



Determination of Polysaccharide Content, Optimization of Extraction Conditions, Monosaccharide Composition and Antioxidant Activity Study in Different Varieties of *Gastrodia elata* Blume

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Abstract

This study investigated the impact of various extraction conditions, such as liquid-to-solid ratio, ultrasonic temperature, and extraction time, on the yield of *Gastrodia elata* polysaccharides through single-factor experiments. The polysaccharide extraction conditions were optimized using response surface methodology by constructing a quadratic polynomial regression model. Monosaccharide composition and content in different varieties of *Gastrodia elata* were determined using high-performance liquid chromatography (HPLC), and the in vitro antioxidant activity of these polysaccharides was analyzed. The results revealed that the optimal conditions for determining the polysaccharide content of *G. elata* using the 3,5-dinitrosalicylic acid method were as follows: a detection wavelength of 540 nm, 2 mL of chromogenic reagent, a chromogenic time of 6 minutes, a chromogenic temperature of 90 °C, a total sugar hydrolysis time of 10 minutes. Through response surface experiments, the optimal extraction conditions for the polysaccharides were determined: *G. elata* polysaccharides were primarily composed of six monosaccharides, with glucose accounting for over 95% of the composition, along with galactose, mannose, rhamnose, and so on. This study provides a theoretical foundation for the environmentally friendly determination of polysaccharide content in *G. elata* and the composition of its nutritional components, specifically its polysaccharides.

Keywords: *Gastrodia elata* polysaccharide; Response surface optimization; Monosaccharide composition; Antioxidant activity; 3,5-dinitrosalicylic acid method.

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1. Introduction

Different from the synthetic polymers like petroleum-based polymers,^[1-5] natural products especially natural polymers have attracted great interests.^[6-9] *Gastrodia elata* Blume, a mycoheterotrophic herbaceous plant belonging to the Orchidaceae family, thrives in temperate forests at altitudes ranging from 400 to 3200 meters. It is predominantly distributed in regions such as Yunnan, Guizhou, Shanxi, and Anhui in China, and is renowned as one of the valuable traditional Chinese medicinal herbs.^[10-12] During the cultivation of *G. elata*, various variations emerged, distinguishing it based on the criteria such as the color of the flower stem and the shape of the tuber, resulting in varieties

such as *G. elata* Bl. F. *Glaucia* S. Chow, *G. elata* Bl. F. *elata*, and *G. elata* Bl. F. *viridis* MaKino, as shown in Fig. 1. *G. elata* is known for its efficacy in alleviating various ailments and disorders, including hypertension, mental disorders, antioxidative effects, wind-dispelling, meridian-opening properties, and anti-aging.^[13-15] The primary active constituents in *G. elata* include gastrodin, parishin, and polysaccharides.^[16,17] In recent years, various biological activities of polysaccharides have gradually come to light.^[18] Polysaccharides are linear polymers connected by glycosidic bonds, possessing a multitude of functions, including immune cell activation and enhancement of the body's immune response.^[19,20] They have demonstrated significant effects in the treatment of conditions such as HIV/AIDS, anti-tumor responses, and anti-aging.^[21,22] Plant-derived polysaccharides exhibit diverse biological activities and are considered highly safe for human consumption. Consequently, the extraction and isolation of polysaccharides from medicinal herbs have

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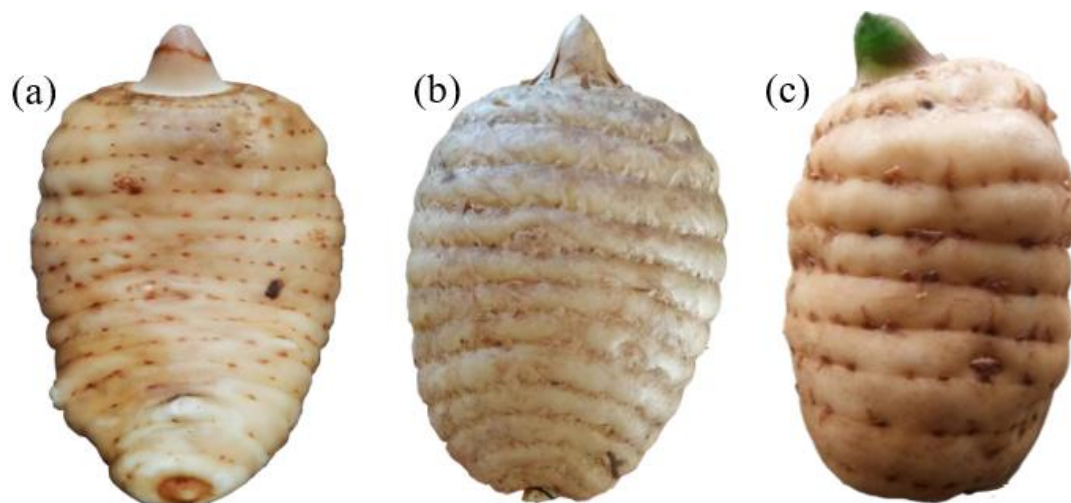


Fig. 1: Various *G. elata* Varieties (a. *G. elata* Bl. F. *elata*; b. *G. elata* Bl. F. *Glauca* S. Chow; c. *G. elata* Bl. F. *viridis* MaKino).

evolved into a pivotal research domain.

Numerous studies have highlighted the significant medicinal properties of *G. elata* polysaccharides. For example, Ming et al.^[23] discovered their lipid-lowering activity, while Chen et al.^[24] found evidence of their anti-cancer properties. Furthermore, *G. elata* polysaccharides have been associated with pharmacological effects such as delaying skeletal muscle aging, immune modulation, and anti-dizziness effects.^[25-27] Importantly, polysaccharides exhibit minimal side effects, garnering extensive attention from researchers. The extraction methods for polysaccharides primarily include hot water extraction, ultrasound-assisted extraction, and enzyme-assisted extraction, fermentation etc.^[28-30] Among others, ultrasound-assisted extraction leverages the mechanical disruption of *G. elata* cell walls using ultrasound energy, combined with thermal effects, to accelerate the release of components from the cells. Compared to other methods, ultrasound-assisted extraction offers advantages such as shorter extraction times, higher extraction efficiency, and the preservation of polysaccharide activity to the maximum extent. It is considered as a favorable method for polysaccharide extraction.^[31]

Currently, the commonly employed methods for determining polysaccharide content include the phenol-sulfuric acid, 3,5-dinitrosalicylic acid (DNS), and anthrone-sulfuric acid methods.^[32,33] Compared to other methods, the DNS method not only enables the simultaneous measurement of reducing sugars and total sugars but also eliminates the need for highly corrosive concentrated sulfuric acid during the measurement process, making it an environmentally friendly

and convenient method. The DNS method was employed for sugar analysis. This method ingeniously combines the two purposes of removing impurity interference and calculating the sugar content into one. It can automatically correct the influence of the original reducing sugars and other reducing impurities in the sample. This is particularly important for Chinese medicinal materials, which may contain endogenous reducing sugars or other reducing substances, as it can effectively prevent the overestimation of the content.

Most of the reported methods for determining *G. elata* polysaccharide content primarily rely on the phenol-sulfuric acid method,^[34] with limited documentation on the use of the DNS method for *G. elata* polysaccharide content determination. Li et al.^[35] found that *G. elata* polysaccharides from Hanzhong mainly consist of rhamnose, galactose, glucose, xylose, and mannose. Ji et al.^[36] in their investigation of *G. elata* from Sichuan reported that it is primarily composed of glucose, with significant variations in content among different varieties. However, there is currently limited research on the monosaccharide composition of *G. elata* polysaccharides from various varieties in Yunnan Province.

In this study, *G. elata* Bl. F. *elata* was used as the raw material to optimize the DNS method for determining *G. elata* polysaccharide content. The impact of extraction conditions, such as the liquid-to-solid ratio, ultrasonic temperature, ultrasonic time, and ultrasonic power on *G. elata* polysaccharide yield was investigated through single-factor experiments. Subsequently, the Box-Behnken response surface experiment was employed to optimize the polysaccharide extraction conditions and a predictive model was constructed. Under these optimized conditions, we determined the polysaccharide content of three *Gastrodia elata* varieties (*G. elata* Bl. F. *Glauca* S. Chow, *G. elata* Bl. F. *elata*, and *G. elata* Bl. F. *viridis* MaKino) and conducted an analysis of the in vitro antioxidant activity of *G. elata* polysaccharides. Finally, the monosaccharide composition and content of various *G. elata* polysaccharides from different species were determined using high-performance liquid

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chromatography (HPLC). This study provides essential data to support the determination of *G. elata* polysaccharides and the analysis of their structural composition.

2. Materials and methods

2.1 Materials and reagents

The *G. elata* Bl. F. *Glaucula* S. Chow, *G. elata* Bl. F. *elata* and *G. elata* Bl. F. *viridis* MaKino used in this experiment were all sourced from Zhaotong City, Yunnan Province, China. Chromatographic grade methanol and trifluoroacetic acid (Shanghai Anpel Experimental Technology Co., Ltd). Chromatographic grade Glucose (Glc), Mannose (Man), Rhamnose (Rha), Glucuronic Acid (GlcA), Galacturonic Acid (GalA), Galactose (Gal), NaOH, and NaAc (Sigma Corporation Co., Ltd). Analytical grade D-anhydrous glucose, hydrochloric acid, concentrated sulfuric acid, sodium hydroxide, potassium ferricyanide, ferric chloride, potassium persulfate, and ethanol (Aladdin Reagent Co., Ltd, Shanghai, China). Reagent grade ABTS (1,1-Diphenyl-2-picrylhydrazyl Free Radical), DPPH (2,2-Diphenyl-1-picrylhydrazyl), and DNS reagent (Shanghai Yuanye Biotechnology Co., Ltd).

2.2 Experimental methods

2.2.1 *Gastrodia elata* polysaccharide content determination using DNS method

2.2.1.1 Preparation of test samples

The weighed 1.0 g of *G. elata* Bl. F. *elata* samples had passed through a 60-mesh sieve, into a 50 mL centrifuge tube. 25 mL of deionized water was added and subjected it to ultrasonication at a frequency of 80 kHz and a temperature of 50 °C for 25 minutes. The extract was then removed and allowed to stand at room temperature, followed by centrifugation at 5000 rpm for 10 minutes. The supernatant was collected to obtain the test sample solution for reducing sugar analysis.

5 mL of the aforementioned reducing sugar test sample solution was transferred into a 50 mL stoppered test tube. 5 mL of 6 mol/L HCl solution was added and heated in a 90 °C water bath for 10 minutes, then rapidly cooled it to room temperature. The mixture was neutralized with a 40% NaOH solution to obtain the total sugar test sample solution.

2.2.1.2 Optimization of conditions

All color development steps followed the same procedure: after adding DNS reagent, the mixture was incubated in a boiling water bath for 6 min, cooled, and diluted to 25 mL with water.

(1) Determination of Detection Wavelength

Following the method outlined by Gao et al.^[37] 2 mL of glucose standard solution and 2 mL of distilled water were precisely transferred into separate 50 mL stoppered test tubes. 2 mL of DNS reagent was added to each tube, mixed thoroughly, and incubated in a boiling water bath for 6 minutes. The glucose color solution (with distilled water as the blank), DNS reagent (with distilled water as the blank), and

glucose color solution (with DNS reagent as the blank) were scanned using a spectrophotometer in the range of 400-800 nm.

(2) Determination of Chromogenic Agent Quantity, Time and Temperature

2 mL of the reducing sugar test sample solution was accurately pipetted into a 50 mL stoppered test tube. A specific volume of chromogenic reagent (DNS reagent) (0.5-3.0 mL) was added and color development was performed in a water bath at varying temperatures (60-100 °C) for different time intervals (2-12 minutes). The absorbance was measured at 540 nm using a spectrophotometer.

(3) Determination of Total Hydrolysis Time

5 mL of the reducing sugar test sample solution was accurately pipetted, 5 mL of 6 mol/L hydrochloric acid was added, and hydrolysis was performed in a boiling water bath for 5, 8, 10, 15, and 20 minutes, respectively. The solution was removed, cooled under running tap water to room temperature, and neutralized with a 40% NaOH solution. 2 mL of the solution was taken into a 50 mL stoppered test tube, 2 mL of DNS reagent was added, mixed well, and reacted in a 90 °C water bath for 6 minutes. The absorbance was measured at 540 nm using a spectrophotometer.

2.2.1.3 Calculation of *Gastrodia elata* polysaccharide content

A standard curve was constructed using glucose as a reference, following the method outlined by Xu Juan.^[38] The mass concentration of glucose (C, mg/mL) was plotted on the x-axis, and the absorbance (A) was plotted on the y-axis. The resulting regression equation was $A = 0.516C + 0.003$, with an R^2 value of 0.9993. This standard curve demonstrates a strong linear relationship for glucose within the range of 0.1 to 0.5 mg/mL.

The calculation of reducing sugar, total sugar, and polysaccharide content was performed using the following formula in conjunction with the standard curve Eq. (1-3).^[37]

$$P_{\text{reducing sugar}}/\% = \frac{C_1 \times V_1}{m \times 1000} \times 100\% \quad (1)$$

$$P_{\text{total sugar}}/\% = \frac{C_2 \times V_2}{m \times 1000} \times \text{Dilution factor} \times 0.9 \times 100\% \quad (2)$$

$$P_{\text{polysaccharide}}/\% = P_{\text{total sugar}} - P_{\text{reducing sugar}} \quad (3)$$

where C_1 : The concentration of reducing sugar/total sugar, mg/L; C_2 : The concentration of total sugar, mg/L; V_1 : Volume of reducing sugar extraction solution, mL; V_2 : Volume of total sugar extraction solution, mL; m : Sample weight, mg.

2.2.2 Exploration of *Gastrodia elata* Polysaccharide Extraction Conditions

2.2.2.1 Single-factor experiments

The effects of liquid-to-solid ratio (10:1, 20:1, 30:1, 40:1, 50:1

Table 1: Factors and level design for response surface experiments.

Factor	Level		
	-1	0	1
Liquid-to-solid ratio (mL/g)	20	30	40
Ultrasonic temperature (°C)	30	40	50
Extraction time (min)	20	25	30

mL/g), extraction time (10, 15, 20, 25, 30 minutes), ultrasonic temperature (20, 30, 40, 50, 60 °C), and ultrasonic frequency (60, 70, 80, 90, 100 kHz) on the yield of *G. elata* polysaccharides from *G. elata* Bl. *F. elata* were investigated using the single-factor experiments. Each experiment was conducted in triplicate.

2.2.2.2 Response surface experiments

Building upon the single-factor experiments, a Box-Behnken experimental design was conducted using the Design Expert 10, with a fixed ultrasonic frequency of 80 kHz. Liquid-to-solid ratio (X_1), ultrasonic temperature (X_2) and extraction time (X_3) were chosen as the influencing factors, with the yield of *G. elata* Bl. *F. elata* polysaccharides as the response variable. The response surface optimization experiment was designed as a 3-factor, 3-level experiment, and the factors and levels of the design are presented in Table 1.

2.2.3 In Vitro antioxidant activity of *Gastrodia elata* polysaccharides

The obtained solution after ultrasonic extraction was subjected to vacuum concentration, alcohol precipitation, followed by the Sevage method to remove proteins, and then subjected to alcohol precipitation again. The resulting material was freeze-dried to obtain crude polysaccharides. Precision was ensured by preparing three *G. elata* polysaccharide solutions with mass concentrations of 1.5 mg/mL, 3.5 mg/mL, 5.5 mg/mL, 7.5 mg/mL, and 9.5 mg/mL as the test solutions.

2.2.3.1 Determination of DPPH radical scavenging capacity

Two milliliters of each polysaccharide sample solution at each concentration were taken in graduated test tubes. Under subdued light conditions, 2 mL of 0.1 mg/mL DPPH ethanol solution was sequentially added to each test tube, and the reaction was allowed to proceed at room temperature in the dark for 30 minutes. The absorbance values of the solutions were measured at 517 nm. Anhydrous ethanol solution was used as a blank control. All experiments were performed in triplicate. The scavenging rate of polysaccharide samples on DPPH free radicals was calculated according to the following Eq.(4).^[39]

$$K_{DPPH}/\% = \left(1 - \frac{A_s - A_r}{A_0}\right) \times 100\% \quad (4)$$

where A_0 : Absorbance value of 2 mL anhydrous ethanol and 2 mL DPPH solution; A_s : Absorbance value after the reaction of 2 mL sample solution and 2 mL DPPH solution; A_r :

Absorbance value of 2 mL sample solution and 2 mL anhydrous ethanol solution.

2.2.3.2 ABTS radical scavenging capacity assay

Precise preparation of a 7 mmol/L ABTS solution and a 2.45 mmol/L potassium persulfate solution were carried out. These solutions were mixed in equal volumes at room temperature and allowed to react in the dark for 12 to 16 hours to obtain the required ABTS working solution. Before use, it was diluted with distilled water to achieve an OD value of 0.7 ± 0.02 at 734 nm. Subsequently, 40 μ L of each polysaccharide sample solution was placed in graduated test tubes. Following this, 160 μ L of ABTS solution was sequentially added to the test tubes under light-avoiding conditions. The reaction was allowed to proceed for 6 minutes at room temperature in the dark. The absorbance values were measured at a wavelength of 734 nm, with distilled water used for zero calibration. All experiments were repeated three times. The scavenging rate of *G. elata* polysaccharides on ABTS free radicals was calculated according to the following Eq. (5).^[40]

$$K_{ABTS}/\% = \left(1 - \frac{A_s - A_r}{A_0}\right) \times 100\% \quad (5)$$

where A_0 : Absorbance value of 40 μ L ethanol and 160 μ L ABTS working solution; A_s : Absorbance value after the reaction of 40 μ L sample solution and 160 μ L ABTS working solution; A_r : Absorbance value of 40 μ L sample solution and 160 μ L ethanol.

2.2.3.3 Reduction capacity determination

0.2 mL of *G. elata* polysaccharide sample solution at each concentration was separately pipetted into test tubes. Subsequently, 0.5 mL of phosphate buffer solution (PBS, pH=6.6) was added to each tube, followed by 0.5 mL of 1% potassium ferricyanide solution. After thorough mixing, the mixtures were incubated at a constant temperature of 50 °C for 20 minutes. Following this incubation, 0.5 mL of 10% trichloroacetic acid solution was introduced. The tubes were then centrifuged at 3000 rpm for 10 minutes, and 0.5 mL of the supernatant was collected. To this supernatant, 0.5 mL of distilled water and 0.1 mL of 0.1% ferric chloride solution were sequentially added. The solutions were allowed to stand for 10 minutes, and the absorbance was measured at 700 nm.

2.2.4 Determination of monosaccharide composition

2.2.4.1 Sample pretreatment

Standard preparation: A 10 mg/mL monosaccharide standard stock solution was prepared. Then, 5 μ L each of Glc, Gal, Man,

Table 2: Regression equations, linear ranges, and detection limits of monosaccharide standards.

Monosaccha	Regression equation	R^2	Linear range ($\mu\text{g/mL}$)	Detection limit ($\mu\text{g/mL}$)
Man	$Y=40112.37X-7018.67$	0.9999	0.5-40	0.5
Rha	$Y=29822X-25770$	0.9995	0.4-32	0.2
GlcA	$Y=39322X-3127$	0.9997	0.6-48	0.5
GalA	$Y=43292X-46544$	1.0000	0.6-48	0.5
Glc	$Y=37523X-65900$	0.9990	0.4-32	0.2
Gal	$Y=47783X-33085$	0.9915	0.4-32	0.1

Fuc, Ara, Xyl, GalA, and Galc monosaccharide stock solutions were taken, followed by the addition of 0.5 mL of a 2 mol/L trifluoroacetic acid ($\text{C}_2\text{HF}_3\text{O}_2$) solution. The hydrolysis was carried out at 120 °C for 120 minutes, followed by simultaneous nitrogen drying with the samples.

Sample preparation: The weighed 5 mg of *G. elata* polysaccharide sample was added to 0.5 mL of a 2 mol/L trifluoroacetic acid solution, and hydrolyzed at 120 °C for 120 minutes, followed by nitrogen drying. The above samples were dissolved in deionized water and adjusted to volume. Subsequently, they were filtered through a 0.22 μm microfiltration membrane before measurement.

2.2.4.2 HPLC conditions

The sample extracts were analyzed by high-performance anion-exchange chromatography (HPAEC) on a CarboPac PA-10 anion-exchange column (4.6 by 250 mm; Dionex) using a pulsed amperometric detector (PAD; Dionex ICS 5000 system).^[41] The conditions for the HPLC are as follows: flow rate of 0.5 mL/min; injection volume of 5 μL ; solvent system, B: (0.1M NaOH, 0.2M NaAc); gradient program, 95:5 V/V at 0 min, 80:20 V/V at 30 min, 60:40 V/V at 30.1 min, 60:40 V/V at 45min, 95:5 V/V at 45.1 min, 95:5 V/V at 60 min.

2.2.4.3 Construction of monosaccharide standard curves

Different gradient concentrations (0.4-50 $\mu\text{g/mL}$) of monosaccharide standard solutions were prepared. Quantification was performed using the external standard method, with the peak area (Y) as the ordinate and various monosaccharide mass concentrations (X) as the abscissa. Standard curves were plotted, and the regression equations, linear relationships, and detection limits for each monosaccharide were obtained, as shown in Table 2. It can be observed that the linear correlation coefficients for all six monosaccha.

3. Results and analysis

3.1 Analysis of polysaccharide content by DNS method

3.1.1 Selection of detection wavelength

As shown in Fig. 2, the wavelength at the maximum absorption peak is 488 nm. It is common to choose the wavelength corresponding to the maximum absorption peak for measurement to improve the sensitivity. However, since DNS reagent has some absorbance at 488 nm, and during the color development period, the DNS content in the blank

sample is higher than that in the test solution, it can have some influence on the absorbance measurement. Therefore, it is not suitable to select 488 nm as the detection wavelength. From Fig. 2, it can also be observed that DNS reagent and distilled water have some absorbance at wavelengths below 540 nm. When the wavelength exceeds 540 nm, the absorbance of DNS reagent and distilled water approaches zero. Therefore, it is advisable to choose 540 nm as the detection wavelength, which is consistent with the wavelength reported in the literature.

3.1.2 Determination of reagent amount, time, and temperature for color development

The effects of key parameters on detection are presented in Fig. 3. As shown in Fig. 3a, absorbance increased with DNS reagent volume up to 2.0 mL, beyond which no significant increase was observed, indicating complete reaction of reducing sugars. Thus, 2.0 mL was selected as the optimal volume. Fig. 3b shows that absorbance increased with color development time up to 6 min, after which it plateaued. Similarly, absorbance increased with temperature up to 90 °C and then leveled off (Fig. 3c). Accordingly, 6 min and 90 °C were chosen as the optimal color development time and temperature, respectively.

3.1.3 Determination of total hydrolysis time

As illustrated in Fig. 4, absorbance increased with hydrolysis time up to 10 min, reaching a maximum, and then slightly decreased, likely due to degradation of the hydrolyzed products upon prolonged heating. These results align with findings by Gao et al. on total sugar determination in sugar beet using the DNS method. Therefore, 10 min was selected as the optimal hydrolysis time.

3.1.4 Reproducibility experiments

Six replicates of the *G. elata* Bl. *F. elata* polysaccharide samples were taken, and repeatability experiments were conducted under the optimal conditions determined in Sections 3.1.2 and 3.1.3. The results are shown in Table 3, with RSD values of 3.81% for reducing sugar, 2.66% for total sugar, and 2.95% for polysaccharides, indicating excellent repeatability and reproducibility of the method.

3.2 Optimization of *Gastrodia elata* polysaccharide extraction conditions

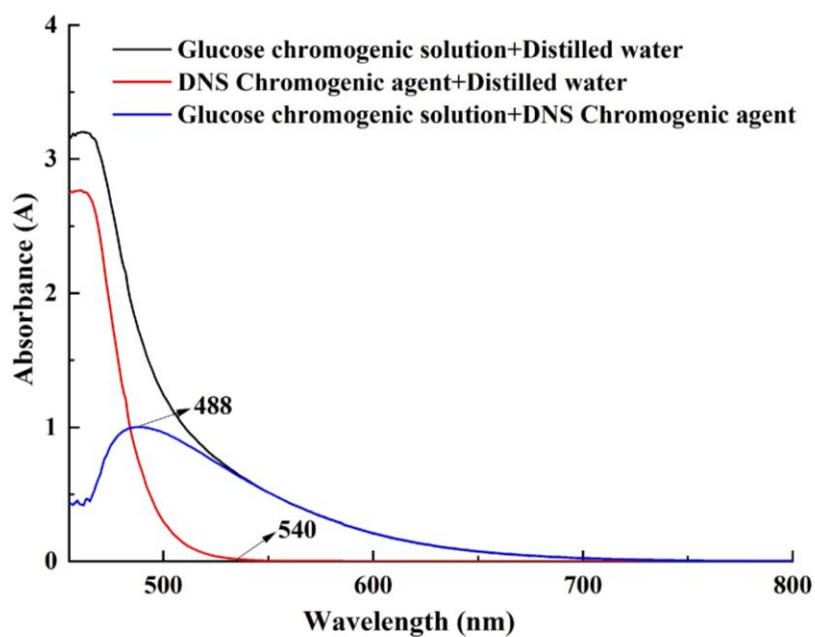


Fig. 2: The absorption spectrum with glucose solution.

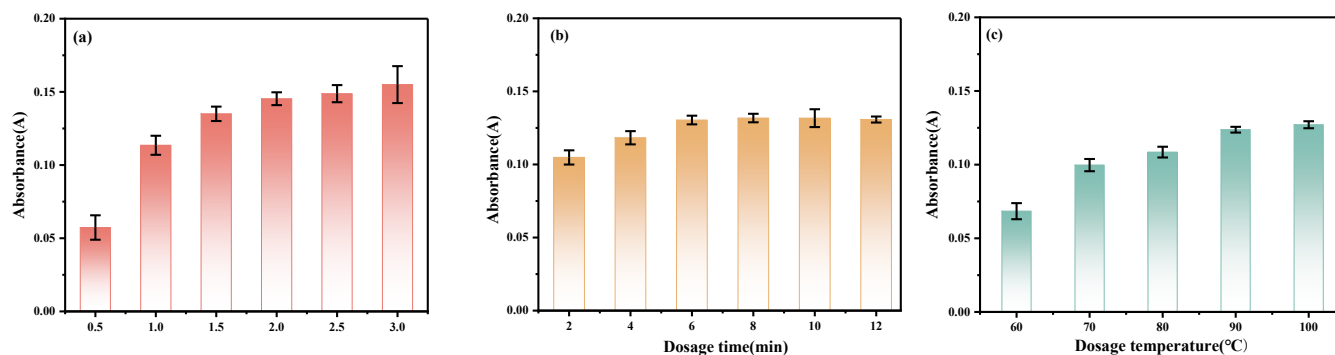


Fig. 3: Influence of Various Factors on Detection Results (a. The amount of chromogenic reagent, b. The color development time, c. The color development temperature).

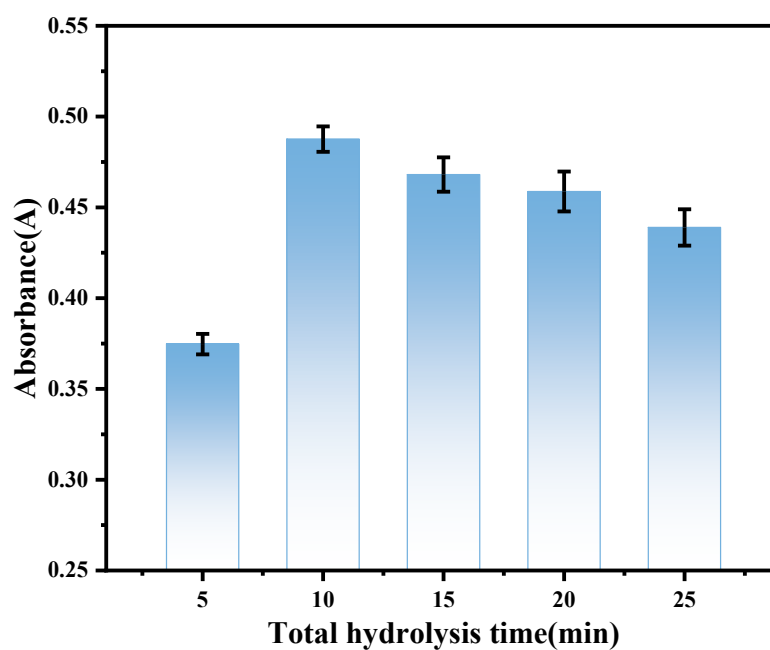


Fig. 4: Influence of total hydrolysis time.

Table 3: Results of reproducibility test.

Component to be tested	Content /%						AVE±RSD/%
Reducing sugar	2.62	2.47	2.47	2.47	2.37	2.62	2.50±3.81
Total sugar	27.47	29.51	28.34	27.76	27.76	28.63	28.25±2.66
Polysaccharide	24.85	27.03	25.87	25.29	25.39	26.02	25.74±2.95

3.2.1 Analysis of single-factor experiments

The impact of liquid-to-solid ratio on the extraction yield of *G. elata* polysaccharides is shown in Fig. 5(a). Within the range of liquid-to-solid ratios from 10 to 50 mL/g, the yield of *G. elata* polysaccharides initially increases and then decreases. When the solvent volume is too small, polysaccharides may not be dissolved completely in water, leading to a high viscosity. On the other hand, when the water volume exceeds 30 times the solid, the transfer and heat transfer effects of polysaccharides in a large amount of solvent are affected.^[42] In addition, excessively high liquid-to-solid ratios may lead to the dissolution of other impurities, thereby inhibiting the extraction of polysaccharides. Therefore, a liquid-to-solid ratio of 30 mL/g is preferred.

The influence of ultrasonic temperature on the extraction yield of *G. elata* polysaccharides is illustrated in Fig. 5(b). As the ultrasonic temperature increases, the polysaccharide yield initially increases and then decreases, with the highest yield observed at 40 °C. This is likely because the elevated temperature accelerates molecular motion, facilitating the penetration of the extracting solvent and the release of intracellular polysaccharides. Most of the polysaccharides have been released into the solution at this temperature. However, excessively high temperatures can disrupt the polysaccharide structure.^[43] Therefore, a temperature of 40 °C is considered optimal.

The impact of ultrasonic frequency on the extraction yield of *G. elata* polysaccharides is depicted in Fig. 5(c). As the ultrasonic frequency increases, the polysaccharide yield shows an initial increase followed by a gradual decrease, with the maximum yield achieved at 80 kHz. This trend may be attributed to the fact that higher ultrasound frequencies can accelerate the disruption of cellular structures, leading to a faster dissolution of polysaccharides. However, excessively high ultrasound frequencies can disrupt the polysaccharide structure, causing degradation.^[44] Therefore, an ultrasound frequency of 80 kHz is considered optimal. However, it should be noted that the ultrasound frequency has a relatively modest impact on the polysaccharide yield.

The influence of extraction time on the extraction yield of *G. elata* polysaccharides is illustrated in Fig. 5(d). With increasing the extraction time, the polysaccharide yield exhibits an initial increase followed by a decrease, with the maximum yield achieved at 25 minutes of extraction. This trend may be attributed to the fact that the polysaccharides present in *G. elata* cannot be completely dissolved and released within a short period. However, as the extraction time prolongs, a greater amount of polysaccharides is gradually dissolved and released into the solvent, leading to an increase

in yield. When the extraction time continues to increase, the sustained high temperature and ultrasound exposure may to some extent disrupt the structure of polysaccharide molecules, causing them to degrade into monosaccharides.^[45] Therefore, the optimal extraction time is chosen as 25 minutes.

3.2.2 Response surface optimization of extraction process

3.2.2.1 Results of box-behnken experiment

Based on the results of single-factor experiments, the extraction process of *G. elata* polysaccharides was further optimized using a Box-Behnken experimental design. The ultrasound power was set at 80 kHz, and three significant factors, namely liquid-to-solid ratio, ultrasonic temperature, and extraction time, were optimized based on the polysaccharide extraction rate as the evaluation criterion. The experimental results are presented in Table 4.

The response surface regression analysis of the experimental data from Table 3 was conducted using Design-Expert 10 software, resulting in the following quadratic regression model describing the relationship between the *G. elata* Bl. *F. elata* polysaccharide extraction rate (Y) and the three factors (X_1 , X_2 , X_3):

$$Y = 32.050 - 0.260X_1 + 0.225X_2 + 0.153X_3 + 0.365X_1X_2 - 0.390X_1X_3 + 0.100X_2X_3 - 4.630X_1^2 - 2.290X_2^2 - 1.510X_3^2$$

In the equation, Y represents the extraction rate of *G. elata* Bl. *F. elata* polysaccharide, X_1 is the liquid-to-solid ratio, X_2 is the ultrasonic temperature, and X_3 is the extraction time.

According to Table 5, the quadratic polynomial fitting model is highly significant ($P < 0.0001$), with an F-value of 62.62, indicating the statistical significance of this model. The lack-of-fit term with a P-value of 0.3445 is not significant, suggesting the model's practical utility within the experimental range. The coefficient of determination (R^2) value is 0.9877, and the adjusted R-squared (Adj- R^2) is 0.9720, indicating a good fit of the model. Additionally, the quadratic terms X_1^2 and X_2^2 both exhibit highly significant impacts on the polysaccharide extraction rate, while X_3^2 demonstrates a significant influence.

3.2.2.2 Analysis of the response surface

The software Design-Expert 10 was used to generate 3D response surface plots and 2D contour plots to investigate the influence of liquid-to-solid ratio, ultrasonic temperature, and extraction time, as well as their interactions, on the yield of *G. elata* polysaccharides. Fig. 6(a) illustrates a significant interaction between liquid-to-solid ratio and ultrasonic temperature at the optimal extraction time of 25.24 minutes. Fig. 6(b) demonstrates some interaction between liquid-to-solid ratio and extraction time at the optimal ultrasonic

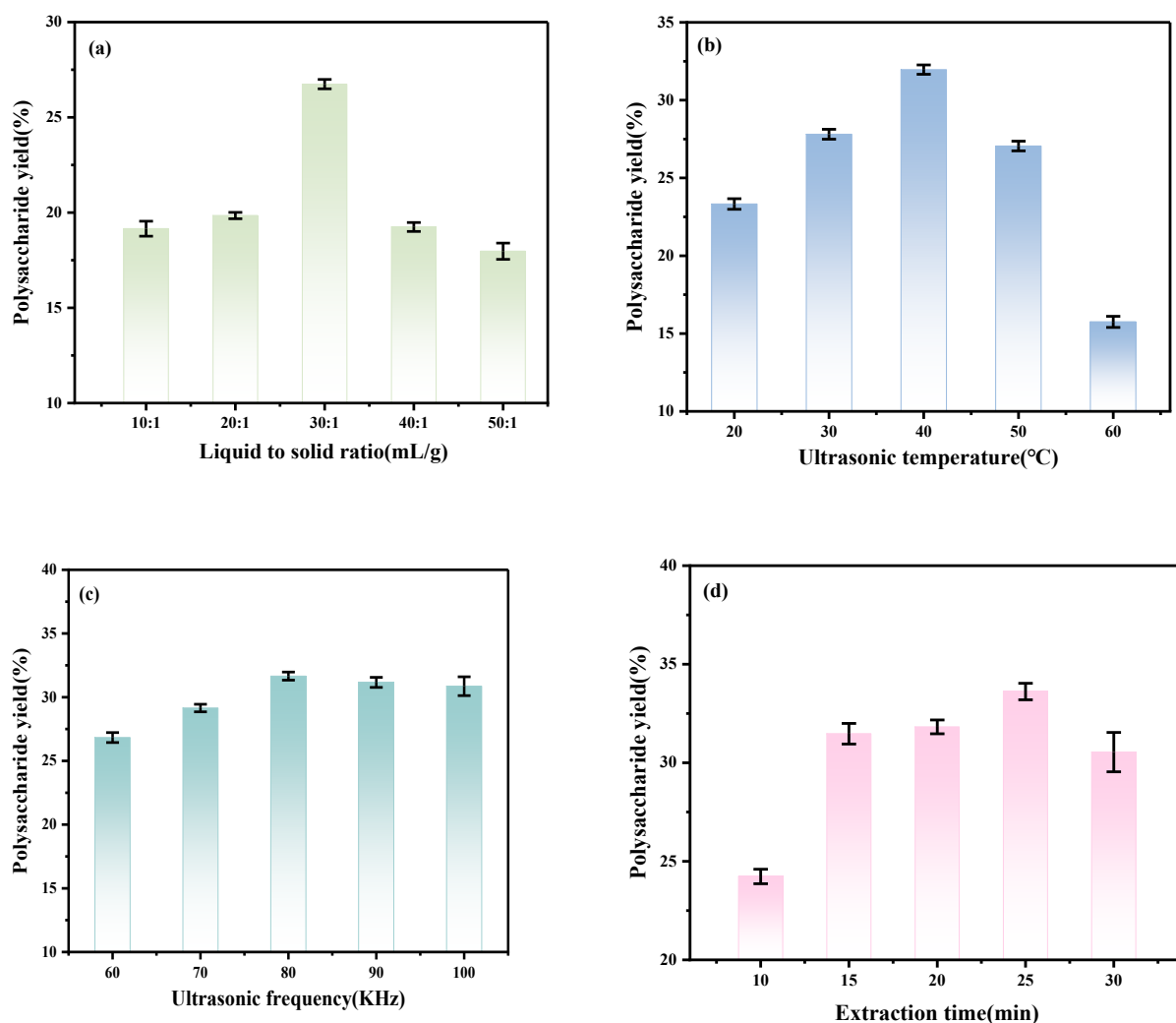


Fig. 5: Influence of liquid-to-solid ratio, ultrasonic temperature, ultrasonic frequency, and extraction time on polysaccharide extraction yield.

Table 4: Box-Behnken experimental results.

Run	X ₁ (Liquid-to-Solid Ratio, mL/g)	X ₂ (Ultrasonic Temperature, °C)	X ₃ (Extraction Time, min)	Yield of polysaccharide (%)
1	0	-1	1	28.13
2	-1	0	1	25.17
3	0	0	0	32.72
4	0	0	0	31.63
5	-1	0	-1	25.80
6	1	0	1	25.19
7	0	1	1	29.17
8	-1	1	0	26.35
9	1	1	0	25.24
10	0	1	-1	28.17
11	0	-1	-1	27.53
12	0	0	0	31.88
13	0	0	0	31.76
14	1	-1	0	26.25
15	-1	-1	0	25.80
16	0	0	0	32.27
17	1	0	-1	24.36

Table 5: Response surface variance analysis.

Source	Sum of Squares	df	Mean Square	F-value	p-value	Significance
Model	134.96	9	15.00	62.62	< 0.0001	**
X ₁	0.5408	1	0.5408	2.26	0.1766	-
X ₂	0.4050	1	0.4050	1.69	0.2346	-
X ₃	0.1861	1	0.1861	0.7769	0.4073	-
X ₁ X ₂	0.5329	1	0.5329	2.23	0.1794	-
X ₁ X ₃	0.6084	1	0.6084	2.54	0.1550	-
X ₂ X ₃	0.0400	1	0.0400	0.1670	0.6950	-
X ₁ ²	90.30	1	90.30	377.07	< 0.0001	**
X ₂ ²	22.10	1	22.10	92.28	< 0.0001	**
X ₃ ²	9.61	1	9.61	40.14	0.0004	*
Residual	1.68	7	0.2395			
Lack of Fit	0.8896	3	0.2965	1.51	0.3412	-
Pure Error	0.7867	4	0.1967			
Cor Total	136.63	16				

R.S.D % = 1.74
R² = 0.9877
R²_{adj} = 0.9720

Note: “–” means no significant; * p-Value<0.05 significant; ** p-Value<0.01 highly significant.

temperature of 40.57 °C. Fig. 6(c) reveals that there is no significant interaction between extraction time and ultrasonic temperature at the optimal liquid-to-solid ratio of 29.72 mL/g. Through software analysis, the optimal extraction conditions for *G. elata* polysaccharides were determined to be a liquid-to-solid ratio of 29.72 mL/g, an ultrasonic temperature of 40.57 °C, and an extraction time of 25.24 minutes. Under these conditions, the predicted yield of *G. elata* polysaccharides was 32.07%.

3.2.2.3 Verification of the model

To validate the model equation, three validation experiments were conducted under the modified optimal conditions of a liquid-to-solid ratio of 30 mL/g, an ultrasonic temperature of 41 °C, and an extraction time of 25 minutes. Under these test conditions, the obtained yield of *G. elata* polysaccharides was (32.14±0.59)%, which is close to the theoretically predicted value. This result indicates that the model can effectively reflect the extraction conditions of *G. elata* polysaccharides.

3.2.3 Determination of polysaccharide content in three different varieties of *Gastrodia elata*

Three different samples of *G. elata* polysaccharides were collected, and their contents of reducing sugars and total sugars were determined using the established testing method. The contents of reducing sugars and total sugars in the samples were calculated based on the standard curve, and the polysaccharide content was also calculated, as presented in Table 6. The results revealed the variations in the content of sugar compounds in *G. elata* among different varieties. For instance, *G. elata* Bl. F. *Glaucua S. Chow* exhibited the highest content of both polysaccharides and total sugars, while the lowest content was observed in *G. elata* Bl. F. *Viridls MaKino*. Interestingly, *G. elata* Bl. F. *Viridls MaKino* had the highest

content of reducing sugars. Relevant research suggests that the content of *G. elata* polysaccharides is influenced by factors such as variety and cultivation environment.

3.3 In Vitro antioxidant activity analysis of *Gastrodia elata* polysaccharides

In Fig.7(a), various *G. elata* polysaccharide extracts demonstrate distinct abilities to scavenge DPPH free radicals. There's a dose-response relationship between the concentration of *G. elata* polysaccharides from different varieties and their DPPH scavenging capacity. Remarkably, *G. elata* Bl. F. *Glaucua S. Chow* polysaccharides exhibit a rapid increase in DPPH scavenging capacity with increasing the concentration. These three types of *G. elata* polysaccharides display varying degrees of DPPH free radical scavenging capacity at different concentrations. At lower concentrations, *G. elata* Bl. F. *viridis MaKino* polysaccharides exhibit the strongest DPPH free radical scavenging capacity, while at a concentration of 9.5 mg/mL, *G. elata* Bl. F. *Glaucua S. Chow* polysaccharides exhibit the highest DPPH free radical scavenging capacity, reaching 98.31%. This suggests that *G. elata* polysaccharides possess significant DPPH free radical scavenging capacity.

As shown in Fig. 7(b), as the concentration of *G. elata* polysaccharides increases, all three types exhibit an enhanced ABTS scavenging capacity. When comparing the scavenging capacity against ABTS free radicals for the three types of *G. elata* polysaccharides at the same concentration, the order is *G. elata* Bl. F. *Glaucua S. Chow* > *G. elata* Bl. F. *elata* > *G. elata* Bl. F. *viridis MaKino*. Fig. 7(a) and Fig. 7(b) show that, for the same variety of *G. elata* polysaccharides at the same concentration, their DPPH free radical scavenging capacity is stronger than their ABTS free radical scavenging capacity.

Fig. 7(c) demonstrates that all three types of *G. elata*

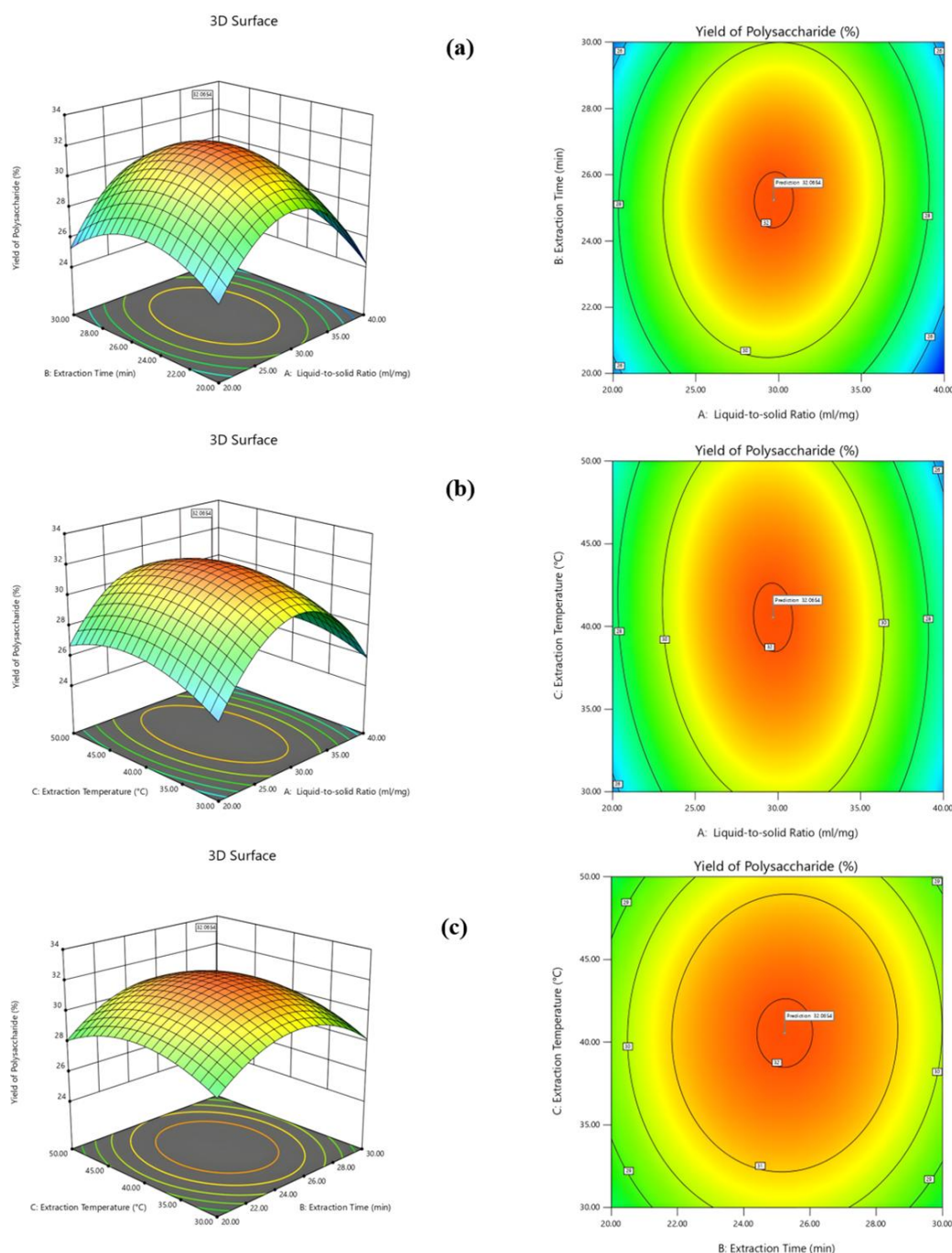


Fig. 6: Response surface and contour plots illustrating the interaction effects of two factors on *G. elata* Polysaccharide Yield. (a. Liquid-to-solid ratio and time, b. Liquid-to-solid ratio and ultrasonic temperature, c. ultrasonic Temperature and extraction time).

polysaccharides possess the ability to reduce Fe^{3+} , and this ability increases gradually with increasing the concentration of *G. elata* polysaccharides. Notably, *G. elata* Bl. *F. elata* polysaccharides exhibit a rapid increase in reducing ability with increasing the concentration. At a concentration of 9.5 mg/mL, the order of total reducing ability for *Gastrodia elata* polysaccharides from different varieties is as follows: *G. elata* Bl. *F. elata* > *G. elata* Bl. *F. Glauca* S. Chow > *G. elata* Bl. *F. viridis* MaKino.

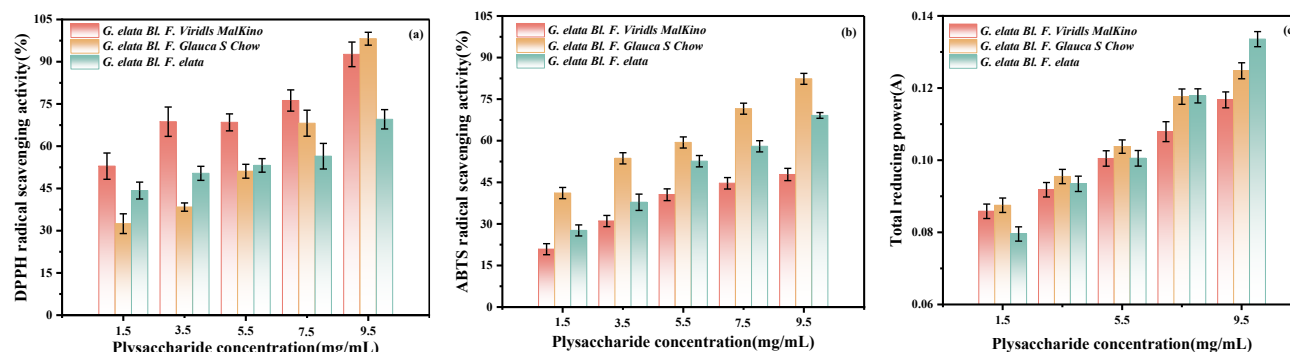
3.4 Monosaccharide composition analysis of *Gastrodia elata* polysaccharides from different varieties

The monosaccharide composition of the three *G. elata* polysaccharides was determined using method 2.2.4. The results, presented in Table 7, reveal that all three varieties of *G. elata* polysaccharides contain glucose, mannose, rhamnose, glucuronic acid, galacturonic acid, and galactose. Glucose is the most abundant monosaccharide, constituting over 95% of the composition in all three polysaccharides. Additionally, *G. elata* polysaccharides contain both glucuronic acid and galacturonic acid, indicating their acidic heteropolysaccharide nature.

Among the three varieties, *G. elata* Bl. *F. elata* and *G. elata* Bl. *F. Glauca* S. Chow exhibit more similar

Table 6: *G. elata* polysaccharide content in different varieties.

Sample	Reducing sugar content /%	Total sugar content /%	Polysaccharide content /%
<i>G. elata</i> Bl. F. <i>Viridis</i> MaKino	7.26	37.32	30.06
<i>G. elata</i> Bl. F. <i>Glaucia</i> S Chow	7.16	41.94	34.78
<i>G. elata</i> Bl. F. <i>elata</i>	6.96	39.10	32.14

**Fig. 7:** *In vitro* Antioxidant Activities of *G. elata* Polysaccharides (a. DPPH, b. ABTS, c. Total Reducing Power).**Table 7:** Monosaccharide composition and content of *G. elata* polysaccharides from different varieties.

Sample	Monosaccharide content /%					
	Man	Rha	GlcA	GalA	Glc	Gal
<i>G. elata</i> Bl. F. <i>Viridis</i> MaKino	0.55±0.015	0.85±0.025	0.48±0.012	1.05±0.017	95.67±0.115	1.25±0.026
<i>G. elata</i> Bl. F. <i>elata</i>	0.40±0.015	1.09±0.211	0.50±0.040	1.21±0.294	95.94±0.469	0.85±0.093
<i>G. elata</i> Bl. F. <i>Glaucia</i> S Chow	0.41±0.035	1.02±0.095	0.53±0.047	1.14±0.190	95.92±0.508	0.82±0.057

Monosaccharide profiles, whereas *G. elata* Bl. F. *viridis* MaKino shows a distinct compositional pattern. These differences may be attributed to a combination of factors, including genetic variability among varieties, local growing conditions (such as soil composition and climate), and post-harvest processing methods, all of which can influence polysaccharide biosynthesis and structural characteristics. Compared with previous literature, the observed variations further highlight the impact of cultivation origin and variety on the monosaccharide composition of *G. elata* polysaccharides. It's noteworthy that monosaccharides can offer a range of health benefits. For example, glucose may promote adhesion between the muscle layer and intestinal mucosa, potentially alleviating constipation. Mannose is recognized for its anti-tumor and anti-obesity properties, while glucuronic acid exhibits detoxifying effects. These findings underscore the potential health advantages of *G. elata* polysaccharides.^[46,47]

4. Conclusion

In this study, the determination conditions for *G. elata* polysaccharides were optimized using the DNS method. The ideal conditions included a detection wavelength of 540 nm, 2 mL of color reagent, a 6-minute color development at 90 °C, and a 10-minute total sugar hydrolysis time. These conditions are convenient, yield stable results, and offer good reproducibility. For polysaccharide extraction, response surface optimization was employed. The optimal conditions

were found to be a liquid-to-material ratio of 30 mL/g, an extraction temperature of 41 °C, and an extraction time of 25 minutes. These settings resulted in a theoretical yield of 32.07%. This yield is considerably higher than that reported for traditional hot water extraction of *G. elata* polysaccharides (8.11%) and is also superior to yields obtained by deep eutectic solvent-based methods (19.88–25.22%). The ultrasonic-assisted extraction method employed in this study offers advantages in terms of extraction efficiency and preservation of polysaccharide bioactivity, consistent with findings on other medicinal plants where UAE outperformed conventional methods in both yield and structural integrity. Using these optimized conditions, the polysaccharide content in *G. elata* samples from three different varieties (*G. elata* Bl. F. *elata*, *G. elata* Bl. F. *Glaucia* S Chow, and *G. elata* Bl. F. *Viridis* MaKino in Yunnan province) was determined. The polysaccharide content varied among the varieties, ranging from 30.06% to 34.78%. Notably, *G. elata* Bl. F. *Glaucia* S. Chow exhibited the highest polysaccharide content (34.78%), consistent with previous reports on variety-dependent variations in *G. elata* tuber polysaccharides. All three *G. elata* polysaccharides primarily consisted of six different monosaccharides, with glucose being the most abundant (over 95%). Their scavenging capabilities against DPPH and ABTS increased with higher polysaccharide concentration. Notably, DPPH free radical scavenging capacity was stronger than ABTS free radical scavenging capacity at the same polysaccharide concentration. All three *G. elata*

polysaccharides also exhibited some reducing ability against trivalent iron. This study provides the basis for the eco-friendly determination of *G. elata* polysaccharide content and lays the groundwork for structural identification and structure-activity relationship analysis of *G. elata* polysaccharides in different varieties. Furthermore, the observed differences in polysaccharide content, antioxidant activity, and monosaccharide composition among varieties offer valuable insights for the targeted cultivation and quality control of *G. elata*. Future research should focus on elucidating the structure-activity relationships of these polysaccharides and evaluating their in vivo bioactivities, which will support the development of *G. elata* polysaccharides into functional foods or therapeutic agents.

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Data Availability

Data will be made available on request.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supporting Information

Not applicable.

CRedit Statement

Guojia Wu: Conceptualization, Methodology, Investigation, Data curation, Writing - Original draft. **Jianwei Li:** Methodology, Validation, Formal analysis, Writing - Original draft. **Lijun Cheng:** Software, Formal analysis, Visualization. **Menghua Tian:** Resources, Investigation, Data curation. **Jie Wu:** Validation, Resources, Project administration. **Ruiqi Zhang:** Software, Data curation, Visualization. **Juan Xu:** Supervision, Writing - Review and editing, Funding acquisition, Corresponding author. **Ying Hou:** Supervision, Writing - Review and editing, Project administration.

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