orthogonal experiment of four factors at three different levels, and the optimal extraction parameters were determined through the experiment. The experimental design scheme is shown in Tables 1 and 2. The K value is the sum of indicators for each factor at each level. The K value is the average of the indicators for each factor at each level. The R value represents the range of the K value.

Table 1. Orthogonal test factor levels and coding by ultrasonic extraction.

Factor	Code	Level		
		1	2	3
Ultrasonic power (W)	A	200	250	300
Extraction time (min)	B	50	60	70
extraction temperature (°C)	C	50	60	70
solid–liquid ratio (g/mL)	D	1:25	1:30	1:35

Table 2. Orthogonal test results by ultrasonic extraction.

Test Number	A	B	C	D	Polysaccharide Extraction Rate/%
1	1	1	1	1	4.23
2	1	2	2	2	4.36
3	1	3	3	3	4.02
4	2	1	2	3	4.63
5	2	2	3	1	4.73
6	2	3	1	2	4.28
7	3	1	3	2	4.40
8	3	2	1	3	4.81
9	3	3	2	1	4.12
K ₁	12.61	13.26	13.32	13.08	
K ₂	13.64	13.90	13.11	13.04	
K ₃	13.33	12.42	13.15	13.46	
k ₁	4.20	4.42	4.44	4.36	
k ₂	4.55	4.63	4.37	4.35	
k ₃	4.44	4.14	4.38	4.49	
R	0.34	0.49	0.07	0.14	
Excellent level	A2	B2	C1	D3	
Optimal combination	A2B2C1D3				

A visual analysis of Table 2 shows that the impact of the four factors involved in ultrasonic extraction on the polysaccharide content of *Epimedium* was B > A > D > C, and the optimal extraction combination is A2B2C1D3. Since this combination was not tested in the experiments, further verification was required, the results of which are shown in Table 3.

Table 3. Ultrasonic extraction verification tests.

Experimental Combination	1	2	3	Average Extraction Rate/%
A2B2C1D3	4.92	4.82	4.81	4.85

Table 3 shows that the polysaccharide yield using the optimal combination of A2B2C1D3 was 4.85%, which was greater than the highest yield of 4.81% seen in the orthogonal experimental table.

Therefore, at an ultrasonic power of 250 W, an extraction time of 60 min, a temperature of 50 °C, and a material-to-liquid ratio of 1:35, the *Epimedium polysaccharide* yield was highest, at 4.85%.

3.2. Results of the Single-Factor Analysis of the Aqueous Enzymatic Method

3.2.1. Effect of the Papain Concentration on *Epimedium polysaccharide* Extraction

For the effect of papain concentration on the extraction of *Epimedium polysaccharides*, the papain concentration ranged from 30–110 U/mL at 20 U/mL intervals, a solid–liquid ratio of 1:30 was used, and ultrasonic extraction was performed at 50 °C for 60 min.

From Figure 5, it can be observed that the effect of enzyme concentration on polysaccharide extraction initially increased and then decreased. The maximum yield, 3.85%, was observed at an enzyme concentration of 70 U/mL, indicating that at this enzyme concentration, the hydrolysis was optimal for the release of polysaccharides. However, further increases in the enzyme concentration did not enhance the polysaccharide yield but instead reduced it. This may have been due to excessive hydrolysis of the *Epimedium* raw material or the generation of inhibitory factors during hydrolysis, adversely affecting the extraction efficiency of the polysaccharides.

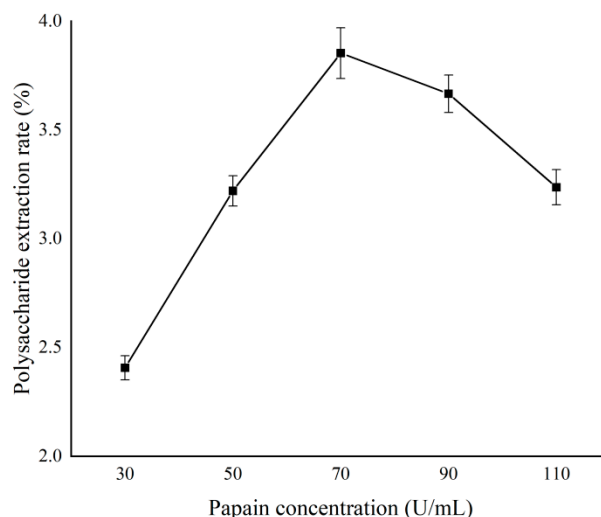


Figure 5. Effect of the papain concentration on Epimedium.

3.2.2. Effect of Extraction Time on the Extraction of *Epimedium polysaccharides* by Aqueous Enzymatic

For the effect of extraction time on the extraction of *Epimedium polysaccharides*, the extraction time ranged from 30–70 min at 10 min intervals, a solid–liquid ratio of 1:30 was used, and papain concentration was added at 70 U/mL and extraction temperature was 50 °C.

As shown in Figure 6, increased extraction time initially enhanced the yield of *Epimedium polysaccharides* after which the yield decreased. At an extraction time of 60 min, the polysaccharide yield reached a peak of 3.85%. Further prolongation of the extraction time did not lead to a continuous increase in polysaccharide yield, but rather a decrease. This may be due to the decomposition or degradation of some polysaccharides as the extraction time increased or reduced enzyme activity, thus affecting the extraction efficiency. In addition, excessively long extractions may also increase production costs without corresponding benefits.

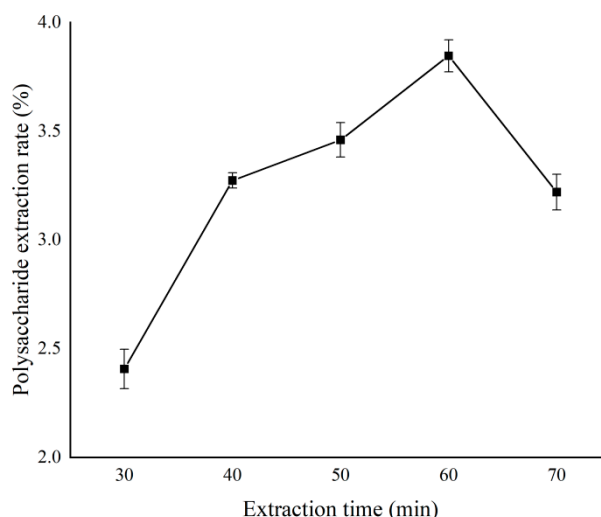


Figure 6. Effect of extraction time on Epimedium polysaccharide extraction by aqueous enzymatic.

3.2.3. Effect of Temperature on the Extraction of *Epimedium polysaccharides* by Aqueous Enzymatic

For the effect of extraction temperature on the extraction of *Epimedium polysaccharides*, the extraction temperature ranged from 30–70 °C at 10 °C intervals, a solid–liquid ratio of

1:30 was used, and papain concentration was added at 70 U/mL and extraction time was 60 min.

Figure 7 shows that increased temperature resulted in an initial enhancement in the *Epimedium polysaccharide* yield, followed by a decline. The maximum yield, 3.74%, was found at a temperature of 50 °C. However, as the temperature continued to rise and exceeded 50 °C, the polysaccharide yield declined. This may have been due to enzyme inactivation or thermal degradation of polysaccharide structures caused by excessive temperature.

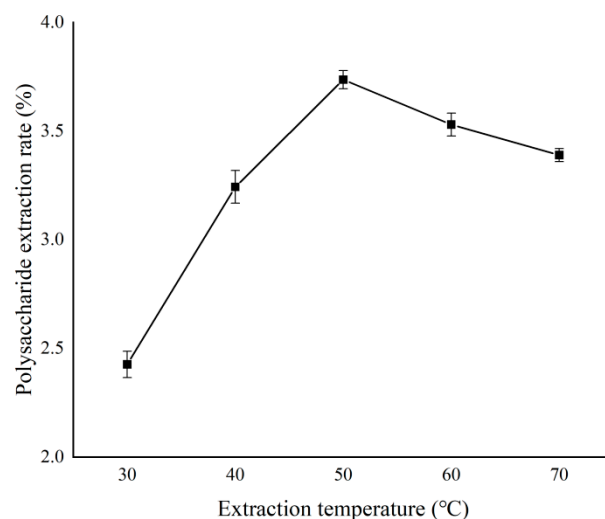


Figure 7. Effect of extraction temperature on *Epimedium polysaccharide* extraction by aqueous enzymatic.

3.2.4. Effect of the Solid–Liquid Ratio on the Yield of *Epimedium polysaccharides* by Aqueous Enzymatic

For the effect of solid–liquid on the extraction of *Epimedium polysaccharides*, the solid–liquid ranged from 1:20–1:40, papain concentration was added at 70 U/mL, and ultrasonic extraction was performed at 50 °C for 60 min.

As shown in Figure 8, increases in the material-to-liquid ratio first increased and then reduced the polysaccharide yield. The maximum yield of 3.73% was found at a material-to-liquid ratio of 1:30. An explanation is that increased material-to-liquid ratios and energy consumption may lead to the dissolution of other substances, thus diminishing the polysaccharide yield.

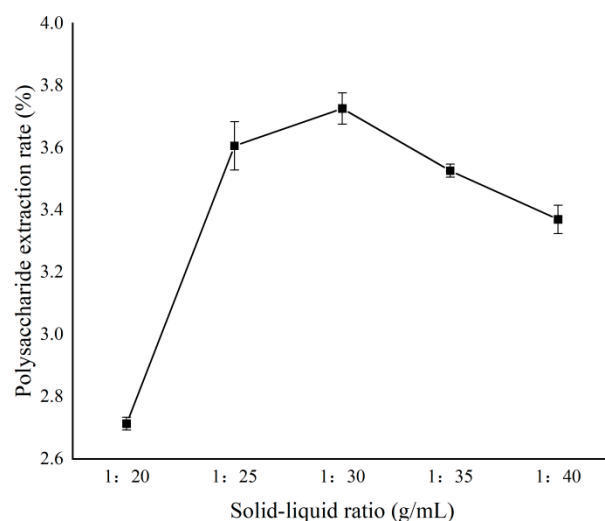


Figure 8. Effect of the liquid ratio on polysaccharide extraction of *Epimedium* by aqueous enzymatic.

3.2.5. Optimization of *Epimedium polysaccharide* Extraction Using Orthogonal Experiments by Aqueous Enzymatic

A single-factor analysis of optimal papain concentration, extraction temperature and time, and solid–liquid ratio was performed by using an orthogonal experiment of four factors at three different levels, and the optimal extraction parameters were determined through the experimental results. The experimental design scheme is shown in Tables 4 and 5.

Table 4. Orthogonal test factor table by Aqueous Enzymatic.

	Code	Level		
		1	2	3
Papain concentration (U/mL)	A	50	70	90
Extraction time (min)	B	50	60	70
Extraction temperature (°C)	C	40	50	60
Solid–liquid ratio (g/mL)	D	1:25	1:30	1:35

Table 5. Orthogonal test results by Aqueous Enzymatic.

Test Number	A	B	C	D	Polysaccharide Extraction Rate/%
1	1	1	1	1	3.60
2	1	2	2	2	3.94
3	1	3	3	3	3.64
4	2	1	2	3	4.31
5	2	2	3	1	4.29
6	2	3	1	2	4.65
7	3	1	3	2	3.76
8	3	2	1	3	3.66
9	3	3	2	1	3.89
K ₁	11.18	11.67	11.91	11.78	
K ₂	13.25	11.89	12.14	12.35	
K ₃	11.31	12.18	11.69	11.61	
k ₁	3.73	3.89	3.97	3.93	
k ₂	4.42	3.96	4.05	4.12	
k ₃	3.77	4.06	3.90	3.87	
R	0.69	0.17	0.15	0.25	
Excellent level	A2	B3	C2	D2	
Optimal combination	A2B3C2D2				

The visual analysis shown in Table 5 indicates that the effects of the four factors in the aqueous enzymatic method for *Epimedium polysaccharide* extraction was A > D > B > C, and the optimal extraction combination was A2B3C2D2. Since this combination was not tested in the above experiments, verification was required, and the results are shown in Table 6.

Table 6. Aqueous Enzymatic Verification tests.

	1	2	3	Average Extraction Rate/%
A2B3C2D2	4.67	4.59	4.89	4.72

As seen in Table 6, the polysaccharide yield using the optimized combination A2B3C2D2 was 4.72%, which was higher than the highest yield of 4.65% found in the orthogonal analysis.

Therefore, the optimal conditions for this method were a papain concentration of 70 U/mL, an extraction time of 70 min, an extraction temperature of 50 °C, and a solid:liquid ratio of 1:30, resulting in a yield of *Epimedium polysaccharides* of 4.72%.

3.3. Results of Single-Factor Analysis of the Microwave Method

3.3.1. Effect of Microwave Power on the Extraction of *Epimedium polysaccharides*

For the effect of microwave power on the extraction of *Epimedium polysaccharides*, the microwave power ranged from 200–800 W at 100 W intervals, a solid–liquid ratio of 1:25 was used, and microwave extraction was performed at 50 °C for 50 min.

As shown in Figure 9, the microwave power affected the yield of *Epimedium polysaccharides*, first increasing and then decreasing. At a power of 500 W, the polysaccharide yield was found to be maximal, with a value of 3.25%.

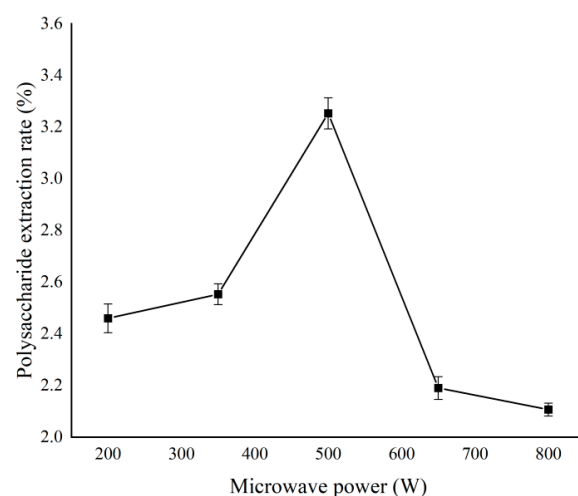


Figure 9. Effect of microwave power on *Epimedium polysaccharide* extraction.

3.3.2. Effect of Extraction Time on the Extraction of *Epimedium polysaccharides* by Microwave Extraction

For the effect of extraction time on the extraction of *Epimedium polysaccharides*, the extraction time ranged from 30–70 min at 10 min intervals, a solid–liquid ratio of 1:25 was used, microwave power was 500 W, and extraction temperature was 50 °C.

From Figure 10, it can be observed that as the extraction time increased, the polysaccharide yield first gradually increased and subsequently decreased. The maximum yield of 3.40% was obtained at an extraction time of 50 min.

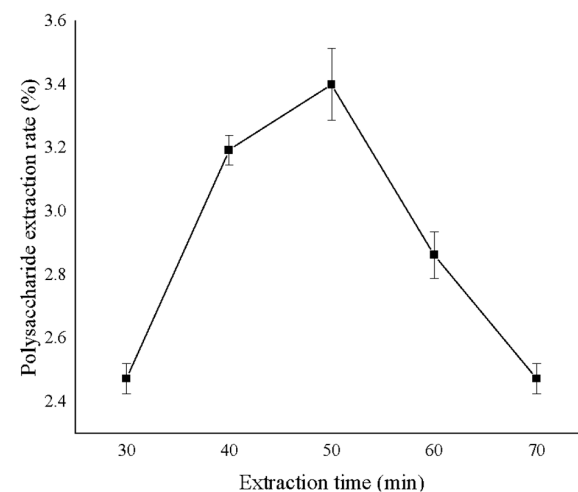


Figure 10. Effect of extraction time on *Epimedium polysaccharide* extraction by microwave extraction.

3.3.3. Effect of Temperature on the Extraction of *Epimedium polysaccharides* by Microwave Extraction

For the effect of extraction temperature on the extraction of *Epimedium polysaccharides*, the extraction temperature ranged from 30–70 °C at 10 °C intervals, a solid–liquid ratio of 1:25 was used, and the microwave power was 500 W for 50 min.

Increasing the temperature first enhanced the polysaccharide yield, which then decreased (Figure 11) with a maximum yield of 4.22% observed at a temperature of 50 °C.

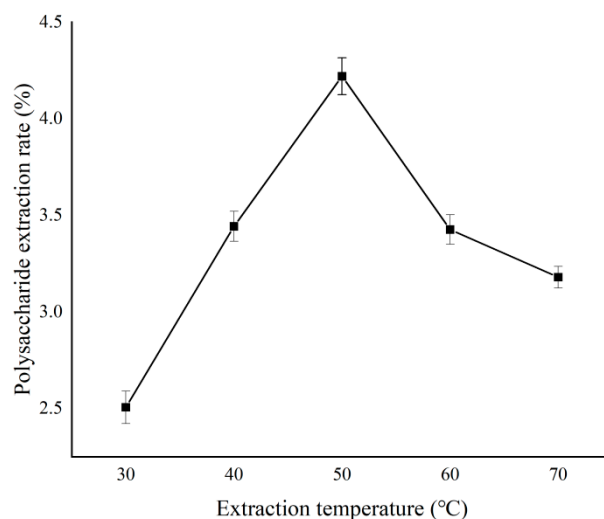


Figure 11. Effect of extraction temperature on *Epimedium polysaccharide* extraction by microwave extraction.

3.3.4. Effect of the Solid–Liquid Ratio on the Yield of *Epimedium polysaccharides* by Microwave Extraction

For the effect of solid–liquid ratio on the extraction of *Epimedium polysaccharides*, the solid–liquid ratio ranged from 1:20–1:40, microwave power was performed at 500 W, and ultrasonic extraction was performed at 50 °C for 50 min.

As shown in Figure 12, while the material-to-liquid ratio influenced the yield of *Epimedium polysaccharides*, it was relatively stable. When the ratio was 1:25, polysaccharide yield was maximal at 3.17%.

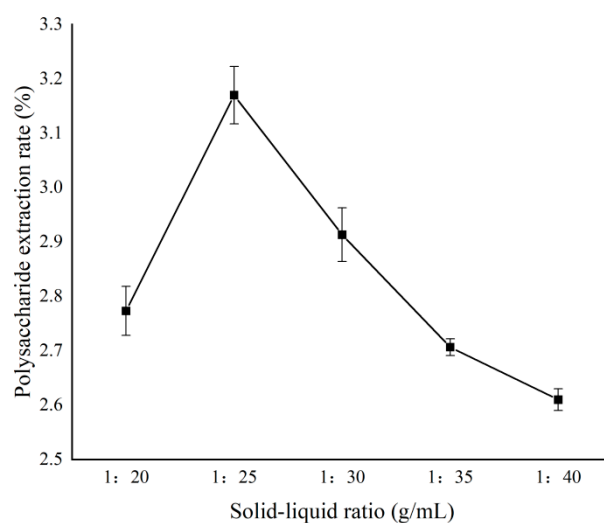


Figure 12. Effect of the solid–liquid ratio on *Epimedium polysaccharide* extraction by microwave extraction.

3.3.5. Optimization of *Epimedium polysaccharide* Extraction Using the Orthogonal Experiment by Microwave Extraction

Based on the results of the single-factor experiments on microwave power, extraction time, extraction temperature, and liquid-to-solid ratio, an orthogonal analysis was performed by using an orthogonal experiment of four factors at three different levels to determine the optimal extraction parameters. The experimental design scheme is shown in Tables 7 and 8.

Table 7. Orthogonal test factor table by microwave extraction.

Factor	Code	Level		
		1	2	3
microwave power (W)	A	350	500	650
Extraction time (min)	B	40	50	60
Extraction temperature (°C)	C	40	50	60
Solid–liquid ratio (g/mL)	D	1:20	1:25	1:30

Table 8. Orthogonal test results by microwave extraction.

Test Number	A	B	C	D	Polysaccharide Extraction Rate/%
1	1	1	1	1	3.46
2	1	2	2	2	3.31
3	1	3	3	3	3.24
4	2	1	2	3	3.22
5	2	2	3	1	3.59
6	2	3	1	2	3.43
7	3	1	3	2	3.86
8	3	2	1	3	3.89
9	3	3	2	1	3.30
K ₁	10.01	10.54	10.78	10.35	
K ₂	10.24	10.79	9.83	10.60	
K ₃	11.05	9.97	10.69	10.35	
k ₁	3.34	3.51	3.59	3.45	
k ₂	3.41	3.60	3.28	3.53	
k ₃	3.68	3.32	3.56	3.45	
R	0.35	0.27	0.32	0.08	
Excellent level	A3	B2	C1	D2	
Optimal combination	A3B2C1D2				

As seen in Table 8, the influence of the four factors in the aqueous enzymatic method on the polysaccharide yield was A > C > B > D, with an optimal extraction combination of A3B2C1D2. Since this combination was not tested in the above experiments, verification was performed (Table 9).

Table 9. Verification tests by microwave extraction.

Experimental Combination	1	2	3	Average Extraction Rate/%
A3B2C1D2	3.88	4.01	4.06	3.98

Table 9 shows that the yield of polysaccharides using the optimal combination of A3B2C1D2 was 3.98%, which was higher than the highest yield of 3.89% shown in the orthogonal analysis table. Therefore, when the microwave power was 650 W, the extraction

time was 50 min, the temperature was 40 °C, and the ratio of material to liquid was 1:25, the yield of *Epimedium polysaccharide* was highest, at 3.98%.

A comprehensive comparison of three different extraction methods for *Epimedium polysaccharides* indicated that the ultrasonic-assisted extraction method performed the best in terms of extraction yield. In addition, the three methods also differed in the purity of the extraction solution. Specifically, the polysaccharide solution obtained by the microwave-assisted extraction method showed poor clarity and transparency, with the presence of a large amount of impurities, seen in the dark color of the solution. This may have been due to thermal degradation or other side reactions occurring during microwave heating, leading to increased impurities in the extract. In contrast, the solution obtained after aqueous enzymatic extraction contained fewer impurities, with a yellowish color, although further improvement is necessary. In contrast, the solution obtained after ultrasonic extraction was light yellow in color, clear and transparent, with a low impurity content. This indicates that ultrasonic extraction had significant advantages in maintaining the purity of the extraction solution, possibly related to its stronger ability to break down plant cell walls, allowing the release of the polysaccharides and reducing the mixing of impurities. Evidence indicates that ultrasound-assisted extraction can not only improve the extraction rate, but also increase the activity of the extracted polysaccharides [23]. Considering the extraction efficiency, clarity, and impurity content of the extraction solutions, ultrasonic extraction is, thus, considered to be more suitable for the extraction of *Epimedium polysaccharides*.

3.4. Purification and Characterization of *Epimedium polysaccharides*

3.4.1. Analysis of Purified *Epimedium polysaccharides*

The yield of polysaccharides extracted using ultrasonication and purified using DEAE-cellulose was determined to be 4.13%, while that obtained by the aqueous enzymatic method was 3.67% and that after microwave extraction was 3.12%, with both the latter purified with DEAE-cellulose.

3.4.2. Infrared Spectroscopy

Figure 13 shows the infrared spectra of *Epimedium polysaccharides*, with characteristic absorption peaks at wavelengths of 3600–3200, 3000–2800, 1400–1200, and 1200–1000 cm^{-1} . The absorption peak at 3374.51 cm^{-1} is attributed to the stretching and vibration of O-H and N-H, which may be related to the hydrogen bonds present in polysaccharides; the absorption peak at 2930.1 cm^{-1} is indicative of C-H stretching vibrations, which are commonly found in the alkyl structure of polysaccharides, while the absorption peak at 1597.98 cm^{-1} represents stretching vibrations of C=O or -CHO, suggesting that the polysaccharide may contain carbonyl or aldehyde groups. The absorption peak at 1219.19 cm^{-1} may be related to in-plane deformation vibrations of C-H.

3.4.3. Molecular Weight Determinations

After dissolving *Epimedium polysaccharides* in deionized water, their molecular weights were measured by size exclusion chromatography (SEC). As shown in Table 10, the number average molecular weight (Mn), weight average molecular weight (Mw), and Z-average molecular weight (Mz) of the obtained *Epimedium polysaccharides* are 1.65×10^5 Da, 6.61×10^5 Da, and 17.3×10^5 Da, respectively, with a PDI of 4.00, indicating a relatively broad molecular weight distribution of *Epimedium polysaccharides* (See Supplementary Materials for details).

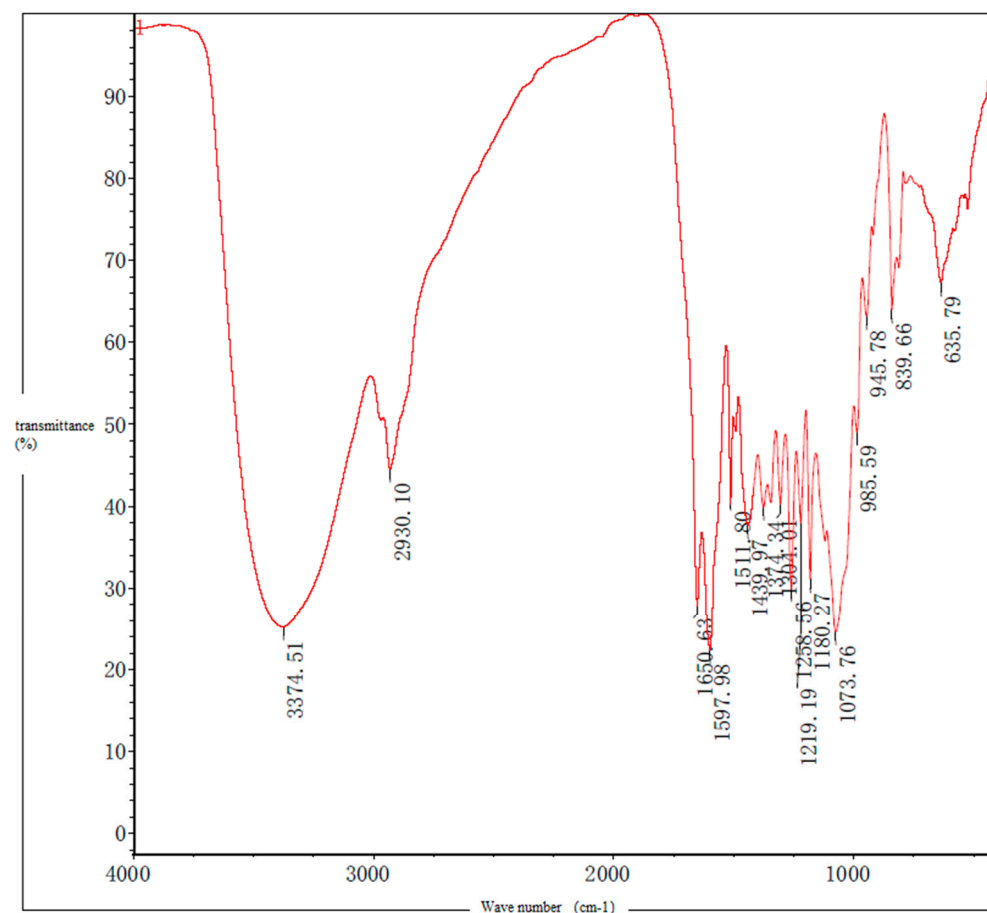


Figure 13. Infrared spectrum analysis results of *Epimedium polysaccharide*.

Table 10. Molecular weight determination results.

Mn/Da	Mw/Da	Mz/Da	PDI
165,318	661,479	1,730,311	4.00125

3.4.4. Liquid Chromatography Results

The monosaccharide composition of the *Epimedium polysaccharide* extract was assessed by HPLC, and the results are shown in Table 11. Analysis of the results showed that the *Epimedium polysaccharide* extract was composed of five monosaccharides, of which glucose was the main monosaccharide component, accounting for 60.2%, followed by galactose, accounting for 30.7% of the total. The contents of mannose, galacturonic acid, and rhamnose were relatively low, accounting for 5.4%, 0.4%, and 0.3%, respectively. The remaining components were impurities such as protein, accounting for 3%.

Table 11. Composition results of five monosaccharides.

Glucose	Galactose	Mannose	Galacturonic Acid	Rhamnose
60.2%	30.7%	5.4%	0.4%	0.3%

4. Conclusions

The extraction yield of *Epimedium polysaccharides* is a direct reflection of the efficiency of the extraction method. This study investigated the extraction and purification of *Epimedium polysaccharides* using different methods. It was found that the optimal method was ultrasonic-assisted extraction using the parameters of 250 W power, 60 min extraction

time, an extraction temperature of 50 °C, and a material-to-liquid ratio of 1:35, with which the *Epimedium polysaccharide* yield was 4.85%. After purification on DEAE-cellulose, the yield of the ultrasonic method was higher than that of the microwave method, and much higher than that of the aqueous enzymatic method. Analysis of the extracts showed the presence of five monosaccharides, namely, glucose, galactose, mannose, galacturonic acid, and rhamnose. The number average molecular weight (Mn) was 16.5×10^4 Da and the weight average molecular weight (Mw) was 17.3×10^5 Da.

In conclusion, the findings of this study underscore the significance of the ultrasonic-assisted extraction method in enhancing the yield of *Epimedium polysaccharides*. The results not only demonstrate the superiority of this method over other tested methods, but also highlight the potential of these polysaccharides in various applications due to their unique composition and molecular weight characteristics. The identification of the monosaccharide constituents and the determination of their molecular weights provide valuable insights into the structural properties of the extracted polysaccharides, which could be instrumental in further research and development of health-promoting products and pharmaceuticals. The relevance of these results extends beyond the scope of this study, contributing to a broader understanding of efficient extraction techniques and their impact on the quality and quantity of bioactive compounds derived from natural sources.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/separations1100296/s1>, Figure S1. Analysis of polysaccharide extract of Epimedium by HPLC. Figure S2. Chromatogram of mixed standard. Figure S3. The data of Molecular weight determinations.

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