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Table: 2, Figs : 3

Optimization of conditions for extraction of ginsenoside from ginseng plant tissues

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ABSTRACT

The conditions of ultrasonic extraction were optimized by response surface methodology. The impact of ethanol volume fraction, extraction time and solvent: solid ratio on the extraction efficiency were determined The total content of 9- ginsenosides in the stems and leaves of ginseng was determined by HPLC. The effects of extracted ginsenosides were evaluated using the tomato leaf spot fungus spore germination test.

Key words: Ethanol, extraction process, fungus, ginseng, ginsenosides, HPLC, leaves, *Panax ginseng*, spore germination, stems, tomato, ultrasonic extraction,

INTRDUCTION

Ginseng (*Panax ginseng* C. A. Mey., family Araliaceae) is a perennial herb (20), whose tubers are extensively used in Chinese medicines (1,6,17). Since the 1970s, the ginsenosides in stems and leaves of this plant has attracted the attention due to their antibacterial activity (5,10,14,29). The total content of ginsenosides in stems and leaves is higher than in the root (11,13,24). Thus developing an efficient process to extract the ginsenosides from stems and leaves can, therefore, greatly reduce the cost of gensenosides and also provide a new approach to the development of natural ginsenosides. Although many methods are known to extract the ginsenosides from the tubers, no simple and efficient method to extract these from the plant tissue is known.

In this study, we used a central composite design-response surface method to optimize the extraction process of ginsenosides from the stems and leaves of ginseng. The quality of the preparation was evaluated by the *Alternaria tenuissima* spore germination test.

MATERIALS AND METHODS

The stems and leaves of ginseng plants were collected from 4-year-old ginseng plants from our experimental field. After air-drying, crushing and sieving through No. 80 mesh, the powder was used for extraction trials.

The efficiency of the extraction process was tested by using the *A. tenuissima* spore gemiantion test. The test fungus *A. tenuissima* was used, which causes the leaf spot disease in tomato. It was earlier isolated and identified in this laboratory (unpublished work).

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Extraction of total ginsenosides

One g ginseng tissue powder was weighed and placed in several 100 ml conical flasks and mixed with ethanol-water solution (10%, 30%, 50%, 70% or 90%) in different solvent : solid ratios (10:1, 20:1, 30:1, 40:1 or 50:1) and soaked for 24 h. The mixtures were then subjected to ultrasonication using (KQ-250DV ultrasonic cleaner, Kunshan Ultrasonic Instruments Co., Ltd, China) 20 kHz for various periods (30, 60, 90, 120 or 150 min) and the extracts were filtered. Extraction was done thrice and the clear filtrates were combined and evaporated to dryness in water bath to obtain the extract (12). The extract was dissolved in 5 mL methanol and filtered through a 0.45-μm membrane for HPLC analysis.

Preparation of standard solutions

The reference standard samples of nine ginsenosides: Rg_1, Re, Rf, Rb_1, Rg_2, Rc, Rb_2, Rb_3 and Rd. (>98% purity) were purchased from the Natural Medicinal Chemistry Laboratory, Jilin University. To prepare standards, 5 mg of each was weighed and mixed with HPLC grade methanol to make final volume of 5 mL, to obtain the reference standard solutions. The standard solutions were filtered through a 0.45-μm membrane filter.

Ginsenoside preparations were analysed using a Shimadzu (Shimadzu Corporation, Tokyo) LC-2010A HPLC system, fitted with a C18 column (4.6 mm×150 mm, 5 μm). The mobile phase A was acetonitrile and B was water. The gradient elution condition was 0-24 min, 18-22% B; 24-26 min, 22-26% B; 26-30 min, 26-32% B; 30-50 min, 32-33.5% B; and 50 -55 min, 33.5-38% B (31). The injection volume was 4 μL, the column temperature was 30 °C, the detection wavelength was 203 nm; the flow rate was 1.0 mL min^{-1}; and the separation time was 55 min.

Tomato leaf spot spore germination test

To test the quality of ginseng tissue extracts, 100 g of the ginsend tissue power was extracted by the standardized procedure given below. The extract was diluted with 0.05% Tween-80 to get 1, 5, 10, 20, 50 and 100 mg mL^{-1} concentrations. The hanging drop method of spore germination (3,9) was used to investigate the effects of extracted ginsenosides on the germination of *A. tenuissima* spores. For this, the fungus was grown on potato dextrose agar (PDA) at 25°C for 5 days in dark. The spores were washed with deionized water, filtered and centrifuged at 1000 rpm for 5 min. The above process was repeated thrice. The spores were re-suspended in deioniszed water to obtain a suspension of 1×10^6 spores /mL. Equal amounts of 10 μL spore suspension and the ginsenosides extract of 6-concentrations were mixed on a glass slip cover and inverted on the concave glass slide. The slides were then incubated in a moistened Petri dish at 28 °C in dark, with three replications per treatment. Tween 80 (0.05%) in water served as control. Spore germination was recorded microscopically (7). Any spore with the sporophyte tube longer than the short radius of the spore was considered as germinated. Three random fields were selected for each treatment and 80-100 spores in each field were monitored. When more than 70% spores in the control group germinated, % germination in all treatments was determined.

Germination (%) = (Germination in treatment group / Germination in control group) × 100%.

Data analysis

SAS 9.4 software was used for the multivariate linear regression, quadratic polynomial, analysis of variance and ridge analysis, while Origin 9.1 software was used for graphing. SPSS 23.0 software was used for the statistical analysis of the experimental data. Multiple factors were compared using Duncan's method. The toxicity regression equation was obtained with the logarithm of concentration of ginsenosides from the stems and leaves of ginseng and the probability of the relative inhibition rate of the spore germination. The EC_{50} value and 95% confidence interval were calculated according to the regression equation.

RESULTS AND DISCUSION

Standardization of extraction procedure- Single-factor test

The extraction procedure was standardized by first using various concentrations of ethanol and varying timings of ultra sound treatment with varying solvent : solid ratio.

When the ultrasonic extraction time was fixed to 60 min and the solvent : solid ratio was fixed at 20:1, the experiment was done 5-times with increasing ethanol concentrations (10%, 30%, 50%, 70% and 90%). The total content of nine ginsenosides extracted from the stems and leaves of ginseng was highest when the ethanol concentration was 70% (Fig. 1A). When the ethanol concentration was > 70%, the content of ginsenosides decreased. It is possible that the higher concentration of ethanol reduced the extraction of some high polarity ginsenosides viz., Ro, Ra_1, Ra_2, Ra_3, Rb_1 (4,18,19,22,23). Therefore, the central composite design was used at ethanol concentration of 40%-90%.

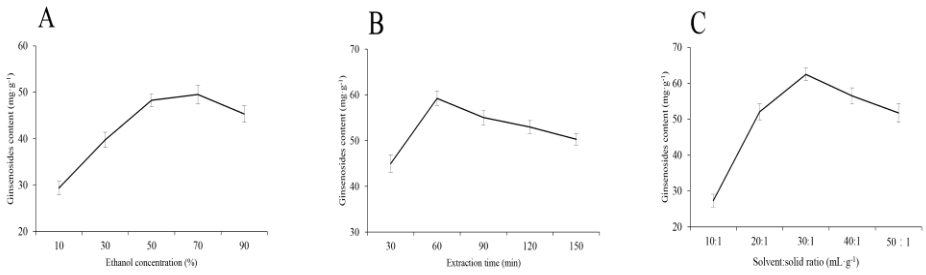

Figure 1. The effects of different factors on ginsensosides content.
A: The effects of ethanol on ginsensosides content. B: The effects of ultrasonic extraction time on ginsensosides content. C: The effects of solvent : solid ratio on ginsensosides content.

With the ethanol concentration of 70% and the solvent: solid ratio of 20:1, the ultrasonic extraction times were varied from 30, 60, 90, 120 and 150 min. As shown in Fig. 1B, when the extraction time was 60 min, the total content of ginsenosides extraction was the highest. After that, it slightly decreased as the extraction time lengthened, but the difference was not significant. Increasing the extraction time, did not significantly increase

the quantity of extracted ginsenosides. Hence, the central composite design was set at extraction time of 30-90 min.

With the ethanol concentration fixed at 70% and the ultrasonic extraction time fixed at 60 min, the solvent : solid ratio was varied from 10:1, 20:1, 30:1, 40:1 and 50:1). As shown in Fig. 1C, when the solvent: solid ratio was < 30:1, the total content of ginsenosides sharply increased with the increase in ethanol concentration. When the solvent:solid ratio was 30:1, the content of ginsenosides was highest. When the solvent : solid ratio was increased to 50:1, the content of ginsenosides decreased. A possible reason is, the non-ginsenoside impurities may have been extracted more than the ginsenosides under these conditions. The central composite design was set at the solvent:solid ratio of 20:1 to 40:1.

Response surface optimization of ginsenoside extraction

The ethanol concentration, the extraction time and the solvent: solid ratio in the ultrasonic extraction were further examined. Based on the results of the single-factor test, a central composite design was developed with the total content of ginsenosides in the stems and leaves of ginseng as the targeted indicator. The impact of the interaction between different factors on the total content of ginsenosides, the influencing parameters of the factors and the conditions of the process were analysed and optimized. The factors and their levels in the central composite design are shown in Table 1 and the central composite design (21,25,27) is shown in Table 2.

Table 1. Factors and their levels in the central composite experimental design

Level	Factor		
	Ethanol conc. (X1) （%）	Extraction time (X2) （min）	Solvent:solid ratio (X3) （$mL \cdot g^{-1}$）
-1.682	40	30	20
-1	50.13674197	42.16409037	24.05469679
0	65	60	30
1	79.86325803	77.83590963	35.94530321
1.682	90	90	40

The RSREG (Quadratic response surface regression) program in SAS software was used for the response surface analysis of the test results. The final regression equation for the total content of the 9-ginsenosides from the stems and leaves of ginseng (Y) with the ethanol concentration (X1), the extraction time (X2), and the solvent-solid ratio (X3) was $Y=-57.950726-0.527955X1+1.644585X2+5.805872X3-0.003730X1^2+0.006059X1X2-0.019061X2^2+0.024264X1X3+0.005026X2X3-0.123565X3^2$.

According to this quadratic regression model, the three-dimensional image and contour map of the response surface were prepared using Origin 9.1 software (Fig. 2). With one of the influencing factors as the central value (coding level of 0), the impacts of the other two factors on the total content of nine ginsenosides in the stems and leaves of ginseng were determined.

Table 2. Central composite design and results of the extraction of ginsenosides from the stems and leaves of ginseng (n=3, x±SD)

No.	Ethanol conce (X1)	Extraction time (X2)	Solvent:solid ratio (X3)	Ginsenosides content ($mg \cdot g^{-1}$)
1	0	0	0	66.35±1.23
2	0	0	0	68.35±1.57
3	0	0	0	68.58±1.45
4	1	1	1	54.29±2.01
5	1	1	-1	50.87±1.49
6	1	-1	1	59.18±1.97
7	1	-1	-1	52.90±2.54
8	-1	1	1	49.25±1.29
9	-1	1	-1	47.15±2.36
10	-1	-1	1	53.30±2.05
11	-1	-1	-1	52.15±2.11
12	-1.682	0	0	55.80±1.38
13	1.682	0	0	62.50±1.14
14	0	-1.682	0	49.15±1.75
15	0	1.682	0	48.10±1.33
16	0	0	-1.682	48.90±1.88
17	0	0	1.682	57.95±1.92

Note: The values of the factors are standardized, the upper level is 1, the lower level is -1, and the zero level is 0.

As shown in Fig 2, the 3-factors [Ethanol concentration(X1), Extraction time (X2) and Solvent:solid ratio (X3)] had certain impacts on the total ginsenoside extraction process with varying degrees of positive correlation and negative correlation between each pair of factors. When the ginsenoside content reached the maximum, the increase in the ethanol concentration inhibited the dissolution of ginsenosides. while the increase in the solvent : solid ratio also decreased the total ginsenoside content of the extracts.

The optimal conditions to extract the ginsenosides from the stems and leaves of ginseng were obtained by overlapping the maps of three response surfaces: Ethanol concentration (X1) of 56.0%-90.0%, Extraction time (X2) of 46.0-71.0 min and Solvent-solid ratio (X3) of 27.8:1-36.5:1. To reduce the energy consumption and save costs, the optimal extraction conditions were ethanol, 65.0% solvent : solid ratio of 29.5:1, and ultrasonic extraction time of 60 min.

Verification of the optimal extraction process

To verify the parameters for extraction, 1.00 g ginseng powder was extracted by 65.0% ethanol, with the solvent : solid ratio of 29.5:1 and the ultrasonic extraction time of 60 min in a final volume of 29.5 mL. The total content of the 9-ginsenosides determined by HPLC were 66.24 $mg \cdot g^{-1}$, 65.96 $mg \cdot g^{-1}$, and 66.37 $mg \cdot g^{-1}$, with mean of 66.19 $mg \cdot g^{-1}$, which was close to the predicted value of 66.25 $mg \cdot g^{-1}$, indicating that the established

model had high credibility. This result is consistent with previous report that the content of ginsenoside in ginseng leaves is about twice that in the roots (24).

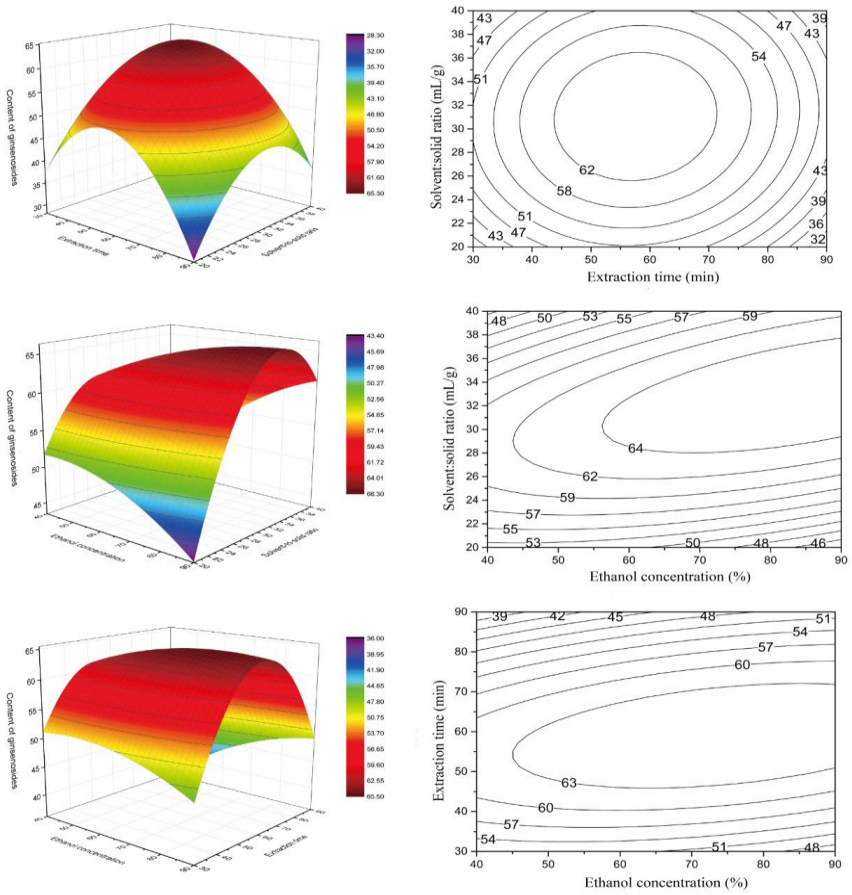

Figure 2. Response surface and contour map of the impact of interaction of two factors on the ginsenoside content in the stems and leaves of ginseng

Effects of ginsenoside extract on the spore germination of leaf spot disease

The effects of ginsenosides extracted from the stems and leaves by the optimum procedure developed herein on the spore germination of tomato leaf spot fungal spores is shown in a Fig. 3**.** It is seen that the extract was inhibitory to the germination of tomato leaf spot fungal spores and as the concentration of the extract increased, the degree of inhibition also increased. The ginsenosides from the stems and leaves of ginseng significantly inhibited the spore germination of the leaf spot disease.

When the concentration of ginsenosides from the stems and leaves of ginseng was 1-100 mg·g^{-1}, spore germination was inhibited. This could be further analyzed through the

toxicity regression analysis with the logarithm of concentration and the probability of the relative inhibition rate of spore germination. The results showed that the regression equation was Y = 1.7896X+2.7188, with the correlation coefficient R^2=0.9281, indicating that the inhibitory effects of the ginsenosides from the stems and leaves of ginseng on spore germination were positively correlated with its concentration in a certain range. The effective inhibitory concentration (EC_{50}) of the ginsenosides was 18.82 mg·mL^{-1} and the 95% confidence interval was 11.31-31.32 mg·mL^{-1}.

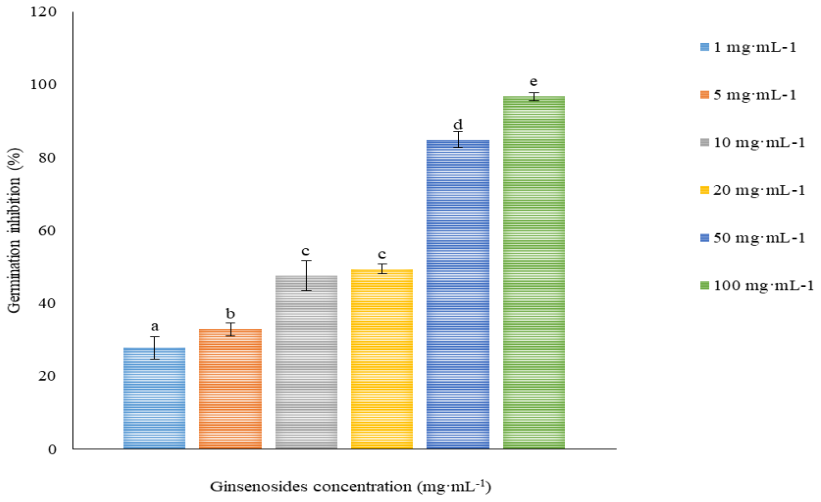

Figure 3. Inhibitory effects of ginsenosides on spore germination of tomato leaf spot fungus (*A. tenuissima*)

In the pilot experiments of this study reflux extraction, soaking**,** microwaving and ultrasonic extraction were applied to extract the ginsenosides from the stems and leaves of ginseng. (2,8,15,16). The results showed that the content of extracted ginsenosides from the stems and leaves of ginseng were highest by ultrasonic treatment in the presence of ethanol. The ultrasonic extraction conditions of extracting the ginsenosides from the stems and leaves of ginseng was determined by central composite design- response surface methodology, which has the advantages of high extraction efficiency, short extraction time and less energy consumption (28,30). Additionally, a pure physical method was applied to ensure the stability of the chemical composition in the extract, which provides a reference for the simple and efficient large-scale industrial production of ginsenosides from the stems and leaves of ginseng.

CONCLUSIONS

In this study, we used a central composite design-response surface method to optimize the extraction process of ginsenosides from the stems and leaves of ginseng. The total content of the 9-ginsenosides detected by HPLC were 66.19 $mg \cdot g^{-1}$. The ginsenosides extracted from tissues of ginseng significantly inhibited the spore germination of tomato leaf spot disease fungus. In ginseng cultivation seasonal shedding of leaves often occurs and the stems and leaves of ginseng are often abandoned in the field. The total content of ginsenosides in the stems and leaves of ginseng is higher than in the root. While effectively utilizing the waste and reducing the cost, our study also supplements the ecological effects of ginsenosides.

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