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Full Length Research Paper

Evaluation of the antifungal activity of total flavonoids extract from *Patrinia Villosa* Juss and optimization by response surface methodology

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The purpose of this work was to assess the antifungal activity of total flavonoids extract from *Patrinia Villosa* Juss. Response surface methodology was employed to optimize the main extraction conditions including extraction time, ethanol concentration and solid-liquid ratio. The effect of total flavonoids extract from *P. villosa* on *Microsporum gypseum*, *Trichophyton mentagrophytes* and *Trichophyton rubrun* were studied. We found that under the conditions of extraction time 25.51 min, solvent concentration 62.82% and solid-liquid ratio to be 27.39:1, it possesses considerable amounts of flavonoids of 8.1987 mg/g rutin equivalent of extract. The effects of antifungal activity of *P. villosa* on *Trichophyton* were more sensitive than *Microsporum*, but less effective than the positive control ketoconazole. In conclusion, total flavonoids extract from *P. villosa* possess potent anti-dermatophyte activities. These activities could contribute, at least in part, to the traditionally claimed therapeutic benefits of *P. villosa*.

Key words: Total flavonoids, antifungal activity, response surface methodology.

INTRODUCTION

Fungal infections, especially ringworm infections and dermatophytosis, can affect various parts of body, such as skin, hair or nails (Ponnusamy et al., 2010). Nowadays, increasing resistance of microorganisms against available antimicrobial agents is of major concern among scientists and clinicians worldwide. To develop new or more efficient and safe antifungal drugs is in urgent requirement. Natural products appear to have relatively mild activities against human pathogenic fungi compared to commercial synthetic antifungal drugs (Ahmadi et al., 2010). To overcome the drawbacks of the current antimicrobial drugs and to obtain more efficacious drugs, an antimicrobial drug having a novel mode of action should be developed (Orhan et al., 2010).

Patrinia villosa is a medicinal herb mainly found in East Asia and northeastern part of North America. The whole plant has antibacterial, anti-inflammatory and hepatic effects (Peng et al., 2006). It has been widely used in the treatment of carbuncles, acute appendicitis, hepatitis, amygdalitis, angina parotidea, anthracia, stasis, intestinal abscess, postpartum pain, dysmenorrhoea and endometriosis (Xie et al., 2008). However, literature search did not yield any more references to early report on study of chemicals from the medicinal herb *P. villosa*. So, further chemical research and discovery from *P. villosa* is warranted for exploiting new traditional Chinese medicine (TCM) products and pharmacological tests (Peng et al., 2006). The aim of this study was to determine the total flavonoids and optimization for ultrasound-assisted extraction of *P. villosa* by response surface methodology (RSM). In addition, evaluation of the antifungal activity of the ethanol extracts of *P. villosa* via diameters of inhibition zones (DIZs) and minimum inhibitory concentration (MIC) against several dermatophytes.

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Table 1. The level of form factors.

Factors	Extraction time (X_1)	Solvent concentration (X_2)	Solid-liquid ratio(X_3)
-1	15	40	20
0	30	60	30
1	45	80	40

MATERIALS AND METHODS

Plant materials and instruments

P. villosa was collected and identified by Professor ZY Wang, College of Life Science and Biopharmacology, Guangdong Pharmaceutical University, Guangdong province, China. In order to avoid degradation, the air-dried plant material was ground just before extraction. BL-2000S Electronic Balance (SETEA Co., Ltd.), KQ-600DE Nc ultrasonic apparatus (Kunshan ultrasonic machine company), PE lambda -35 UV spectrophotometer (Perkin Elmer Co., Ltd.) were used in this study. All other chemicals and solvents used in this study were analytical grade and obtained from Tianjin Reagent Company (Tianjin, China).

The test species were: *Microsporum gypseum* ATCC 14683, *Trichophyton mentagrophytes* ATCC 1481, *Trichophyton rubrun* ATCC 28189. Various microbial strains were obtained from National institute for the Control of Pharmaceutical and Biological Products (NICPBP). The fungi were maintained on Sabouraud dextrose agar (SDA) slants at 10°C and sub-cultured monthly.

Extraction of total flavonoids from *P. villosa*

P. villosa crushed, with 24 mesh screen. The dried powder 5.0 g was added solvent (ethanol concentration 40, 60 and 80%) in proportion (solid-liquid ratio 20:1, 30:1, 40:1) and extracted with ultrasonic wave (15, 30 and 45 min) at 50 kHz. The filtrate was collected and the residue was extracted again as the protocol above. Then, the filtered solution was stored at 4°C for further analysis.

Determination of total flavonoid content

The total flavonoid content of the extract was determined by the method described in the Chinese Pharmacopoeia (Chinese Pharmacopoeia Committee, 2005). The extraction and different concentrations of the rutin standards solution were diluted appropriately and mixed with 1 ml $NaNO_2$ (5%). After standing for 6 min, 1 ml of 10% $AlCl_3$ and 10 ml of NaOH (1 M) were added to the mixture. The mixture was adjusted to 25 ml with distilled water and allowed to rest for 15 min. The absorbance (A) was measured at 510 nm, with distilled water as a blank control. Rutin was used as a reference standard and the total flavonoid content was expressed as rutin equivalents (RE, l g/mg extract). All determinations were performed in triplicate.

Optimization of total flavonoid by Box-Behnken design for RSM

In order to obtain suitable extraction conditions for *P. villosa*, Box-Behnken designs (BBD, Design Expert software, Trial Version 8.0.4, Stat-Ease Inc., Minneapolis, MN, USA) was applied to experimental design, data analysis and model building. Based on the preliminary tests, a total of 17 runs from BBD were employed to optimize the main extraction conditions including extraction time (X_1), ethanol concentration (X_2) and solid-liquid ratio (X_3) as Table 1 shows.

Agar-well diffusion assay

The agar-well diffusion method was used to determine the antifungal activities of the tested phytochemicals. A 200 µl fungal suspension (10^8 CFU/ml) was poured and uniformly spread. One of four antimicrobial susceptibility test discs (6 mm diameter, Oxoid Limited, Hampshire, England) was dropped with 10 µl of extract (Equivalent to the concentration of crude herbs 50 mg/ml). Ketoconazole 500 µg/ml was used as positive controls for the tested fungi. Blank solvent was used as a negative control. The plates were incubated at 30°C for 48 to 96 h. Antifungal activity was evaluated by measuring the diameter of the inhibition zones. Each assay in this experiment was repeated three times.

Determination of minimum inhibitory concentrations (MICs)

Minimum inhibitory concentrations of the extracts were tested by standard NCCLs (National Committee for Clinical Laboratory Standard, 2000) in sterile 96-well microplates. 90 µl of RPMI 1640 liquid medium was distributed from the 2nd to the 12th well, a volume of 180 µl compounds initially prepared (the highest concentration 50 mg/ml) was pipetted into the first test wells of each microtitre line and then 90 µl of scalar dilution were transferred from the 1st to the 12th well. Finally, 10 µl of bacterial suspension (10^8 CFU/ml) were added to each well, to achieve a concentration of 10^7 CFU/ml. The final concentrations of the compounds adopted to evaluate antibacterial activity ranged from 0.0244 to 50 mg/ml. Two columns in each plate were used as controls: one column with Ketoconazole in a serial dilution of 0.244 to 500 µg/ml and one column containing the solvent as negative control (Nenaah, 2010). The plates were incubated at 30°C for 48 to 96 h, and each treatment was performed in duplicate thrice. The lowest concentrations of the test samples, which did not show any visual growth of test organisms after macroscopic evaluation, were determined as MICs, which were expressed in mg/ml.

Statistical analysis

All experiments data in Tables and Figures represent mean values±standard deviation (n=3). Results were evaluated for statistical significance using one-way ANOVA by SPSS V.13 (SPSS Inc., Chicago, USA). The confidence level for statistical significance was set at a probability value of 0.05. The response obtained from each set of experimental design was subjected to multiple non-linear regressions using the Design Expert software, Trial Version 8.0.4. The quality of the fit of the polynomial model equation expressed by the coefficient was checked using F-test and p-value.

Table 2. Results of response surface methodology.

No.	X_1	X_2	X_3	Y (%)
1	1	0	-1	8.1649
2	0	0	0	7.5008
3	1	0	1	7.8665
4	-1	1	0	8.16285
5	0	0	0	7.9516
6	0	-1	-1	8.18365
1	1	0	-1	8.1649
2	0	0	0	7.5008
3	1	0	1	7.8665
4	-1	1	0	8.16285
5	0	0	0	7.9516
6	0	-1	-1	8.18365
7	-1	0	-1	7.6996
8	1	-1	0	8.012
9	-1	0	1	8.1621
10	0	-1	1	8.1644
11	1	1	0	7.99015
12	0	1	1	7.98005
13	0	0	0	7.7918
14	0	0	0	7.93695
15	-1	-1	0	7.6286
16	0	1	-1	7.9146
17	0	0	0	7.74675

RESULTS AND DISCUSSION

Total flavonoids content

Calibration curves were constructed by linear regression of total flavonoids content (*Y*), versus the concentration (*x*). For linearity validation, rutin standard solutions at a concentration range of 0.036～2.839 mg/L, $Y=2185.22X_1+3.18$ *(r=0.9999).* The total flavonoid content of *P. villosa* extraction was expressed as rutin equivalents in mg/g of extracts. The extracts contained 7.933±0.206 mg/g total flavonoids as shown in Table 2.

Optimization of RSM

Box-Behnken design for multivariate optimization is a class of rotatable or nearly rotatable second-order designs based on three-level incomplete factorial designs (Li et al., 2011). The results indicated that the model used to fit response variable was significant (p<0.0001) and adequate to represent the relationship between the response and the independent variables (Ballard et al., 2009; Pierozan et al., 2009). The *F*-test suggested that model had a very high model *F*-value (*F*=37.1), indicating that this model was highly significant. R^2 adj (adjusted determination coefficient) is the correlation measure for testing the goodness-of-fit of the regression equation (Yang et al., 2009). The R^2 value of this model is 0.9795, which indicates that only 2.05% of the total variations were not explained by model. Meanwhile, a relatively lower value of coefficient of variation (*CV*=0.56) showed a better precision and reliability of the experiments carried out (Thana et al., 2008).

It can be seen in Table 3 that extraction yield was affected most significantly by solid-liquid ratio (X_3, p=0.0002), followed by solvent concentration (X_2, p=0.0021) and extraction time (X_1, p= 0.0788). It was evident that two quadratic parameters (X_2^2, X_3^2) and one interaction parameters (X_1X_2) were significant at the level of p<0.0001 or p<0.05. The predicted response *Y* for the yield of extraction could be expressed by the following second-order polynomial equation in term of coded values:

$$Y = 8.17-0.032X_1+0.075X_2-0.11X_3+0.057X_1X_2-0.011X_1X_3-0.041X_2X_3-0.036X_1^2-0.24X_2^2-0.22X_3^2.$$

The regression equation was graphically represented by 3D response surface and 2D contour plots (Li et al., 2011). From 3D response surface curves and contour plots shown in Figures 1 to 3, the effect of the independent variables and their mutual interaction on the extraction yield can be seen. Response surfaces were

Table 3. Response surface regression analysis results.

Source	DF	SS	MS	F	P
X_1	1	0.00844	0.00844	4.229067	< 0.0001
X_2	1	0.045128	0.045128	22.6116	0.0788
X_3	1	0.099927	0.099927	50.06927	0.0021
$X_1 X_2$	1	0.012905	0.012905	6.46615	0.0002
$X_1 X_3$	1	0.000457	0.000457	0.228929	0.0385
X_2X_3	1	0.006622	0.006622	3.31796	0.6469
X_1^2	1	0.005503	0.005503	2.757415	0.1113
X_2^2	1	0.25058	0.25058	125.5556	0.1408
X_3^2	1	0.201646	0.201646	101.0366	< 0.0001
Model	9	0.666897	0.0741	37.12833	< 0.0001
Error	7	0.01397	0.001996		
Total	16	0.680867			

plotted to study the effects of parameters and their interactions on extraction yield. Figure 1 is the response surface and contour plot showing the effect of extraction time and solvent concentration on the response at the fixed value of the ratio of solid-liquid to material. It can be seen that by increasing the extraction time, the extraction yield increased as well, reached a maximum value while the further increase of extraction time had slightly effect. Figure 2 depicts the interaction effect of extraction time and ratio of solid-liquid on the response at the fixed value of solvent concentration. The increase of solid-liquid ratio can significantly enhance the response, and then a maximum response was obtained, and beyond this level, no obvious increase was observed. Figure 3 describes the interaction effect of solvent concentration and solid-liquid ratio on the response at the fixed value of extraction time.

In this study, the aim of optimization was to find the conditions which gave the maximum extraction yield in *P. villosa*. The software predicted the optimum extraction time and solvent concentration and solid-liquid ratio to be 25.51 min, 62.82%, 27.39:1. Under these conditions, the extraction yield was 8.19 mg/g. To test validity of response surface analysis method, the extraction was carried out under the proposed conditions and the extraction yield was 8.1987 mg/g (n=3). The good correlation between these results confirmed that the response model was adequate to reflect the expected optimization.

Antifungal activity

As shown in Table 4, the extraction of *P. villosa* showed antifungal effects against all of the tested microorganisms. Diameters of inhibition zones (DIZs) ranged from 7.0 to 12.0 mm. The scale of measurement comprised the following: >15 mm zone was strongly inhibitory, 10~15 mm zone was moderately inhibitory and <10 mm zone was weakly inhibitory. *T. mentagrophytes* and *T. rubrun* were the most susceptible with inhibition zones of 11.0 and 12.0 mm, respectively, while active against *M. gypseum* with inhibition zones reaching 7.0 mm.

MIC was tested for concentrations ranging from 0.0244 to 50 mg/ml and all extraction methods exhibited antifungal activity against all tested strains. It was considered that if the crude extract displayed a MIC equal or less than 100 µg/ml, the antifungal activity was strong; from 100 to 500 µg/ml the antifungal activity was moderate; from 500 to 1000 µg/ml the antifungal activity was weak; over 1000 µg/ml they were considered inactive. Data in Table 4 revealed that the antifungal activity of *Trichophyton* were more sensitive than the *Microsporum* for *P. villosa.*

Conclusion

Flavonoids are very important constituents of plants because of the scavenging ability conferred by their hydroxyl groups. The flavonoids may contribute directly to antioxidative action (Zheng et al., 2011). In the present study, we demonstrated and optimization the total flavonoids extract from *P. villosa* possesses considerable amounts of flavonoids by response surface methodology (8.1987 mg/g rutin equivalent of extract after optimization). The data obtained clearly indicate that the antifungal activity of *Trichophyton* were more sensitive than the *Microsporum* for *P. villosa.* These antifungal activities could have contributed, at least partly, to the therapeutic benefits of the certain traditional claims for *P. villosa*. It is the first time to report antifungal activity from the plant of *Patrinia genus* in the world. Its therapeutic benefits and bioactive compounds warrant further investigation.

Table 4. Antibacterial DIZs (mm) and MIC (µg/ml) of *P. villosa* and Positive control

Antibacteria	*P. villosa*		Positive control	
	DIZs	MICa	DIZs	MICb
Mic.gyp.	7.0±0.05	3.125±0.00	40.0±0.20	1.953±0.00
Tri. men.	11.0±0.32	0.781±0.00	32.1±0.30	0.488±0.00
Tri.rub.	12.0±0.10	0.781±0.00	25.3±0.50	0.976±0.00

amg/mL; bµg/mL; Positive control: ketoconazole 500 µg/mL; *Microsporum gypseum* ATCC 14683, *Trichophyton mentagrophytes* ATCC 1481, *Trichophyton rubrun* ATCC 28189.

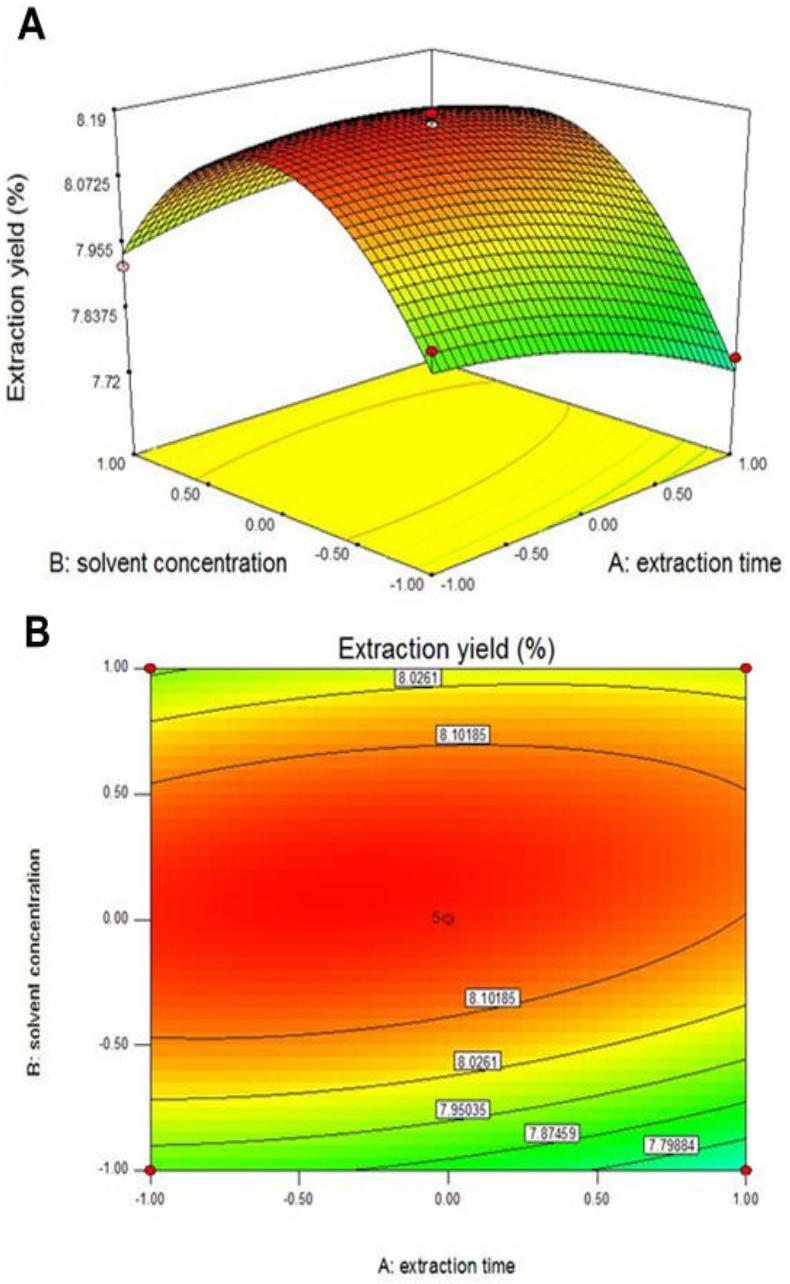

Figure 1. Response surface plot and contour plot of extraction time and solvent concentration on extraction yield.

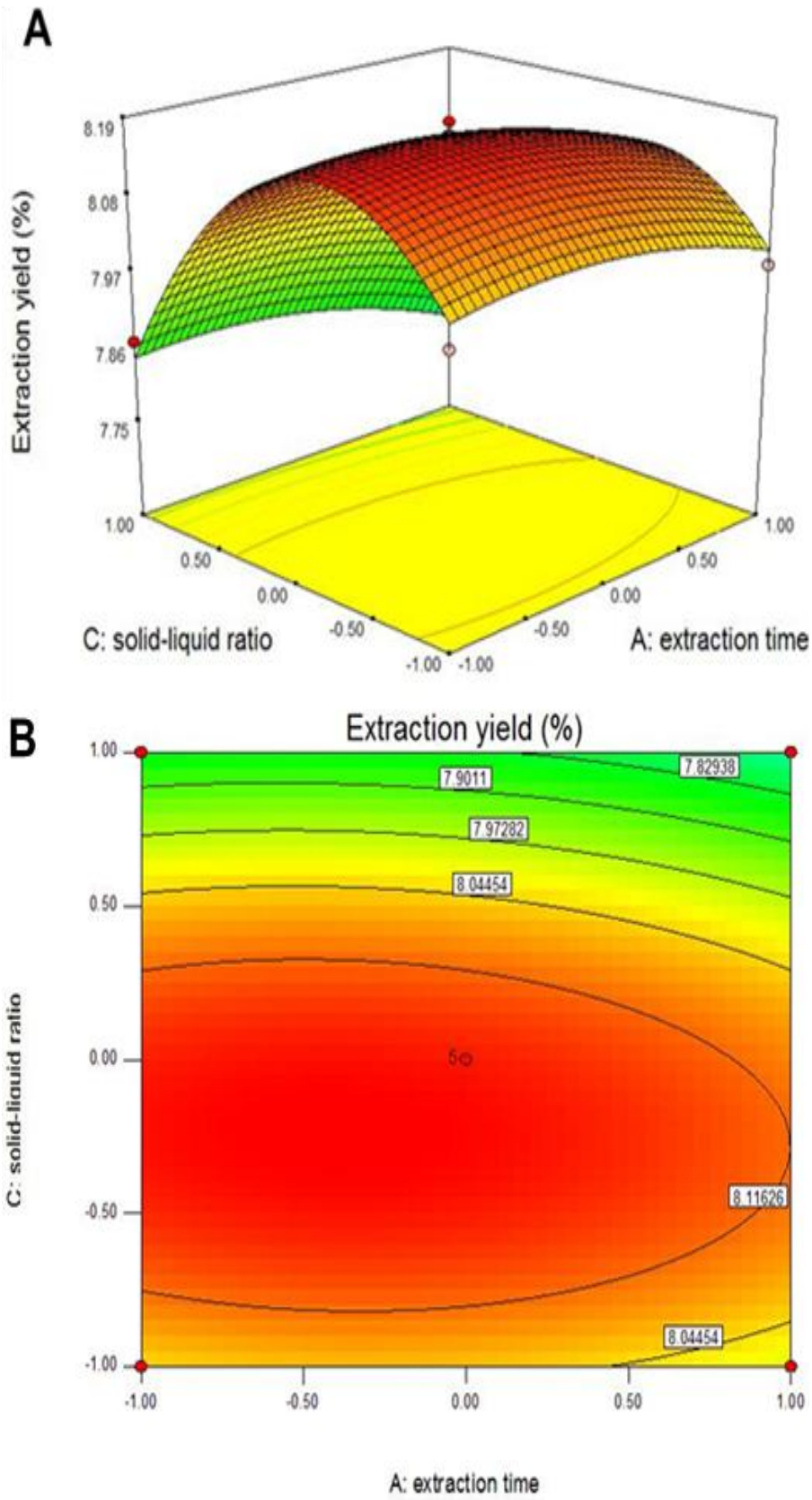

Figure 2. Response surface plot and contour plot of extraction time and solid-liquid ratio on extraction yield.

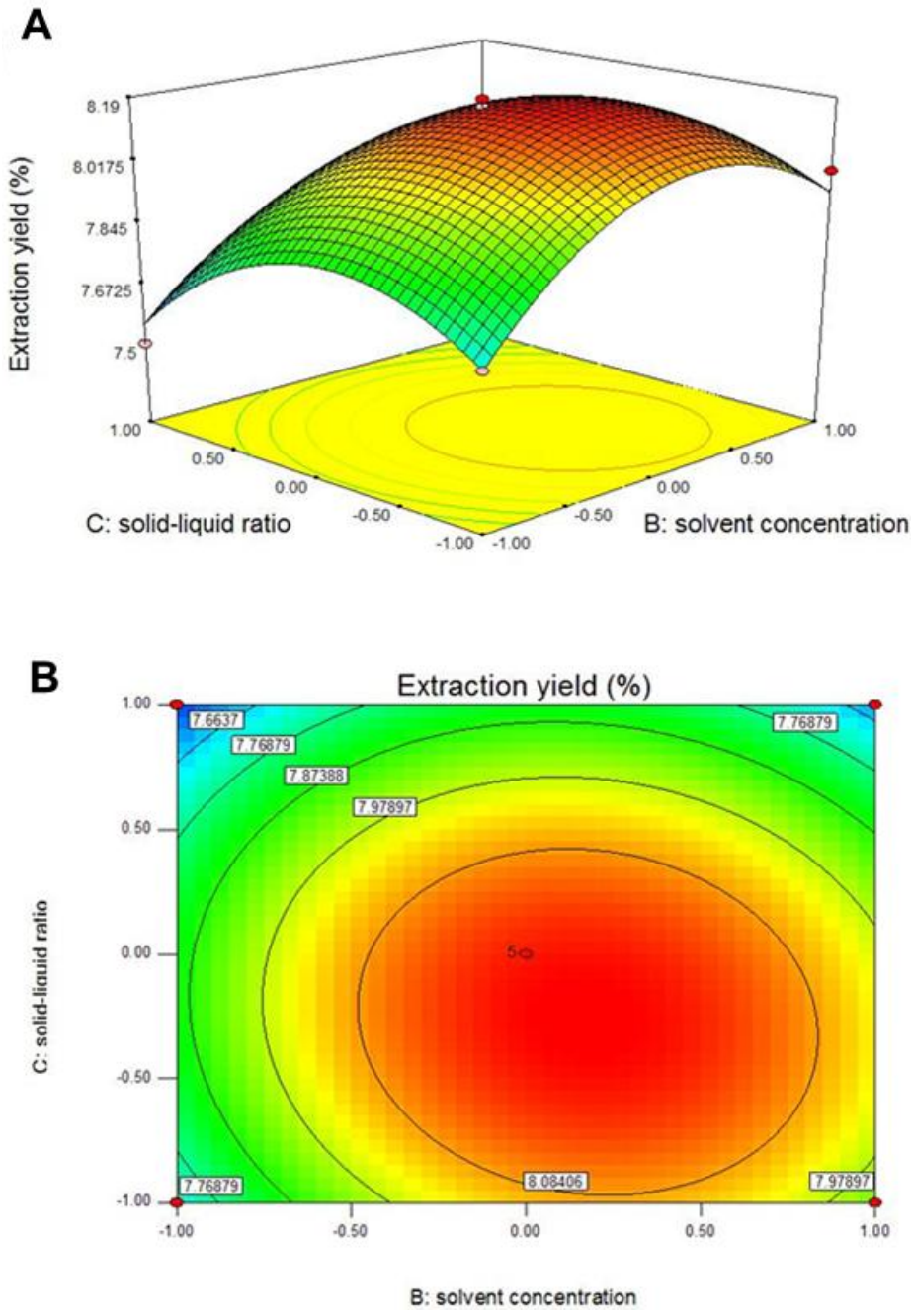

Figure 3. Response surface plot and contour plot of solvent concentration and solid-liquid ratio on extraction yield.

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