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Optimization of ultrasound-assisted extraction of two saponins from *Paris polyphylla* var. *yunnanensis* leaves using response surface methodology

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Paris polyphylla var. *yunnanensis* is one of the famous Chinese herbs, in which two saponins (polyphyllin II and polyphyllin VII) have anticancer effects. The endangerment of *Paris polyphylla* var. *yunnanensis*, makes the study of optimizing the extraction of polyphyllin II and polyphyllin VII from the leaves of *Paris polyphylla* var. *yunnanensis* more important. The study established and optimized the process of ultrasound-assisted extraction for polyphyllin II and polyphyllin VII using the Box Behnken Design method of response surface methodology. The results showed that the optimum extraction conditions for polyphyllin II and polyphyllin VII are ethanol concentration of 73 and 70%, extraction temperature of 43 and 50°C, and number of extraction 3, respectively. Under the above conditions, the contents of polyphyllin II and polyphyllin VII were measured to be 6.427 and 19.015 mg/g (DW). The results showed that the experimental model fitted well, and the response surface methodology (RSM) was feasible to optimize the extraction process of polyphyllin II and polyphyllin VII from *Paris polyphylla* var. *yunnanensis* leaves. This method provides an effective approach for the comprehensive development and utilization of non-medicinal parts of *Paris polyphylla* var. *yunnanensis*.

KEYWORDS

***Paris polyphylla* var. *yunnanensis*, saponins, ultrasound-assisted extraction, response surface methodology (RSM), optimization of the extraction process**

1 Introduction

The rhizome of *Paris polyphylla* var. *yunnanensis* (*P. polyphylla* var. *yunnanensis*) is the raw material of traditional Chinese medicine – Paridis Rhizoma. *P. polyphylla* var. *yunnanensis* is mainly distributed in the Gaoligong Mountain Area of Tengchong, Southwest Yunnan and Sichuan Province of China, and the north of Myanmar, including evergreen broad-leaved forest, coniferous forest, and broad-leaved mixed forest at an altitude of 600–2,300 m. It usually grows in hillside shade and shrubs (Wu, 2020). Studies have found

that the main medicinal components of Paridis Rhizoma are steroidal saponins (Negi et al., 2014; Thapa et al., 2022). Polyphyllin II and polyphyllin VII (Figure 1) are critical steroidal saponins with anti-cancer, anti-tumour, anti-inflammatory, and analgesic effects (Ding et al., 2021; Liu et al., 2021; Yan et al., 2023). Recent studies have shown that polyphyllin II can physiologically induce apoptosis and treat lung cancer as an adjuvant drug (Wang et al., 2019; Yang et al., 2021; Pang et al., 2023). Polyphyllin VII can inhibit tumour cell proliferation, invasion, and migration, induce tumour cell apoptosis, and reverse tumour drug resistance through multiple ways and mechanisms (Chen et al., 2016; Hsieh et al., 2016). With the discovery of the pharmacological effects of *P. polyphylla* var. *yunnanensis*, the demand for Paridis Rhizoma as a medicinal material is increasing. The wild *P. polyphylla* var. *yunnanensis* was excavated wantonly, so the growth environment was damaged, and *P. polyphylla* var. *yunnanensis* was on the verge of extinction (Cunningham et al., 2018; Shicai et al., 2020). Simultaneously, due to exacting growth conditions, long growth cycles, and immature artificial cultivation technology, Paridis Rhizoma supply is insufficient (Song et al., 2015; Wang and Li, 2018; Yue et al., 2021). In recent years, studies have reported that polyphyllins are mainly synthesized in chloroplasts, transported down through stems, and accumulated in roots, which proves that leaves are important organs for the biosynthesis of steroid saponins. Therefore leaves contain medicinal components similar to those in the rhizoma (Qin et al., 2018; Liang et al., 2019; Guo et al., 2021). However, there is nearly no utilization for the leaves of *P. polyphylla* var. *yunnanensis* (Guo et al., 2008; Deb et al., 2015).

The effective chemical components contained in medicinal plants are very complex. How to maximize the extraction of effective ingredients from medicinal plants is crucial to further research on medicinal ingredients, and improve the quality of medicinal materials and clinical efficacy. Therefore, it is of great significance to find suitable extraction methods and optimize the extraction process for the full utilization of medicinal materials. Compared with traditional extraction methods such as boiling, cold soaking and Soxhlet extraction, ultrasound-assisted extraction (UAE) has shown greater advantages in the extraction of effective components of medicinal plants due to its high efficiency, high speed, low temperature and other characteristics (Luo et al., 2010; Lee et al., 2013; Cheok et al., 2014). The response surface methodology (RSM) is a method integrating mathematics and statistics. It seeks the best result through regression equation analysis, solves multivariable problems, and evaluates the nonlinear relationship between indicators and factors. It is easy to use and has good condition predictability. At present, it is often used in the research of extraction of plant components (Heleno et al., 2016; Aydar, 2018; Kumar et al., 2021). Meng et al. (2015) used the central design-response surface method to optimize the ultrasonic extraction of total saponin from the stems and leaves of *P. polyphylla* var. *yunnanensis*; Ju et al. (2015) used the central composite de-sign-response surface method to optimize the reflux extraction of total saponins from *P. polyphylla* var. *yunnanensis*, however, there are fewer reports on the extraction of single saponin components from *P. polyphylla* var. *yunnanensis*. Therefore, based on the single-factor experiment, the ultrasonic extraction for polyphyllin II and polyphyllin VII from the leaves of *P. polyphylla* var. *yunnanensis* was optimized using response surface methodology (RSM) in this study. The extraction efficiency of these two saponins is improved, and using the non-medicinal part of *P. polyphylla* var. *yunnanensis* alleviates the resource shortage. Finally, it laid a foundation for the comprehensive utilization of *P. polyphylla* var. *yunnanensis*.

2 Results and discussion

2.1 Analysis of high-performance liquid chromatography (HPLC)

According to the HPLC analysis, a linear regression was performed with the concentration as the horizontal coordinate (X, mg/mL) and the peak area as the vertical coordinate (Y), and the equations of the standard curves obtained as follows: $y = 2{,}899x + 25.202$ ($R^2 = 0.9992$) for polyphyllin II and $y = 3078.7x + 37.704$ ($R^2 = 0.9998$) for polyphyllin VII were shown in Figure 2. They showed a good linearity over the range of 0.0625–1 mg/mL.

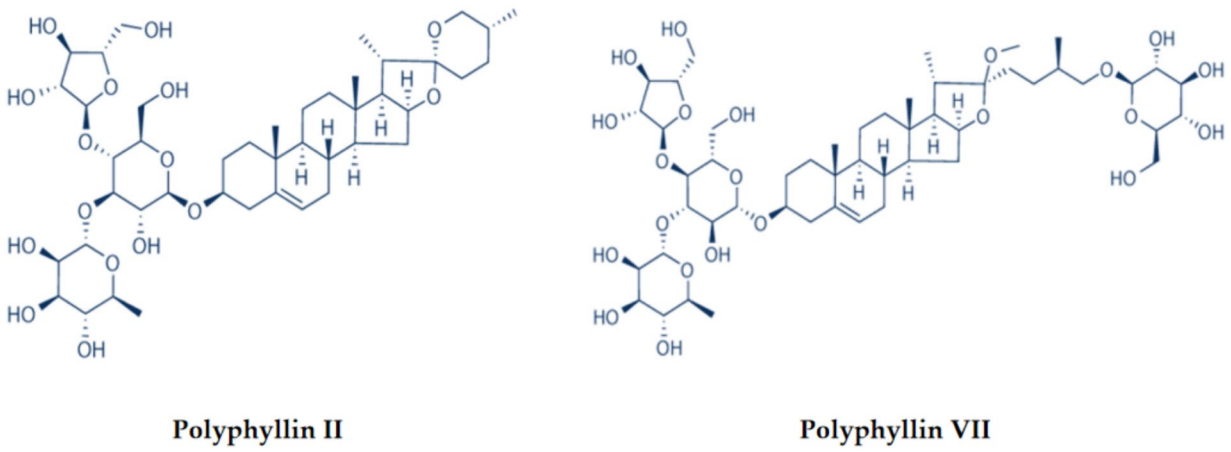

FIGURE 1
Chemical structures of polyphyllin II and polyphyllin VII.

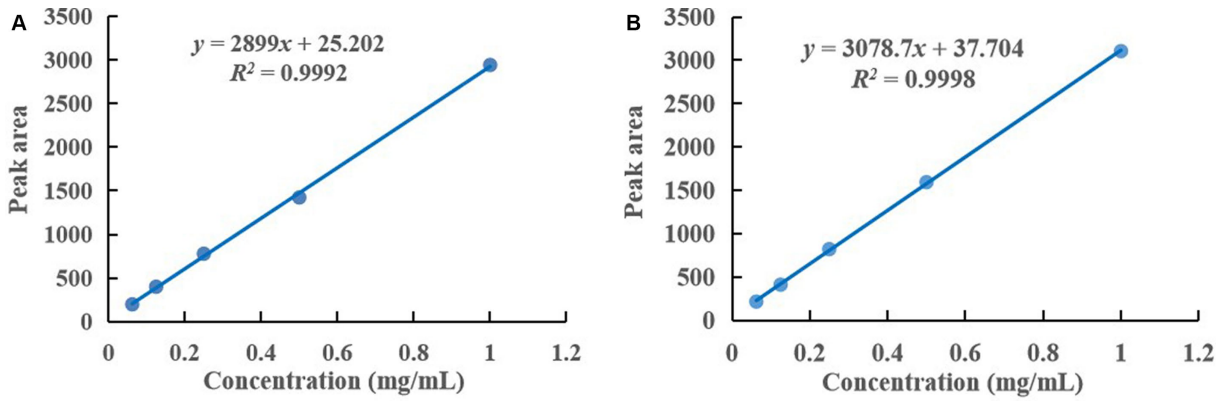

FIGURE 2
Standard curves of polyphyllin II **(A)** and polyphyllin VII **(B)**.

2.2 Single-factor experiment

Extracting bioactive secondary metabolites from medicinal plants is influenced by factors such as extraction method, solid–liquid ratio, time, temperature, solvent, and others (Cheok et al., 2014; Meng et al., 2015; Rodrigues et al., 2017; He et al., 2022). Therefore, in order to improve the extraction efficiency of medicinal components, this study investigated the main factors affecting polyphyllin II and polyphyllin VII content in the leaves of *P. polyphylla* var. *yunnanensis*. The results are shown in Figure 3.

2.2.1 Effect of solid–liquid ratio on the content of polyphyllin II and polyphyllin VII

Increasing the solid–liquid ratio allows better solubilization of the target components into the solvent, and the amount of extraction solvent has an important effect on the extraction content of saponins (Meng et al., 2015). Therefore, the influence of the solid–liquid ratio on the content of the two saponins is investigated. The results are shown in Figure 3A. There is no significant difference among the treatments for polyphyllin II and polyphyllin VII. The two saponins are effectively extracted into the solvent, and the solid–liquid ratio of 1:5 (g/mL) with less material consumption is appropriate.

2.2.2 Effect of extraction time on the content of polyphyllin II and polyphyllin VII

The increase in extraction time is conducive to the full extraction of active components, but the cost of time and energy also increases. Excessive ultrasonic time may also lead to the degradation of target components (Sarvin et al., 2018; He et al., 2022). The effect of extraction time on the contents of the two saponins was examined, and the results, shown in Figure 3B, indicated no significant changes in the contents of polyphyllin II and polyphyllin VII. The reason for this phenomenon is that the solvent produces cavitation bubbles under the action of ultrasound, causing the plant cell wall to weaken, thus improving the extraction efficiency of the active compounds and making the two saponins completely extracted from the solvent in a relatively short time (Rodrigues et al., 2017). In summary, considering the energy problem, it is more appropriate when the extraction time is 0.5 h.

2.2.3 Effect of extraction temperature on the content of polyphyllin II and polyphyllin VII

Temperature can not only accelerate the dissolution rate of saponins but also can affect the efficiency of UAE. Therefore, it is necessary to choose the appropriate extraction temperature according to the corresponding target compounds to achieve the highest extraction rate (Sarvin et al., 2018). The influences of temperature on the contents of the two saponins were investigated. The results are shown in Figure 3C. The extraction temperature has a significant impact on the extraction of polyphyllin II and polyphyllin VII. As the temperature increased, the content of polyphyllin II did not change significantly between 20 and 30°C, while the content of polyphyllin VII increased significantly and reached the maximum extracted content. As the temperature continued to increase up to 50°C, the contents of the two saponins gradually decreased, which may be due to the degradation of saponins, resulting in the reduction of the extraction content of the two saponins (Shen et al., 2014). Therefore, the extraction temperature in subsequent response surface experiments was chosen to be 30–50°C.

2.2.4 Effect of the number of extraction on the content of polyphyllin II and polyphyllin VII

The more number of extraction, the more solvents are used and the more energy is consumed (Chen et al., 2012), so it is important to choose the most appropriate number of extraction. The effect of the number of extraction on the content of the two saponins is investigated. The results are shown in Figure 3D. The contents of both polyphyllin II and polyphyllin VII increased significantly with the number of extraction increasing from 1 to 2. However, there was no significant difference in the contents of the two saponins number of extraction 2, 3 and 4. It indicated that the two saponins had been fully extracted through twice extraction. Therefore, the number of extraction in subsequent response surface experiments was chosen to be 1–3.

2.2.5 Effect of ethanol concentration on the content of polyphyllin II and polyphyllin VII

The concentration of the extraction solvent affects the solubility of the solvent to the saponins, which is one of the most important factors affecting the extraction rate of steroidal saponins (Shamprasad et al., 2022). The experiment investigated the influence of different

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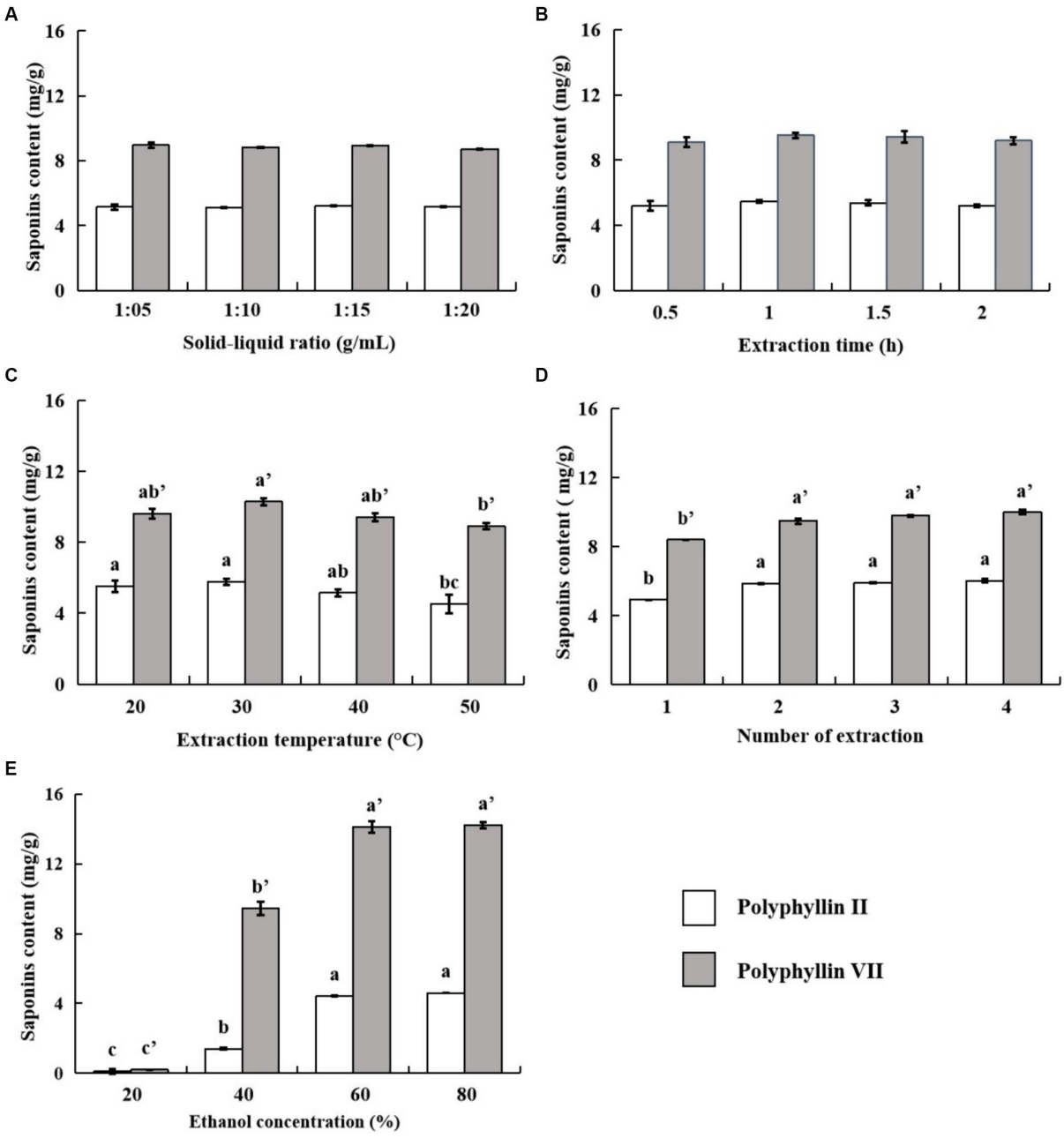

FIGURE 3
Effects of solid–liquid ratio **(A)**, extraction time **(B)**, extraction temperature **(C)**, number of extraction **(D)** and ethanol concentration **(E)** on the extraction content of polyphyllin II and polyphyllin VII. a, b and c represent significant differences among treatments for polyphyllin II; a', b' and c' represent significant differences among treatments for polyphyllin VII ($p < 0.05$), $n = 3$.

ethanol concentrations on the contents of the two saponins. The results are shown in Figure 3E. Both saponins can hardly be extracted with 20% ethanol. With the increase of ethanol concentration (below 80%), the contents of polyphyllin II and polyphyllin VII increase significantly ($p < 0.05$). However, there was no significant difference between the contents of both saponins extracted with 60 and 80% ethanol, the content of both saponins increased significantly, probably due to the higher polarity of the extracted solution at higher water content, resulting in the insolubility of low-polarity saponins. After 60% ethanol is increased to 80% ethanol, the content of the two saponins does not decrease significantly, indicating that the ethanol with too high concentration is not suitable for the extraction of the two saponins. To sum up, the content of two saponins is significantly higher in the ethanol concentration range of 40–80% ethanol. Therefore, ethanol concentration was chosen to be 40–80% in subsequent response surface experiments.

2.3 Establishment of RSM

The true value and coding level of the independent variable in the response surface method Box–Behnken design (BBD) method are

shown in Table 1. Seventeen sets of experiments were obtained by response surface experimental design with five centroids (0, 0, 0) to estimate the pure error, and the results obtained after the experiments are shown in Table 2.

2.4 Model fitting and statistical analysis

The distributions of the predicted and actual contents for both polyphyllin II (Figure 4A) and polyphyllin VII (Figure 4B) are close to the lines, suggesting that the model fits well. The RSM-optimized UAE process is a good process for extracting steroid saponins from medicinal plants, and other researchers have also made similar reports and have been widely applied (Hadidi et al., 2020; Khoang et al., 2022).

The quadratic multiple regression model between the contents of polyphyllin II and polyphyllin VII and various factors is obtained after multiple regression fitting analyses of the BBD experimental results, as shown in Table 3. The influence of each factor on the response value is directly reflected in each coefficient in the equation. Within the scope of experimental design, the absolute values of the partial regression coefficients of the dependent variables A, B, and C for polyphyllin II and polyphyllin VII are A > C > B respectively, indicating that the most significant factor affecting the content of polyphyllin II and polyphyllin VII is ethanol concentration, followed by number of extraction and extraction temperature. A higher statistical correlation coefficient ($R^2 > 90\%$) indicates a good fit between experimental values with those obtained by the models (Kefi et al., 2022). The coefficients of determination (R^2) of the quadratic multiple regression models vary between 0.9805 and 0.9726, and values of predicted coefficients of determination vary from 0.9374 to 0.9553 for polyphyllin II and polyphyllin VII, respectively (Table 3). The lack of fit shows a close agreement that exists between the experimental results and the theoretical values predicted by the quadratic multiple regression model (Carasek and Pawliszyn, 2006; Chávez-Moreno et al., 2018; Zhang et al., 2020).

The analysis of variance (ANOVA) for each of the factors concerning polyphyllin II and polyphyllin VII contents was obtained by the analysis of the Design Expert 12.0 software. The p-value representing the significance of the coefficient is important for understanding the interaction patterns between variables, and a value below 0.05 (0.01 or 0.0001) indicates that the test parameter is significant (highly significant or extremely significant) at a significance level of 5% (1% or 0.01%) (Zhong et al., 2016). It can be concluded from Table 4 that the polyphyllin II model is extremely significant ($F = 39.0700$, $p < 0.0001$), and the lack of fit in the model is not significant ($F = 4.11$, $p = 0.1027 > 0.0500$), indicating that the regression equation does not demonstrate lack of fit, i.e., there are no significant influence factors other factors except those factors considered in this experiment, and the regression model can fit the true response value (Zhang et al., 2022). It can be observed that the linear term of ethanol concentration ($p < 0.0001$) had a higher effect on the polyphyllin II content than that of the number of extraction ($p = 0.0004$). The two-level interaction between ethanol concentration and extraction

TABLE 1 Independent variables and their levels for BBD experiments.

Independent variables	Unit	Symbols	Coded levels		
			−1	0	1
Ethanol concentration	%	A	40	60	80
Extraction temperature	°C	B	30	40	50
Number of extraction	-	C	1	2	3

TABLE 2 Box–Behnken experimental design and results for the content of polyphyllin II and polyphyllin VII.

Run	A: Ethanol concentration	B: Extraction temperature	C: Number of extraction	Content (mg/g)	
				Polyphyllin II	Polyphyllin VII
1	−1	−1	0	1.85 ± 0.02 j	12.96 ± 0.35 j
2	1	−1	0	6.08 ± 0.01 abc	17.62 ± 0.06 cd
3	−1	1	0	3.37 ± 0.12 i	15.43 ± 0.25 h
4	1	1	0	6.18 ± 0.04 ab	17.85 ± 0.23 bc
5	−1	0	−1	2.00 ± 0.05 j	11.39 ± 0.30 k
6	1	0	−1	5.15 ± 0.04 f	14.63 ± 0.13 i
7	−1	0	1	3.68 ± 0.10 h	15.08 ± 0.16 hi
8	1	0	1	6.36 ± 0.03 a	18.84 ± 0.12 a
9	0	−1	−1	4.68 ± 0.02 g	15.01 ± 0.06 hi
10	0	1	−1	4.73 ± 0.03 g	15.12 ± 0.17 hi
11	0	−1	1	5.82 ± 0.10 cd	17.91 ± 0.06 bc
12	0	1	1	6.00 ± 0.08 bc	18.32 ± 0.21 ab
13	0	0	0	5.60 ± 0.06 de	17.21 ± 0.16 de
14	0	0	0	5.67 ± 0.04 de	17.15 ± 0.18 def
15	0	0	0	5.57 ± 0.06 de	16.97 ± 0.15 ef
16	0	0	0	5.20 ± 0.03 f	16.61 ± 0.12 fg
17	0	0	0	5.38 ± 0.02 ef	16.32 ± 0.20 g

Different lowercase letters indicate significant differences among the contents of polyphyllin II or polyphyllin VII ($p < 0.05$). Data are expressed as the mean ($n = 3$) ± S.D.

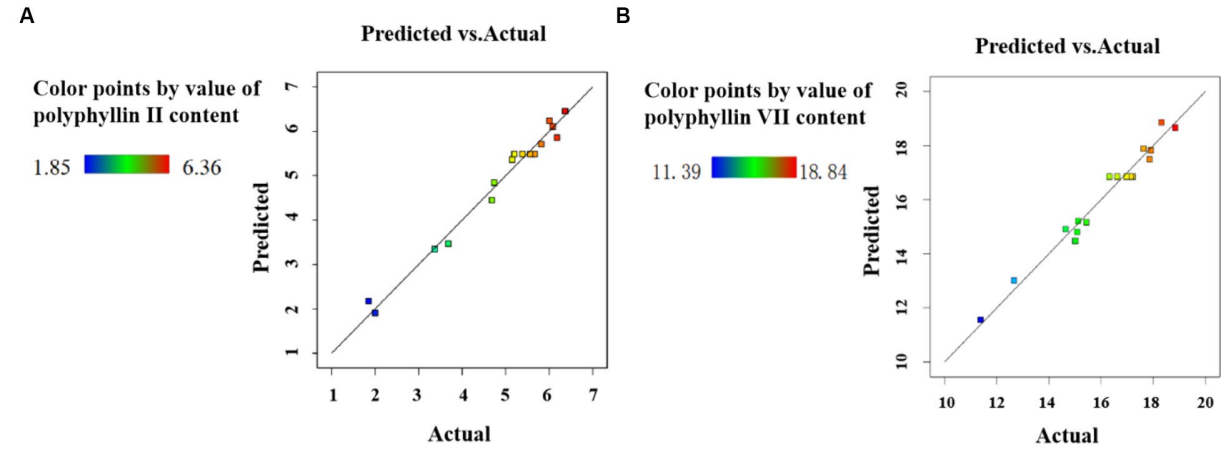

FIGURE 4
Predicted value and actual value of the contents for polyphyllin II **(A)** and polyphyllin VII **(B)**.

TABLE 3 Second-order polynomial equation for the relationship between the response variable and the test variable.

Response variable	Model equation*	R^2	R^2adj
Polyphyllin II content (Y_1)	$Y_1 = 5.4840 + 1.6088\ A + 0.2313\ B + 0.6625\ C - 0.3550\ AB - 0.1175\ AC + 0.0325\ BC - 1.0620A^2 - 0.0520\ B^2 - 0.1245\ C^2$	0.9805	0.9554
Polyphyllin VII content (Y_2)	$Y_2 = 16.8520 + 1.7600\ A + 0.4025\ B + 1.7500\ C - 0.5600\ AB + 0.1300\ AC + 0.0750\ BC - 1.2460A^2 + 0.3590\ B^2 - 0.6210\ C^2$	0.9748	0.9423

*A, B, and C are the independent variables.

temperature had a significant effect on the polyphyllin II content. The quadratic term of ethanol concentration ($p = 0.0001$) had a highly significant effect on the polyphyllin II content.

In Table 5, the F-value of the polyphyllin VII model is extremely significant ($F = 30.04$, $p < 0.0001$), and the lack of Fit ($F = 2.33$, $p = 0.2160$) was insignificant, indicating that the experimental model has a high fit with the measured results. It can be observed that the linear term of ethanol concentration ($p < 0.0001$) and number of extraction ($p < 0.0001$) had a higher effect on the polyphyllin VII content than that of extraction temperature ($p = 0.0473$). The two-level interaction had no significant effect on the polyphyllin VII content. The quadratic term of ethanol concentration ($p = 0.0010$) and number of extraction ($p = 0.0311$) had a significant effect on the polyphyllin VII content.

2.5 Response surface interaction analysis of polyphyllin II and polyphyllin VII

Three-dimensional (3D) response surface plots and two-dimensional (2D) isopleth plots are useful tools to visually represent the behavior of each variable at different experimental levels, as well as the type of interaction between two variables to determine the optimal conditions for each factor when the response values are at their maximum. These plots are commonly used in experimental design and analysis (Lee et al., 2013; He et al., 2022). The Figures 5A, 6A show that there are convex surfaces with steep slopes and the isopleths have an elliptical shape. This indicates that there are very high response values present and that the interaction between ethanol concentration and extraction temperature has a significant impact on the extraction content of two saponins. The contour lines of the ethanol concentration axis are dense, which indicates that the ethanol concentration has a significant effect on the content of two saponins. As the concentration of ethanol increases from 40 to 80%, the extraction of two saponins initially increases to the highest level and then decreases. This could be because steroidal saponins are usually present in a complex mixture with a higher polarity than ethanol. Therefore, if the ethanol concentration is too high or too low, it can reduce solubility, leading to a decrease in the extraction of the two saponins (Sahu et al., 2008). The sparse isopleths of the extraction temperature axis indicate that the effect of ethanol concentration on two saponins is greater than the extraction temperature. Figures 5B, 6B show steeper surfaces and large slopes, which indicate an interaction between ethanol concentration and the number of extraction. The isopleths are dense along the axes, indicating that the content of two saponins is significantly affected by the ethanol concentration and number of extraction. As shown in Figures 5C, 6C 3D surfaces have small slopes and the 2D isopleth plots are sparse, so the interaction between the two factors does not have a significant effect on the content of the two saponins. However, axial isopleths of the number of extraction are dense, so the number of extraction has a greater effect on the content of the two saponins than the extraction temperature. Simultaneously the extraction temperature axial isopleths are dense in Figure 6C, indicating that the extraction temperature has a significant effect on the content of polyphyllin VII. The solubility of polyphyllin VII increased with increasing extraction temperature due to cell wall disruption, solvent

TABLE 4 Analysis of variance for the fitted quadratic polynomial model of extraction of polyphyllin II.

Source	Sum of squares	df	Mean square	F-value	*p*-value
Model	30.1500	9	3.3500	39.0700	< 0.0001***
A-Ethanol concentration	20.7000	1	20.7000	241.4600	< 0.0001***
B-Extraction temperature	0.4278	1	0.4278	4.9900	0.0606
C-Number of extraction	3.5100	1	3.5100	40.9500	0.0004**
AB	0.5041	1	0.5041	5.8800	0.0458*
AC	0.0552	1	0.0552	0.6440	0.4486
BC	0.0042	1	0.0044	0.0493	0.8307
A^2	4.7500	1	4.7500	55.3800	0.0001**
B^2	0.0114	1	0.0114	0.1328	0.7263
C^2	0.0653	1	0.0653	0.7611	0.4119
Residual	0.6002	7	0.0857		
Lack of Fit	0.4533	3	0.1511	4.1100	0.1027
Pure Error	0.1469	4	0.0367		
Cor Total	30.7500	16			

*indicates significant difference ($p < 0.05$), **indicates highly significant ($p < 0.01$), ***indicates extremely significant ($p < 0.0001$).

TABLE 5 Analysis of variance for the fitted quadratic polynomial model of extraction of polyphyllin VII.

Source	Sum of squares	df	Mean square	*F*-value	*p*-value
Model	60.7200	9	6.7500	30.0400	< 0.0001***
A-Ethanol concentration	24.7800	1	24.7800	110.3200	< 0.0001***
B-Extraction temperature	1.3000	1	1.3000	5.7700	0.0473*
C-Number of extraction	24.5000	1	24.5000	109.0700	< 0.0001***
AB	1.2500	1	1.2500	5.5800	0.0501
AC	0.0676	1	0.0676	0.3010	0.6003
BC	0.0225	1	0.0225	0.1002	0.7609
A^2	6.5400	1	6.5400	29.1000	0.0010**
B^2	0.5427	1	0.5427	2.4200	0.1641
C^2	1.6200	1	1.6200	7.2300	0.0311*
Residual	1.5700	7	0.2246		
Lack of Fit	0.9999	3	0.3333	2.3300	0.2160
Pure Error	0.5725	4	0.1431		
Cor Total	62.3000	16			

*indicates significant difference ($p < 0.05$), **indicates highly significant ($p < 0.01$), ***indicates extremely significant ($p < 0.0001$).

penetration into the plant matrix, and higher mass transfer rates. The analysis of the response surface plot above was consistent with the findings of the ANOVA results.

2.6 Verification of the models

Predictive model analysis showed that the optimal extraction processes for polyphyllin II and polyphyllin VII were ethanol concentrations of 72.9659 and 70.663%, extraction temperatures of 43.218 and 50.000°C, and number of extraction 3, respectively. The theoretical predicted values of extraction were 6.548 and 19.172 mg/g (DW), respectively. Considering the practical feasibility, the extraction processes of polyphyllin II and polyphyllin VII were adjusted to ethanol concentrations of 73 and 70%, extraction temperatures of 43 and 50°C, and the number of extraction 3, respectively. Under the adjusted process conditions, after five parallel experiments, the content of polyphyllin II was 6.427 mg/g (DW, RSD of 1.40%), with a deviation of 1.84%; and the content of polyphyllin VII was 19.015 mg/g (DW, RSD of 1.97%), with a deviation of 0.82%.

Currently, due to the scarcity and high demand for Paridis Rhizoma, many scholars have optimized the extraction of effective medicinal components from the rhizomes of *P. polyphylla* var. *yunnanensis* to alleviate the situation. Tian et al. (2024) extracted polyphyllins from *Paris polyphylla* var. *chinensis* rhizomes by using ultrasonic-assisted extraction technology, and the final optimized process was a

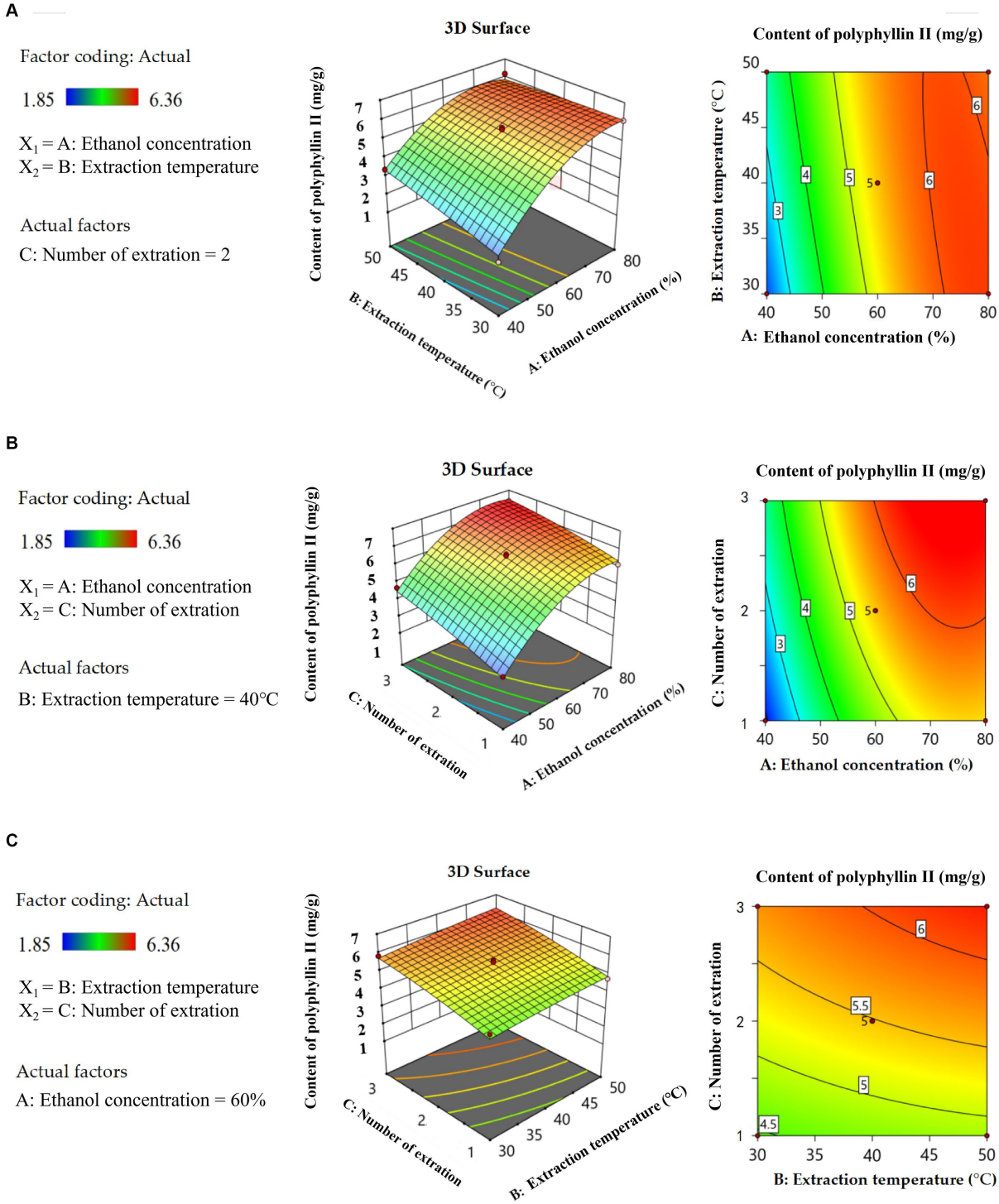

FIGURE 5
3D response surface curve plots and 2D isopleth plots of the influence of the interaction between ethanol concentration and extraction temperature **(A)**, ethanol concentration and number of extraction **(B)**, and extraction temperature and number of extraction **(C)** on the content of polyphyllin II.

liquid–solid ratio of 41.72 mL/g, extraction temperature of 55.97°C, extraction time of 30.21 min, and the total polyphyllins extraction yield was 52.56 mg/g. Liu et al. (2022) also used ultrasonic-assisted extraction technology to extract the polyphyllins from *Paris polyphylla* Smith var. *chinensis* rhizomes. However, there has been no study on the extraction process optimization of *P. polyphylla* var. *yunnanensis* leaves by using ultrasound-assisted extraction technology. Therefore this study was carried out to optimize the extraction process of saponins from non-medicinal parts, leaves of *P. polyphylla* var. *yunnanensis*. The utilization of *P. polyphylla* var. *yunnanensis* was improved.

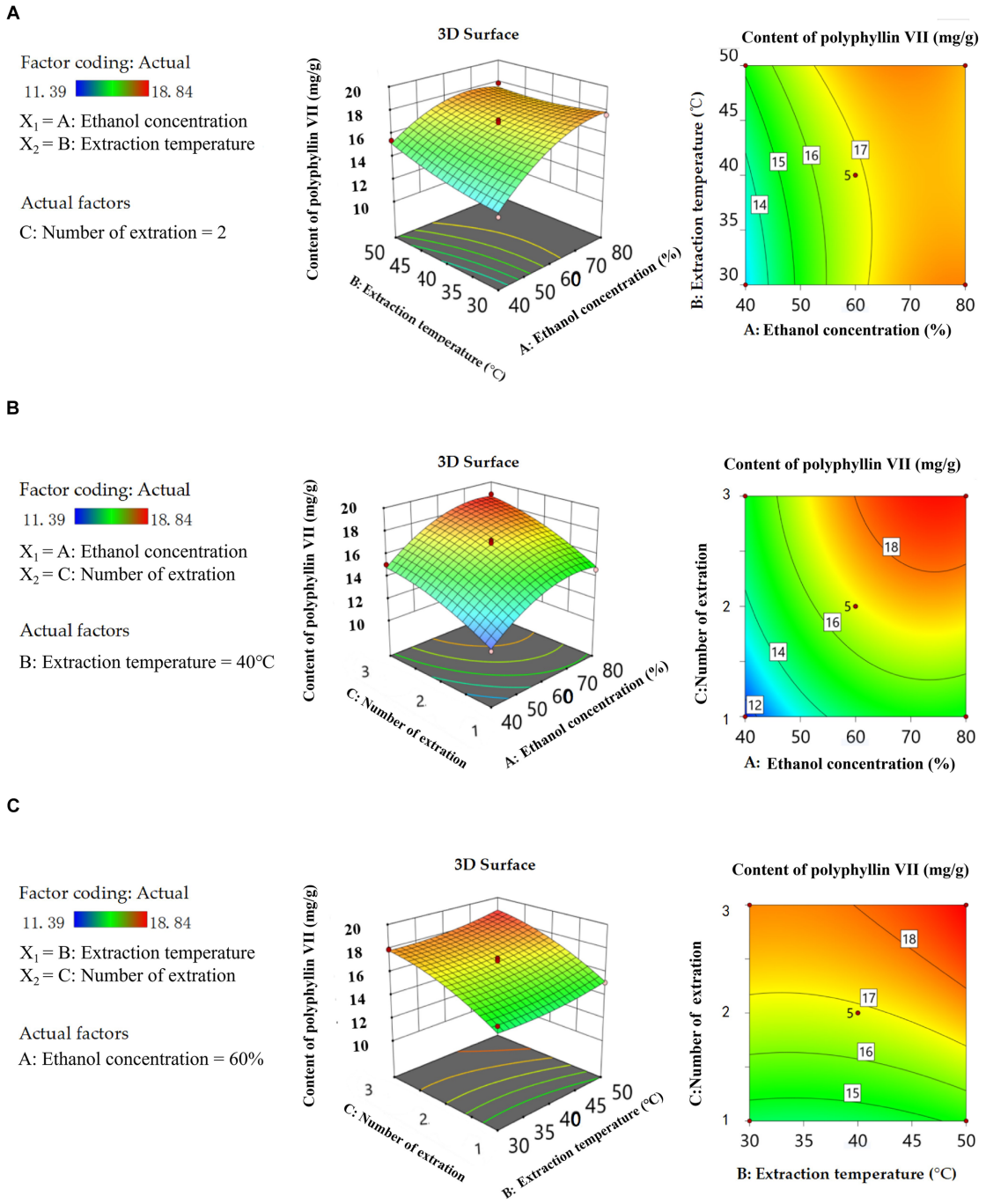

FIGURE 6
3D response surface curve plots and 2D isopleth plots of the influence of the interaction between ethanol concentration and extraction temperature **(A)**, ethanol concentration and number of extraction **(B)**, extraction temperature and number of extraction **(C)** on the content of polyphyllin VII.

3 Materials and methods

3.1 Materials and chemicals

Seedings of *P. polyphylla* var. *yunnanensis* (7 to 8 years old) were purchased from Yunnan Kunming Jiange Herbal Cultivation Co., Ltd., The fresh leaves of the *P. polyphylla* var. *yunnanensis* seedings were dried to constant weight at 50°C in the thermostatic blast drying oven (DHG-9145A, Shanghai Yiheng Technology Co., Ltd., Shanghai, China) and were crushed with a grinder. The leaf powder used in this study was passed through a 60-mesh sieve.

Polyphyllin II and polyphyllin VII standards with 98% purity were purchased from Shanghai NatureStandard Bio-Technology Co.,

Ltd., (Shanghai, China). Chromato-graphic acetonitrile (Beijing Bailingwei Technology Co., Ltd., Beijing, China) was used for HPLC analysis. Ultrapure water was made by a water purifier (PP010XXM1, ELGA, United Kingdom). All solvents prepared for HPLC were filtered through a syringe filter (0.22 μm pore size) before use. Ethanol was of analytical grade and purchased from Guangdong Guanghua Technology Co., Ltd., (Guangdong, China).

3.2 Ultrasound-assisted extraction of polyphyllin II and polyphyllin VII

Polyphyllin II and polyphyllin VII were extracted from the leaf of *P. polyphylla* var. *yunnanensis* by UAE, which was performed by using ethanol as the extraction solvent at the given ethanol concentration, solid–liquid ratio, extraction time, extraction temperature and number of extraction. That is to say, a 0.2 g sample powder was extracted by 2 mL of 75% ethanol at 30°C in an ultrasonic bath at 53 kHz (SK5210HP, Shanghai Kudos Ultrasonic Instrument Co., Ltd., Shanghai, China) for 0.5 h, and the process was repeated three times. Subsequently, the extracted solutions were combined and filtered through a syringe filter (0.22 μm pore size) for HPLC analysis. When a single-factor experiment was carried out, other factors were set as above condition. The single-factor experiments were carried out on five factors: solid–liquid ratio (1:5, 1:10, 1:15, 1:20 g/mL), extraction time (0.5, 1, 1.5, 2 h), extraction temperature (20, 30, 40, 50°C), number of extraction (1, 2, 3, 4) and ethanol concentration (20, 40, 60, 80%). Each treatment consisted of three repetitions. The single-factor experiment scheme for the extraction of polyphyllin II and polyphyllin VII is shown in Figure 7.

3.3 Response surface methodology (RSM)

BBD is one of the most commonly used design methods in RSM, which can evaluate multiple independent variables and even their interactions (Tirado-Kulieva et al., 2021). Through the regression model obtained, the extracted content of two saponins can be predicted when the factors within the design range are in different combinations at different levels. Based on the single-factor experiment, three important factors, namely, ethanol concentration (A), extraction temperature (B) and number of extraction (C), had a significant influence on the extraction content of two saponins. These three factors with three levels were used for the RSM-BBD experiment design.

A second-order polynomial model was developed to determine the regression coefficients and the significance of the model and the dependent variable ($p < 0.05$) was tested by ANOVA after the content of the two saponins was determined according to the experimental design. Then, 2D isopleth plots and 3D response surface curve plots were drawn to analyze the effect of the interaction of the factors on the response values. Finally, the optimal ultrasonic extraction process parameters were determined and validated using the response surface prediction model.

3.4 Quantification of polyphyllin II and polyphyllin VII by HPLC

An Agilent high-performance liquid chromatography system (Agilent Technologies, Santa Clara, CA, United States), consisting of a manual injector, a quarternary pump, a DAD UV detector and an EC-C18 column (250 mm × 4.6 mm; 4 μm) was used for the determination of polyphyllin II and polyphyllin VII. The mobile phase consisted of A (water) and B (acetonitrile) using the gradient elution method (Table 6) at a flow rate of 1 mL/min. The column temperature was maintained at 30°C, the detection wavelength was set to 203 nm, and the injection volume was 10 μL. A series of standard solutions of five concentrations, 0.0625 mg/mL, 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL and 1.0 mg/mL, were prepared by diluting the mixed standard solution with chromatographic methanol (Beijing Bailingwei Technology Co., Ltd., Beijing, China) for the determination of the standard curves.

TABLE 6 HPLC mobile phase procedure.

Time (min)	A: water (%)	B: acetonitrile (%)
0.0	57.0	43.0
13.0	57.0	43.0
14.0	45.0	55.0
25.0	45.0	55.0

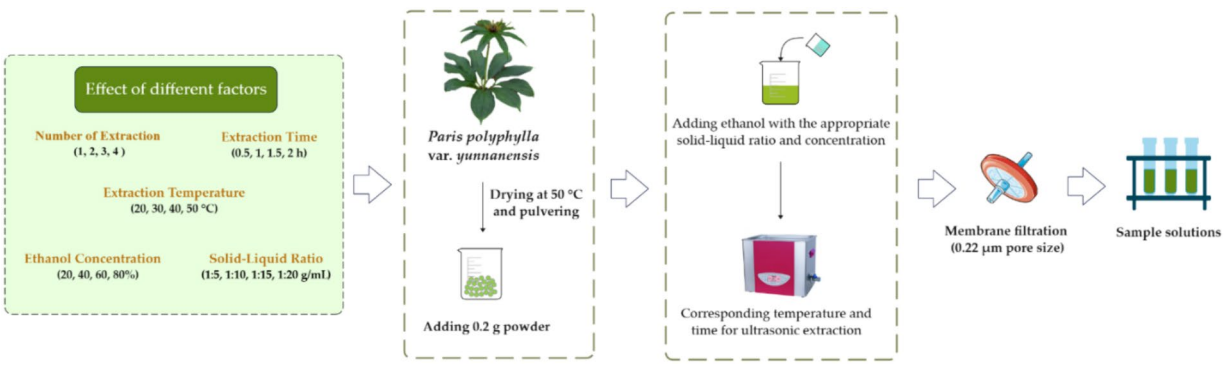

FIGURE 7
Experimental scheme for extraction of polyphyllin II and polyphyllin VII.

3.5 Statistical analysis

The data obtained in the experiments were counted using Excel software (2013, Microsoft Office) and then subjected to *T*-test and one-way ANOVA using IBM SPSS Statistics 25.0 (Statistical Product Service Solutions, United States). The statistical significance level was set at $p < 0.05$. Design Expert 12.0 software (Stat Ease, Inc., Minneapolis, MN, United States) was used for experimental design, ANOVA, modelling and prediction optimization.

4 Conclusion

On the basis of a single-factor experiment, three extraction factors (ethanol concentration, extraction temperature, and number of extraction) were optimized for the extraction process of polyphyllin II and polyphyllin VII from *P. polyphylla* var. *yunnanensis* leaves using the BBD method of the RSM in this study. The optimal extraction process for polyphyllin II and polyphyllin VII are ethanol concentration of 73 and 70%, extraction temperature of 43 and 50°C, and number of extraction 3, respectively. Under the above conditions, the contents of polyphyllin II and polyphyllin VII were measured to be 6.427 and 19.015 mg/g (DW). The predicted values by response surface methodology were basically consistent with the actual results, and the model fit was good. Therefore, the RSM can be applied to optimize the extraction process of polyphyllin II and polyphyllin VII from *P. polyphylla* var. *yunnanensis* leaves. This method provides an effective approach for the comprehensive development and utilization of non-medicinal parts of *P. polyphylla* var. *yunnanensis*.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

XG: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. QQ: Writing – review & editing, Visualization, Validation, Investigation, Formal analysis. YJ: Writing – review & editing, Visualization, Validation, Investigation, Formal analysis. HL: Writing – original draft, Visualization, Validation, Investigation. KG: Writing – original draft, Visualization, Validation, Investigation. ZZ: Writing – original draft, Visualization, Validation, Investigation. PL: Writing – review & editing, Project administration. AL: Writing – review & editing, Resources, Project administration, Funding acquisition. RS: Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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