A Simple and Economic Limewater-based Conversion of Spirostanol Saponins: Using Paridis Rhizoma as a case study

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Research

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Abstract

Background

Green and economic conversion of spirostanol saponins in the plant is considered as an attracting area in pharmaceutical applications. We aimed to provide a practical paradigm named limewater-based conversion of spirostanol saponins (LCSS), meanwhile, a widely-used traditional Chinese herbal medicine, *Paridis Rhizoma* (PR), was selected as a case study.

Methods

Based on single factor experiments, response surface methodology (RSM) using a Box-Behnken design (BBD) was employed to optimize processing time, limewater concentration and solvent volume to obtain a maximum total saponins yield from PR. H1299, A549 and HeLa cell lines was carried out to check pharmacological effect of Crude *Paridis Rhizoma* (CPR) and Processed *Paridis Rhizoma* (PPR), and the technology was reconfirmed by another herbal medicine, *Anemarrhenae Rhizoma* (AR).

Results

The optimal conditions were: processing time of 24 h, limewater concentration of 0.12% limewater and solvent volume of 60 mL/30 g. Under these conditions, the contents of polyphyllin VII, polyphyllin II, dioscin, gracillin, and polyphyllin I had 1.131 ± 0.448 , 1.835 ± 0.479 , 1.430 ± 0.550 , 1.761 ± 0.571 and 1.668 ± 0.360 times increasing in four batches of PR, which was responsible for the increasing of total spirostanol saponins (TSS) in PPR. In addition, the extracts of PPR exhibited stronger antitumor potential than that of CPR on H1299, A549 and HeLa cell lines based on MTT test and cell scratch test. The efficiency of proposed LCSS was then reconfirmed by *Anemarrhenae Rhizome* (AR), indicating its capacity in broader application.

Conclusion

This study depicted a LCSS strategy that may have great potential in achieving effective and economic improvement of spirostanol saponin accumulation in herbal medicines.

Background

Steroidal saponins, which are a type of defense compound in the plant kingdom, are exclusively distributed in the monocotyledonous families of Liliaceae, Dioscoreaceae, Alliaceae, Taccaceae, Asparagaceae, Dracaenaceae, and Agavaceae [1], and have been exploited for the production of lead compounds in drug discovery for their diverse pharmacological properties [2]. Steroidal saponins have

been reported to exerts many biological activities, such as cardioprotective [3], antimicrobial [4], anticancer [5], anti-inflammatory [6], anti-diabetes [7], anti-oxidative effect [8], and so on.

PR, known as Chonglou(重楼) in Chinese, is an well-established herbal medicine in China, often used to treat fevers, headaches, burns, wounds, and cancers [9]. *Paris polyphylla* Smith var. *yunnanensis* (Franch.) Hand.-Mazz and *Paris polyphylla* Smith var. *chinensis* (Franch.) Hara are the two legal species according to the CP 2015 edition [10], which is the main raw material of Chinese medicine including “Yunnan Baiyao” and “Gongxuening”. Studies have shown that PR has diverse pharmacological effects including antitumor [11–13], antimicrobial [14], anthelmintic activities [15], promoting hemostasis [16], antifungal [17], and immuno-stimulating properties [18]. The major active components in PR are spirostanol saponins including polyphyllin VII, VI, I, II, which are as the markers for quality control of PR [19]. Besides the spirostanol glycosides, furostanol glycosides with an open side-chain at C-22 is another type of steroidal saponins in PR [20].

In order to increase potency and efficiency or to avoid side effects and toxicity, traditional Chinese herbs are often processed before prescription [21]. Limewater, as a traditional processing method, is employed to process some herbal medicines such as *Pinelliae Rhizoma Praeparatum*, *Strychni Semen* and *Fritillaria anhuiensis* [22–24]. In order to improve the utilization of PR, limewater was used to transform Colloid *Paridis Rhizoma* to *Starchy Paridis Rhizoma* [25], and we found that the content of spirostanol saponins, which are the quality marker of crude herbs and prepared slices, increased significantly after being processed, but the processing technology is not set up, and underlying mechanism was unclear. In this study, five typical spirostanol saponins including polyphyllin I, polyphyllin II, polyphyllin VII, gracillin and dioscin were selected to investigate the processing technology and the consequence of limewater-based conversion of spirostanol saponins (LCSS). Subsequently, their antitumor activity against H1299, A549 and HeLa cell lines was carried out to check pharmacological effect. In order to verify the processing technology, *Anemarrhenae Rhizoma* (AR), another herbal medicine rich in both furostanol and spirostanol saponins, was investigated. Thus, we set up a simple, green LCSS strategy, and these findings might provide paradigm for transformation of spirostanol steroidal compounds.

Materials And Methods

Reagents and materials

HPLC-grade acetonitrile was obtained from Tedia Company Inc. (Fairfield, OH,USA). Deionized water was purified using the Milli-Q system (Millipore, Bedford, MA,USA). All other chemicals were of analytical grade and commercially available. Standard compounds, including polyphyllin I, polyphyllin II and polyphyllin VII were supplied by China Food and Drug Inspection and Research Institute (Beijing, China), gracillin, dioscin, timosaponin BII (TS BII) and timosaponin AIII (TS AIII) were obtained from Chengdu Must Biotech Co., Ltd (Chengdu, China), the purity was higher than 98.0% which was determined by HPLC analysis, the chemical structure of polyphyllin I, polyphyllin II, polyphyllin VII, gracillin and dioscin were shown in figure S1. The crude *Paridis Rhizoma* was collected from Juhuacun Chinese herbal medicine

market located in Kunming and Chinese medicinal herbs supplier in Simao, Yunnan Province, and *Anemarrhenae Rhizoma* was purchased from Bozhou County, Anhui Province, which was identified by Dr. Shuyun Bao from Wannan Medical College. The voucher specimen was deposited in School of Pharmacy, Wannan Medical College, Wuhu, Anhui Province.

H1299, A549 and HeLa cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cell lines were maintained in Dulbecco's modified Eagle medium (DMEM, Gibco) with 10% fetal bovine serum (FBS, Gibco) at 37 °C in a humidified atmosphere with 5% CO₂.

HPLC analysis for PR

Quantitative analysis of Polyphyllin VII, Polyphyllin II, gracillin, dioscin and Polyphyllin I was implemented by HPLC on a Shimadzu Prominence LC-20AT liquid chromatographic system (Shimadzu Instruments Company, Japan) composed of quaternionic pumps, a SPD-20A UV/Visible detector, a SIL-20AC auto-sampler, CTO-20A column oven and LC solution software. A ZORBAX Eclipse XDB-C18 (4.6 × 250mm, 5 µm) and a ZORBAX Eclipse XDB C₁₈ guard column (10 mm × 4 mm, 5 µm) were used. The mobile phase consisted of water (A) and acetonitrile (B) with the following gradient program: 0–8 min, 32–34% of B; 8–15 min, 34–40% of B; 15–25 min, 40–40% of B; 25–27 min, 40–43% of B; 27–60 min, 43–45% of B, 60–65 min, 45–60% of B. The column oven temperature was fixed at 30 °C, and the online monitor wavelengths were set at 203 nm. The flow rate was 1.0 mL/min and the injection volume was 10 µL. Calibration curves were established for the five saponins by plotting peak areas of standard solutions against their concentrations. The regression line were $Y = 2655.5X - 7441.1$ ($R^2 = 0.9998$) for Polyphyllin VII, $Y = 2994.9X - 17495$ ($R^2 = 1.0000$) for Polyphyllin II, $Y = 3329.3X - 11082$ ($R^2 = 0.9998$) for gracillin, $Y = 3328.3X - 17690$ ($R^2 = 0.9999$) for dioscin, and $Y = 3155.1X - 18750$ ($R^2 = 0.9999$) for Polyphyllin I. The linear ranges were 8.80–440.00 µg/mL for polyphyllin VII, 18.24–912.00 µg/mL for polyphyllin II, 8.20–410.00 µg/mL for gracillin, 16.48–824.00 µg/mL for dioscin, and 19.72–986.00 µg/mL for Polyphyllin I.

HPLC analysis for AR [26]

Qualitative analysis of TS BII and TS AIII was executed according to the Shimadzu LC-20AT liquid chromatographic system, containing an ELSD. A Sepax GP C₁₈ reverse phase column (250 mm × 4.6 mm, 5 µm) and a Shimadzu Inertsil ODS-SP C₁₈ guard column (10 mm × 4 mm, 5 µm) were used. The mobile phase consisted of water (A, with 0.1% formic acid) and acetonitrile (B) with the following gradient program: 0–21 min, 23–24% of B; 21–24 min, 24–42% of B; 24–30 min, 42–55% of B; 30–43 min, 55–65% of B; 43–44 min, 65–90% of B; 44–49 min, 100–100% of B. The flow rate, the injection volume and the column oven temperature was 1.0 mL/min, 10 µL and 30 °C, respectively. The ELSD conditions were set as follows: drift tube temperature was 35 °C, gain value was 2 and carrier gas pressure was 350 bar.

Preparation of CPR

The crude materials were moistened with water and cut into 0.3 cm pieces. After dried in oven at 50 °C, then was used as CPR in the following experiment.

Single-factor experiments

Investigation of limewater concentration

60 mL of water, clarified limewater, 0.1%, 0.5% and 1% limewater were added into 30 g pieces of CPR (purchased from Simao) respectively, then the sample were processed for 24 h, followed by removing the residual solvent and drying in oven at 50 °C.

Investigation of processing time

60 mL 0.1% limewater was added into 30 g pieces of CPR (purchased from Simao), then the samples were processed for 6 h, 12 h, 24 h and 48 h, respectively. Next, the residual solvent was discarded and the samples were dried in oven at 50 °C.

Response surface methodology experiments

Response surface method (RSM) was employed to optimize the conditions of processing technology. According to the results of single factor optimization, three variables including processing time, limewater concentration and solvent volume were selected, coded as X_1 – X_3 and examined in three levels, then the Box-Behnken design includes 17 experiment runs were performed in random in order to evaluate the main effects of the factors. The factors, high and low levels, the obtained results are presented in Table 1.

Scale-up experiment

500 g pieces of PR was immersed in 1000 mL 0.12% limewater for 24 h, then the solvent was removed and PR was dried in oven at 50 °C. The treated PR was used as PPR.

Preparation of sample solutions

The CPR and PPR were grounded into powder and sieved (50 mesh). 0.5 g of sample powder was accurately weighed and transferred to a 50 mL glass-stoppered conical flask. 25 mL 80% ethanol (v/v) was added and the filled flask was weighed with a precision of ± 0.01 g. The sample solution was sonicated with ultrasound for 40 min, and cooled to room temperature. Then adjusted the flask to the initial weight by adding 80% ethanol (v/v) as needed, and the solution was filtered through a 0.22 μ m Millipore filter before injection for HPLC analysis.

Preparation of extracts for pharmacological experiment in vitro

About 200 g powder of CPR and PPR (purchased from Simao) were transferred to a 5000 mL round-bottomed flask equipped with a reflux condenser, followed by adding 3000 mL 80% ethanol (v/v) into the flask. The solutions were refluxed twice in heating jacket for 2 h. The first and second filtrates were combined then concentrated using the vacuum rotary evaporator and lyophilized to powders.

MTT assay

Inhibition effect of CPR and PPR on H1299, A549 and HeLa cell lines was evaluated by MTT assay. A total quantity of 1×10^4 cell lines were seeded in 96-well plates (Corning) and incubated overnight. Then, cell lines in each well were treated with different concentrations (1, 5, 20, 50, 100 ng/ μ L) of CPR or PPR, respectively, and the cell lines were further cultured for 24 h. Next, 50 μ L of MTT reagent was added into each well according to the user manual of the reagent. The supernatants were then removed 4 h later, and 150 μ L of DMSO was added to dissolve formazan crystals. Absorbance at 550 nm was determined by a microplate reader.

Cell scratch test

H1299, A549 and HeLa cell lines were planted in 6-well plates (Corning) for scratch test. When the cell lines grew to a confluency of 90%, a sterile pipette tip was used to create linear wounds on the surface of each well. Then the cell lines in plates were washed with PBS three times to remove the floating cell lines. Next, the cell lines were cultured in DMEM supplemented with 1% FBS in an incubator. And different concentrations of CPR and PPR were added into each well, respectively. Cell migration was observed at 0, 6, 12, 24 h by an inverted microscope. Image J was used to analyze the wound healing rate.

Statistical Analysis

Statistical analyses were conducted using SPSS 19.0 (IBM, Armonk, NY, USA), and statistical analysis of RSM was executed using Design Expert 8.06.1. The count data were presented as the mean $\pm$ standard deviation. $p < 0.05$ was considered statistical significance.

Results

Analysis of HPLC

Based on previous findings [27, 28], the chromatograms conditions of HPLC were optimized and established, the baseline resolution of the thirteen peaks was obtained and good peak shapes were observed without tailing (Fig. 1). In the chromatogram, peak 6, peak 9, peak 10, peak 11, and peak 12 were identified as polyphyllin VII, polyphyllin II, dioscin, gracillin, and polyphyllin I with authentic standards by comparing their retention times. Peak 6 was pennogenyl sanponin, and peak 9, peak 10, peak 11, and peak 12 were diosgenyl sanponins, and peak 1, peak 2, peak 3 and peak 4 were predicted to be prototype saponins according to the previous report [29].

Single Factor Experiment

The key factors of convert technology are limewater concentration and processing time, so in order to investigate these two conditions, a series of single factor experiments were carried out based on the contents of five saponins (total spirostanol saponins, TSS) in the pieces as the evaluation index. Keeping processing time at 24 h, the concentration of limewater was investigated. The results showed that the

contents of polyphyllin II, polyphyllin I, dioscin, gracillin and TSS ranked the highest in five groups when limewater concentration was 0.1%, presenting 4.39 ± 0.43 , 1.37 ± 0.13 , 16.79 ± 2.04 , 15.01 ± 1.25 and 38.74 ± 0.66 mg/g (Table S1). Later on, the processing time was investigated while the limewater concentration was set at 0.1%. We found that when processing time was less than 12 h, the pieces can't be moistened thoroughly. When processing time was over 12 h, the contents of polyphyllin II, polyphyllin I, dioscin, gracillina and the TSS peaked when processing time was 24 h (Table S2).

Response Surface Methodology

Experimental Design and Results of BBD

Based on single factor experiments, RSM using a BBD was employed to optimize processing time, limewater concentration and solvent volume to obtain a maximum yield of TSS including five known polyphyllins. As seen in Table 1, the results showed that the yields of TSS ranged from 15.12 mg/g to 20.06 mg/g.

Fitting the Model

The analysis of variance (ANOVA), goodness-of-fit and the adequacy of the regression model were summarized in Table 2. In this model, the second-order polynomial model for the yield of TSS was statistically significant with a small model p-value ($p < 0.0001$) and satisfactory coefficient of determination ($R^2 = 0.9721$). In this case, interaction parameters (X_1X_3 , X_2X_3) and quadratic parameters (X_1^2 , X_2^2 , X_3^2) were significant at the level of $p < 0.01$, the linear parameter of X_2 was significant at the level of $p < 0.05$. The "Lack of Fit-Value" of the model is not significant with a p-value of 0.4725. The significant regression and non-significant lack of fit indicated that the regression equation is adequate to represent the actual relationship between the response values (Y) and three independent variables. The quadratic regression equation was obtained as follows Eq. (1):

$$Y = 4.374 + 0.824 * X_1 + 23.729 * X_2 + 0.129 * X_3 + 0.356 * X_1X_2 - 0.003 * X_1X_3 + 0.661 * X_2X_3 - 0.014 * X_1^2 - 300.400 * X_1^2 - 0.001 * X_1^2 \quad (1)$$

Analysis of Response Surfaces

Response surface plots were shown in Figure S2. The interaction between various factors can be seen directly from the response surfaces plots. Figure S2A shows the effect of the interaction of processing time and limewater concentration on the yield of TSS at a fixed solvent volume of 60 mL. An increase of limewater concentration (X_2) resulted in an increase of yield of TSS to a maximum at a certain level, while an increase of processing time (X_1) resulted in an initial increase of yield of TSS and then decreased as the processing time continued to increase. Figure S2B shows the effect of the interaction of the volume and processing time on the yield of TSS at a fixed limewater concentration of 0.1%. It could be observed that the volume and processing time resulted in similar effects on yield of TSS as in Figure S2A. Figure S2C shows the effect of the interaction of volume and limewater concentration on the yield of TSS at a fixed processing time of 24 h. It could be observed that both volume and limewater

concentration demonstrated a positive influence on the yield of TSS. The combination of the analysis of variance (ANOVA) (Table 2) and response surfaces (Figure S2) indicated that the interaction effect between processing time and solvent volume interaction parameters (X_1X_3), and limewater concentration and solvent volume (X_2X_3) were statistically significant, but the interaction effect between processing time and limewater concentration (X_1X_2) was non-significant. Furthermore, it was concluded that the effect of limewater concentration was more significant than solvent volume and processing time on the content of TSS.

Optimization of LCSS

Optimized conditions for the system were processing time 24.48 h, 0.12% limewater concentration and solvent volume 59.99 mL, aimed to maximize the yield of TSS. Considering the operability, the optimal conditions were slightly modified as follows: extraction time, 24.5 h; limewater concentration, 0.12%; and solvent volume, 60 mL. To confirm the suitability of the model equation, three confirmation experiments were conducted under the optimized conditions. Under these conditions the experimental the yield of TSS was 19.69 ± 0.14 mg/g, which matched well with the predicted values of 19.75 mg/g. This confirmed that the model was adequate for optimization of the LCSS.

Verification of LCSS

Based on above optimized parameters, 500 g of four batches of PR collected from Yunnan Province were verified under the conditions of 0.12% limewater, processing time of 24 h and solvent volume of 1000 mL. The results showed that the content of TSS in PPR increased distinctly, from 16.65 ± 0.48 mg/g to 35.97 ± 0.96 mg/g in batch one, from 21.63 ± 0.24 mg/g to 30.05 ± 0.32 mg/g in batch two, from 22.02 ± 0.27 mg/g to 40.72 ± 0.57 mg/g in batch three, and from 16.39 ± 0.23 mg/g to 20.24 ± 0.68 mg/g in batch four (Table 3). The ratio of areas of peak 1, 2, 3, 4, 5, 7, 8, 13 and the content of five known polyphyllins in PPR and CPR were calculate to investigate the content variation of compounds. The average ratios of peak 1, 2, 3, 4, 5 and 7 in four batches of PPR/CPR were less than 1, while that of polyphyllin VII, polyphyllin II, dioscin, gracillin, and polyphyllin I were 1.131 ± 0.448 , 1.835 ± 0.479 , 1.430 ± 0.550 , 1.761 ± 0.571 and 1.668 ± 0.360 (Fig. 2), indicating that spirostanol saponins can be converted after limewater processing.

MTT test

To evaluate LCSS, the cytotoxicity of CPR and PPR on H1299, A549 and HeLa cell lines were carried out. We calculated the inhibition rate by the OD value of cell lines treated by different concentrations of CPR and PPR comparing with control group using MTT assay. The results revealed that both CPR and PPR can significantly inhibit the activity of three tumor cell lines in a dose-dependent manner. What's more, PPR exhibited a stronger inhibition than CPR at the same concentration in most groups (Fig. 3).

Cell scratch test

Cell scratch test is usually used in studies to find out the effect of drugs on the ability of migration of cell lines *in vitro*. We compared the migration ability of H1299, A549 and HeLa cell lines treated with CPR and

PPR, respectively. The results illustrated that the migration distance of H1299, A549 and HeLa cell lines treated with CPR and PPR was considerably less than that of control cell lines after 24 h culture, suggesting that both samples can suppress the metastasis of three tumor cell lines. Furthermore, the migration ability of cell lines treated with PPR were significantly decreased than cell lines treated with CPR on the H1299 cell lines at the concentration of 10 ng/ μ L and on the HeLa cell lines at the concentration of 10 ng/ μ L and 20 ng/ μ L ($p < 0.05$), implying that PPR presented stronger inhibitory activity of migration on all tumor cell lines than CPR (Fig. 4).

Discussion

As a comprehensive mathematical and statistical method, RSM has been applied to optimize multiple factors [30–32], and BBD is a traditional method that is used in the experimental design [33, 34]. In the article, the factors of limewater concentration, processing time and solvent volume were listed to optimize the technology of LCSS based on BBD. In order to lessen the error of contents in sample, the crude materials was cut into 0.3 cm pieces, and 30 g of sample was employed in single processing experiment. When the solvent volume was 45 mL, the sample was moistened felicitously, so the levels of solvent volume were selected as 30 mL, 60 mL and 90 mL, considering that excessive solvent caused the composition dissolving out.

Steroidal saponins are classified into two subfamilies, spirostanol type saponins and furostanol type saponins [35], there are furostanol and corresponding spirostanol type compounds simultaneously in plants [36], and furostanol saponins can be converted into spirostanol form when catalyzing by furostanol glycoside 26-O- β -glucosidase [37, 38]. In the study, the contents of TSS increased after limewater processing, and the increasing amount of polyphyllin II, gracillin, dioscin, and polyphyllin I are responsible for the increasing of TSS. PR also contains these two types of compounds [39, 40], the transformation mechanism was speculated that C-22 hydroxy furostanol saponins were converted to the corresponding spirostanol saponins by deglycosylation reactions, cleavage of 26 aglycone bonds and F-cyclization after limewater processing, in accordance with that the area of peak 1, peak 2, peak 3 and peak 4 decreased in the HPLC chromatograms of four batches PR. In order to demonstrate the hypothesis, we selected *Anemarrhenae Rhizome* (AR), another herbal medicine comprising furostanol saponin of timosaponin BII (TS BII) and spirostanol form of timosaponin AIII (TS AIII), to verify the LCSS based on the optimized parameters. After processing, the area of TS BII peak decreased dramatically, from 837823 to 256393, while the area of TS AIII increased markedly, from 8455 to 1397972 (Fig. 5a). Accordingly, the transformation from furostanol saponins to spirostanol form was probably the important mechanism in limewater processing (Fig. 5b). The amount of spirostanol saponins fluctuated after processing in three batches of PR, which may be related to the content of corresponding precursor saponins.

The compounds of polyphyllin I, polyphyllin II, gracillin and dioscin, which are quality control markers of PR, present excellent antitumor activity in recent reports [41–46]. In this work, the content of polyphyllin I, polyphyllin II, gracillin and dioscin increased significantly in PR after processing, showing consistence

with the fact that PPR exhibited a stronger effect on tumor cell lines than CPR in both MTT assay and scratch test. Taken together, the stronger anticancer effect of PPR can be attributed the increased content of spirostanol saponins, suggesting that it might be useful for quality promotion of PR.

Conclusion

The processing technology of LCSS was established, with the content of spirostanol saponins increased significantly in the PPR. The mechanism of transformation is assumed that furostanol type saponins is converted into corresponding spirostanol saponin *via* deglycosylation in LCSS, which was reconfirmed by AR. The PPR had a notable advantage of higher content of active ingredients and stronger antitumor activity, meaning that the application of LCSS improved the quality of PR, and represented a more valuable alternative to CPR. Thus, a simple, economic and effective LCSS strategy of spirosteranol saponins was set up in raw materials based on activity evaluation and constituent analysis. The results of this research might provide paradigm for elucidating the processing mechanism of PR, and also will expand its application in pharmaceutical industry for the preparation of other spirostanol steroid saponin in herbal materials.

Abbreviations

LCSS, limewater-based conversion of spirostanol saponins; PR, Paridis Rhizoma; PPR, Processed Paridis Rhizoma; CPR, Crude Paridis Rhizoma; Total spirostanol saponins, TSS; RSM, Response surface methodology; BBD, Box-Behnken design; AR, Anemarrhenae Rhizome; TS BII, timosaponin BII; TS AIII, timosaponin AIII.

Declarations

Authors' Contributions

SN, LL and FB designed the experiments. LY, LL and XY performed the experiments. XS and FB analyzed the data. LY and FB wrote the manuscript. XS, LL, YF and SN discussed the results and revised the final manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Availability of data and materials

All data used to support the findings of this study are available from the corresponding author upon request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Tables

Table 1 Coded levels, conditions runs with the experimental used in BBD

Run	Time (h) X_1	Concentration (%) X_2	Volume (mL) X_3	Yield of TSS (mg/g) Y
1	24(0)	0.1(0)	60(0)	20.06
2	24(0)	0.1(0)	60(0)	19.73
3	36(1)	0.2(1)	60(0)	15.92
4	24(0)	0.2(1)	90(1)	18.62
5	12(-1)	0.1(0)	90(1)	16.86
6	24(0)	0.05(-1)	30(-1)	18.31
7	36(1)	0.1(0)	30(-1)	18.13
8	24(0)	0.1(0)	60(0)	19.89
9	24(0)	0.1(0)	60(0)	19.56
10	12(-1)	0.1(0)	30(-1)	15.66
11	24(0)	0.1(0)	60(0)	18.88
12	12(-1)	0.05(-1)	60(0)	16.79
13	12(-1)	0.2(1)	60(0)	15.12
14	24(0)	0.05(-1)	90(1)	15.69
15	36(1)	0.1(0)	90(1)	15.37
16	36(1)	0.05(-1)	60(0)	16.09
17	24(0)	0.2(1)	30(-1)	15.33

Table 2. ANOVA for linear, square, and interactions of factors in RSM model for LCSS

Source	Sum of Squares	df	Mean Square	F Value	p-value	
Model	51.07	9	5.67	27.14	0.0001	significant
X_1 -Time	0.16	1	0.16	0.77	0.4084	
X_2 -Concentration	1.46	1	1.46	6.96	0.0335*	
X_3 -Volume	0.42	1	0.42	2.03	0.1972	
X_1X_2	0.43	1	0.43	2.07	0.193	
X_1X_3	3.92	1	3.92	18.75	0.0034**	
X_2X_3	9.35	1	9.35	44.69	0.0003**	
X_1^2	17.92	1	17.92	85.72	< 0.0001**	
X_2^2	8.97	1	8.97	42.92	0.0003**	
X_3^2	4.69	1	4.69	22.44	0.0021**	
Residual	1.46	7	0.21			
Lack of Fit	0.63	3	0.21	1.02	0.4725	not significant
Pure Error	0.83	4	0.21			
Cor Total	52.53	16				
R^2	0.9721					
R^2_{Adj}	0.9363					
C.V. %	2.63					

Table 3 Content of saponins in four batches of CPR and PPR (mg/g, n=3)

program		Content					
		Polyphyllin VII	Polyphyllin II	Dioscin	Gracillin	Polyphyllin I	TSS
Batch 1	CPP	1.07 ± 0.03	5.14 ± 0.16	0.76 ± 0.06	1.29 ± 0.05	8.39 ± 0.37	16.65 ± 0.48
	PPP	0.96 ± 0.06	12.57 ± 0.41	1.67 ± 0.05	2.88 ± 0.06	17.9 ± 0.46	35.97 ± 0.96
Batch 2	CPP	3.31 ± 0.06	4.30 ± 0.07	2.10 ± 0.10	0.76 ± 0.02	11.17 ± 0.31	21.63 ± 0.24
	PPP	2.41 ± 0.04	8.22 ± 0.16	1.90 ± 0.07	1.25 ± 0.04	16.27 ± 0.36	30.05 ± 0.32
Batch 3	CPP	0.96 ± 0.07	3.18 ± 0.09	1.19 ± 0.02	6.83 ± 0.20	9.86 ± 0.31	22.02 ± 0.27
	PPP	1.68 ± 0.04	5.31 ± 0.09	1.49 ± 0.02	14.88 ± 0.29	17.36 ± 0.61	40.72 ± 0.57
Batch 4	CPP	0.54 ± 0.02	3.25 ± 0.08	0.72 ± 0.03	4.09 ± 0.08	7.81 ± 0.10	16.39 ± 0.23
	PPP	0.62 ± 0.02	4.25 ± 0.04	0.97 ± 0.04	4.10 ± 0.06	10.31 ± 0.15	20.24 ± 0.68

Figures

Figure 1

HPLC chromatograms of CPR (a), PPR (b) and five standards (c) peak 6, polyphyllin VII; peak 9, peak polyphyllin II; peak 10, dioscin; peak 11, gracillin; peak 12, polyphyllin I.

Figure 2

Ratios of area peak (a) and contents (b) of five saponins in four batches of PPR and CPR. PP VII, polyphyllin VII; PP II, polyphyllin II; G, gracillin; D, Dioscin; PP I , polyphyllin I.

Figure 3

CPR and PPR inhibit the growth of H1299 (a), A549 (b) and HeLa (c) cell lines. “*” indicates the significance of difference of inhibition status between CPR and PPR at the same concentration. **, $p < 0.01$; ***, $p < 0.001$.

Figure 4

Effects of CPR and PPR on wound healing of H1299 (a, b), A549 (c, d) and HeLa (e, f) cell lines. "*" indicates the significance of difference of inhibition status between CPR and PPR at the same concentration. *, $p < 0.05$.

Figure 5

The transformation from TS BII to TS AIII with cleavage of 26 aglycone bonds and F-cyclization processed by limewater. a, HPLC chromatograms of AR before and after processing; b, The transformation mechanism of furostanol saponins to spirostanol form.

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