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ORIGINAL ARTICLE

Efficient Preparation of Narcissin from *Opuntia ficus-indica* Fruits by Combination of Response Surface Methodology and High-speed Countercurrent Chromatography

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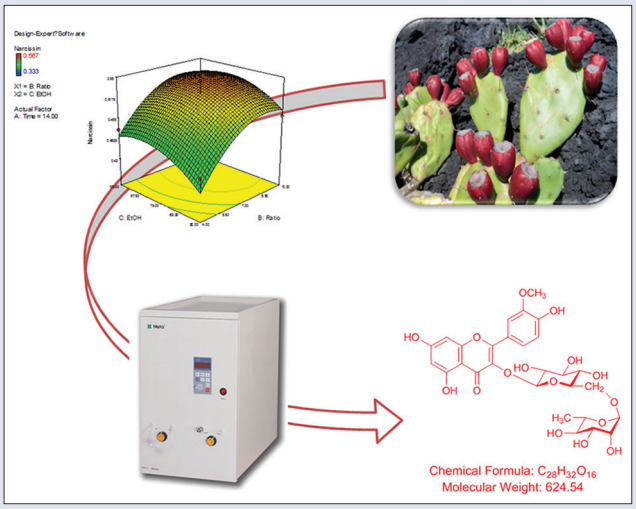
ABSTRACT

Background: Narcissin is well known for their various biological activities. **Objective:** To recover narcissin from the *Opuntia ficus-indica* fruits (OFIF), an efficient method was developed by a combination of response surface methodology (RSM) and high-speed countercurrent chromatography (HSCCC). **Materials and Methods:** Optimization of extraction conditions of narcissin from OFIF was determined using RSM with three-level-three-factor Box-Behnken design (BBD). Then, a rapid and efficient method for the isolation of narcissin from the rich narcissin extracts was developed using HSCCC. **Results:** Regression analysis showed a good fit of the experimental data and the optimal condition was obtained as extraction time (X_1), 6.02 h; solvent to material ratio (X_2), 8.16 mL/g; and ethanol concentration (X_3), 93.48%. Then, the rich narcissin extracts were separated by HSCCC with a two-phase solvent system composed of *n*-hexane: ethyl acetate: methanol: water (1.5:5:5:1.5, v/v/v/v) in one step within 60 min. As a result, 12 mg of narcissin was isolated from 100 mg of crude extract with purities of 98.5%, as determined by high-performance liquid chromatography (HPLC). **Conclusion:** This study can be useful to the development of industrial extraction processes, including further studies concerning the optimal number of sequential steps to enhance the efficacy of a large-scale extraction system.

Key words: Box-Behnken design, high-speed countercurrent chromatography, *Opuntia ficus-indica*, narcissin, response surface methodology

SUMMARY

- The optimum conditions for the extraction of narcissin from *Opuntia ficus-indica* fruits were determined using response surface methodology
- Box-Behnken design was utilized to evaluate the effects of three-independent variables
- Rapid and efficient method for the isolation of narcissin from the rich narcissin extracts was developed using high-speed countercurrent chromatography.



Abbreviations used: BBD: Box-Behnken design; HPLC: High-performance liquid chromatography; HSCCC: High-speed countercurrent chromatography; OFIF: *Opuntia ficus-indica* fruits; RSM: Response surface methodology

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INTRODUCTION

Opuntia ficus-indica var. saboten Makino commonly called prickly pear, tuna, or nopal cactus, belongs to the dicotyledonous angiosperm *Cactaceae* family, a family that includes about 1500 species of cactus.[1] They are distributed throughout most of the warm weather parts of America, Africa, Southern Europe, and Asia.[2] Among the *Opuntia* species, *O. ficus-indica* is found on Jeju Island in Korea, which is widely cultivated for use in food additives and supplements such as juice, jam, and tea. Its fruits and stems have been traditionally used as oriental folk medicine for edema, burns, wounds, bronchial asthma, indigestion, and diabetes.[3] It also has been reported to have biological activities such as reduction of gastric damage, antitumoral, anti-inflammatory, and anti-allergic effects.[4-11]

Previous studies on the chemical constituents of the *O. ficus-indica* fruits (OFIF) revealed the presence of alkaloids, flavonoids, terpenoids, polysaccharides, and organic acids.[12-14] Among the constituents, flavonoid such as narcissin was known as the most characteristic constituent of OFIF.[15] Narcissin, as a kind of the major compositions in OFIF, is well known for their various biological activities, such as hepatoprotective activities, α-glucosidase inhibitory activities,

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antioxidative activities, and apoptosis-inducing activities against human myelogenous erythroleukemia cells.[16-19] Due to these pharmacological effects, it is necessary to establish an efficient method for separation of narcissin from this plant.

To recover bioactive compounds from plant raw materials, extraction is widely used and constitutes the first important step for natural product researches.[20] The optimum conditions for extraction of narcissin from the OFIF were determined using response surface methodology (RSM). RSM is effective statistical technique for optimizing the extraction process variables, thereby deduce optimal conditions for certain process.[21-23]

Then, a rapid and efficient method for the separation and purification of narcissin from the rich narcissin extracts was developed using high-speed countercurrent chromatography (HSCCC). HSCCC is a liquid–liquid separation technique, where the mobile phase flows over the stationary phase that is retained within a spinning coil.[24,25] HSCCC does not need any solid-phase support, so loss of sample due to irreversible adsorption to solid absorbents can be prevented. Moreover, it is rapid and more reproducible than conventional column chromatography. In addition, advantages such as higher loading capacities and ease of scaling up have been well documented.[26,27]

In this study, efficient method was developed for preparation of narcissin from OFIF by combination of RSM and HSCCC and separated narcissin was identified through quadrupole-time of flight liquid chromatography/mass (Q-TOF LC/MS) and nuclear magnetic resonance (NMR) spectra.

MATERIALS AND METHODS

Plant materials

The OFIF were collected from Jeju Island (Republic of Korea) and the certificate of identity and quality is also provided. A voucher specimen (YIPS-OP-161114) was deposited at the Herbarium of College of Pharmacy, Yonsei Institute of Pharmaceutical Sciences, Yonsei University, Incheon, Korea.

Reagents and apparatus

All organic solvents, such as hexane, chloroform, ethyl acetate, ethanol, methanol, and n-butanol used for extraction and column chromatography were of analytical grade and purchased from Duksan Chemical (Anseong, Korea). HSCCC was carried out with a model TBE-300A (Shenzhen, Tauto Biotech, China). Thin-layer chromatography was performed on a precoated silica gel 60 F_{254} (0.25 mm, Merck, USA). High-performance liquid chromatography (HPLC) was carried out using an Agilent 1260 HPLC system. ESI-MS spectra were obtained using an Agilent 6550 iFunnel Q-TOF LC/MS system (Agilent Technologies, Santa Clara, USA). ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on an Agilent 400-MR NMR spectrometer (Agilent Technologies, Santa Clara, USA) and TMS was used as an internal standard. Data processing was carried out with the MestReNova 6.0.2 program. All other chemicals and reagents were of analytical grade.

Preparation of *Opuntia ficus-indica* fruits

The OFIF were finely chopped. The pulp was separated from the seeds and crushed in a grinding mill. The obtained mash was filtered and then sterilized at 65°C for 30 min. The extracted juice was mixed with dextrin at a ratio of 7:1 and freeze-dried.

Experimental design

An optimization of extraction conditions for the extraction of narcissin from the OFIF was conducted using RSM. For RSM, the levels of independent variables for the extraction of narcissin were selected based on the results obtained from our preliminary experiments. Briefly, 15 experimental runs were conducted with three independent variables and three levels were developed according to the Box-Behnken design (BBD) as shown in Table 1. The BBD was used for designing the experiments to evaluate the nonlinear relationship between response values and factors. It has the advantage of being able to reduce the number of combinations compared with the other designs.[28] The independent variables were extraction time (X_1, h), solvent-to-material ratio (X_2, mL/g), and ethanol concentration (X_3, %), while the response variable was the yield of narcissin from OFIF. The generalized second-order polynomial model used in the response surface analysis was as following equation,[29]

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j + \sum_{i=1}^{k} \beta_{ii} X_i^2$$

where β_0 is the intercept; β_i, β_{ii}, and β_{ij} are the linear, quadratic, and interaction terms, respectively, and X_i and X_j are the independent variables.[30] The three-dimensional surface response plots were generated showing the relationship between the response and independent variables.

Table 1: Experimental design and responses of the dependent variables to extraction conditions

Standard order[a]	Run order[b]	Coded variables			Independent variables			Dependent variable
		X_1	X_2	X_3	Extraction time (h)	Solvent ratio (mL/g)	Ethanol concentration (%)	Narcissin (mg)
12	1	−1	0	−1	4	7	50	0.483
14	2	−1	−1	0	4	4	75	0.47
6	3	−1	1	0	4	10	75	0.584
15	4	0	0	0	14	7	75	0.57
1	5	0	0	0	14	7	75	0.525
7	6	0	1	1	14	10	100	0.528
2	7	0	1	−1	14	10	50	0.515
3	8	0	−1	1	14	4	100	0.443
8	9	−1	0	1	4	7	100	0.592
13	10	1	−1	0	24	4	75	0.308
9	11	1	1	0	24	10	75	0.452
10	12	1	0	1	24	7	100	0.453
5	13	0	0	0	14	7	75	0.569
4	14	0	−1	−1	14	4	50	0.417
11	15	1	0	−1	24	7	50	0.477

[a]No randomized; [b]Randomized

Statistical analysis

The Design Expert software (Version 9.0, Minneapolis, USA) was used to conduct the statistical analysis. Results for the yield of narcissin were expressed as means ± standard deviations. A response surface analysis and an analysis of variance (ANOVA) were employed to determine the regression coefficients, statistical significance of the model terms, and to fit the mathematical models of the experimental data that aimed to optimize the overall region for response variables.

Selection of high-speed countercurrent chromatography solvent system

The partition coefficient (*K*) of the target compound is critical for the isolation by HSCCC. A small amount of the rich narcissin extracts was added to a test vial, to which 3 mL of preequilibrated two-phase solvent system was added. The test vial was shaken vigorously to equilibrate the sample between two phases thoroughly. The upper and lower phases were separated and evaporated under N_2 gas, respectively. Dried residues of each phase were dissolved in methanol of 1 mL and analyzed by HPLC. The *K* value was obtained by the ratio of peak area upper and lower phase.

Preparation of two-phase solvent system and sample solutions

Based on the partition coefficient (*K*) value, a two-phase solvent system composed of n-hexane: ethyl acetate: methanol: water (1.5:5:5:1.5, v/v/v/v) was selected for the HSCCC separation. The upper and lower were separated, and then degassed by sonication for 30 min before use. The sample solution was prepared by dissolving 100 mg of the rich narcissin extracts in 10 mL mobile phase and then filtered through a 0.2 μm membrane.

High-speed countercurrent chromatography separation procedure

The multilayer coil column was first entirely filled with the upper phase as stationary phase, then the lower phase as mobile phase was pumped into the column at the flow rate of 3 mL/min in the head-to-tail elution mode; the column was rotated at 800 rpm. Once the mobile phase front emerged and the hydrodynamic equilibrium was established within the column, the sample solution was injected; effluent was continuously monitored with a UV detector at 254 nm.

Identification of high-speed countercurrent chromatography peak fractions

The peak fractions from the HSCCC separation were analyzed by HPLC. The analysis was performed with a Shiseido C_{18} column (150 mm × 4.6 mm, 5 μm) at column temperature of 30°C. The gradient elution system consisted of solvent A (acetonitrile) and solvent B (0.1% formic acid). The gradient elution was performed as follows: 0–10 min, 0%–12% A; 10–20 min, 12%–20% A; 20–30 min, 20% A; 30–35 min, 20%–40% A; 35–40 min, 40%–90% A; 40–42 min, 90%–100% A; 42–45 min, 100% A. The sample injection volume was 10 μL. The detection wavelength was 254 nm and the flow rate was 1 mL/min. All solvents were filtered through a 0.2 μm membrane before use. The HSCCC peak fractions were further identified by Q-TOF LC/MS [Figure 1d], NMR and literature searches.

RESULTS AND DISCUSSION

Model fitting

Optimization of the extraction condition was performed by BBD. According to the BBD design, 15 experiments were performed. Extraction time (hr, X_1), solvent-to-material ratio (mL/g, X_2), and ethanol concentration (%, X_3) were chosen as three variables, which could potentially affect contents of narcissin. As shown in Table 1, the narcissin contents were varied notably depending on extraction condition. ANOVA was statistically significant ($P < 0.05$) and suggested that at least one of the parameters of the model can explain the experimental variation of narcissin contents. Correlation coefficient, adjusted correlation coefficient, and lack-of-fit values for both dependent variables are shown in Table 2. The results suggested that the model fitted well for the experimental data.

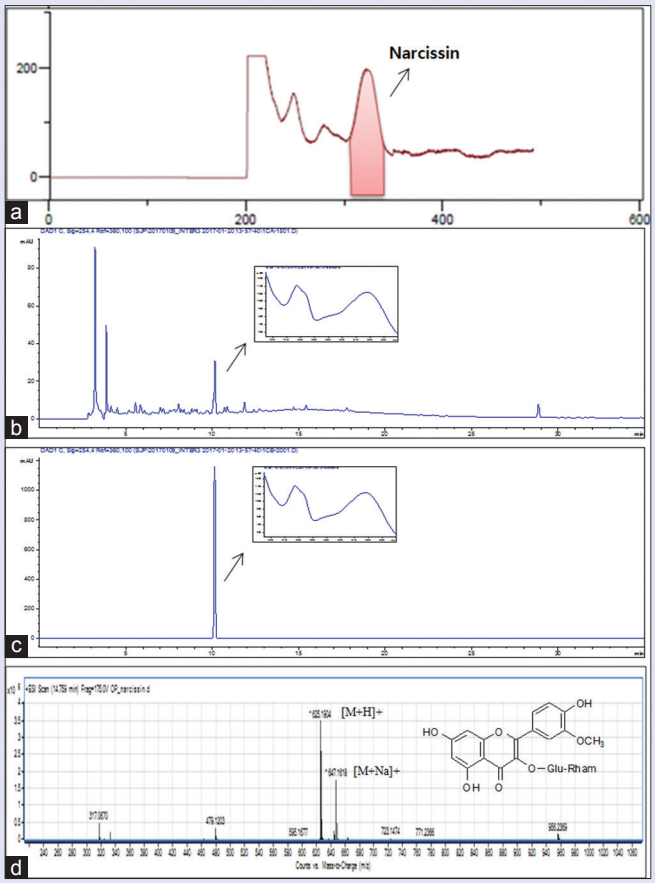

Figure 1: (a) High-speed countercurrent chromatography chromatogram of the rich narcissin extracts from *Opuntia ficus-indica* fruits, (b) High-performance liquid chromatography chromatogram of the rich narcissin extracts from *Opuntia ficus-indica* fruits (c) High-performance liquid chromatography chromatogram of narcissin isolated from *Opuntia ficus-indica* fruits by High-speed countercurrent chromatography, and (d) mass spectrum and chemical structure of narcissin isolated from *Opuntia ficus-indica* fruits

Table 2: Analysis of variance for the response surface quadratic models

	Degree of freedom	Sum of square	Mean square	*F*	*P*
<Model>	9	0.046	0.005	5.31	0.04
Linear	3	0.031	0.01	10.99	0.012
Square	3	0.008	0.003	2.82	0.147
Interaction	3	0.006	0.002	2.12	0.216
<Residual error>	5	0.004	0.001		
Lack-of-fit	3	0.003	0.001	1.74	0.386
Pure error	2	0.001	0.001		
Total	14	0.050			

R^2=0.9053

Effect of extraction parameters on the yield of narcissin

The mathematical expression for the relationship of narcissin contents with variables X_1, X_2, and X_3 is given in an equation as follows.

$$\text{Narcissin contents} = 0.53 - 0.049X_1 + 0.036X_2 + 0.016X_3 + 0.02X_1X_2 - 0.033X_1X_3 - 0.003X_2X_3 - 0.032X_1^2 - 0.032X_2^2 - 0.022X_3^2$$

In the models [Table 3], the linear term of extraction time (X_1) had the most significant effect ($P < 0.01$) on narcissin contents. The linear term of solvent-to-material ratio (X_2) also showed a significant effect ($P < 0.05$). However, other linear variable X_3; interaction terms of variables X_1X_2, X_1X_3, and X_2X_3; and quadratic terms of X_1^2, X_2^2 and X_3^2 were shown not to be significant. Table 2 shows the ANOVA of the fitted quadratic polynomial model for narcissin contents. The fitness of the predicted model for narcissin contents was supported by a $F = 5.31$ and a $P = 0.04$. The value of coefficient determination (R^2) of the predicted model in this response was 0.9053, which suggested the high degree of correlation between observed and predicted value. In addition, P value for lack of fit was 0.386 which is insignificant relative to the pure errors. In general, lack-of-fit test for the model describes the variation in the data around the fitted model.[31] If the model does not fit the data well, the value of lack of fit will come out to be statistically significant, and when investigation proceeded with the model, the optimization of the fitted response surface is likely to give misleading results. In this study, statistical analysis supported the good fit of experimental values and the predicted ones and availability of this polynomial model for further optimization. To visualize the relationship between the response and experimental levels of the independent variables for the narcissin contents, three-dimensional surface plots were constructed according to the quadratic polynomial model equations [Figure 2]. As shown in Figure 2a, when ethanol concentration (%) was fixed at the center point (75%), narcissin contents decreased as the extraction time increased from 14 h to 24 h and reached the maximum value when ethanol concentration was elevated from 5.5 mL/g to 10 mL/g. Figure 2b shows the effect of the interaction of extraction time and ethanol concentration on the narcissin contents at a fixed solvent to material ratio of 7 mL/g. Narcissin contents increased when ethanol concentration increased from 60% to 100%; however, the narcissin contents decreased when extraction time exceeded 9 h. As shown in Figure 2c, when extraction time was fixed at the center point (14 h), narcissin contents increased as the solvent-to-material ratio increased from 5.5 mL/g to 10 mL/g and reached the maximum value when ethanol concentration increased from 60% to 100%.

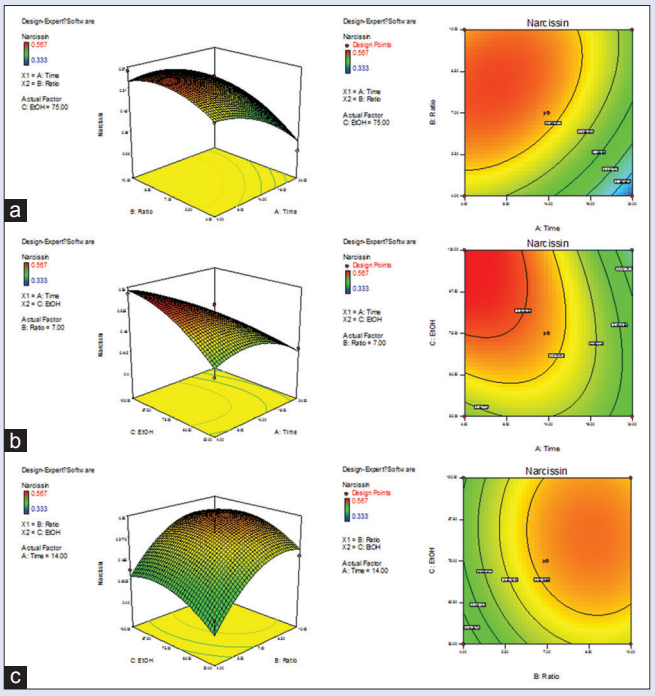

Figure 2: Three-dimensional response surface graph and contour plots for the effects of extraction conditions for narcissin from *Opuntia ficus-indica* fruits: (a) extraction time and solvent-to-material ratio, (b) extraction time and ethanol concentration, and (c) solvent-to-material ratio and ethanol concentration

Experimental validation of the optimum conditions

The predicted maximum contents and experimental contents of narcissin were presented in Table 4. To ensure that the predicted model was similar to the practical value, further trials were carried out under following modified optimal conditions: the extraction time of 6 h, solvent-to-material ratio of 8 mL/g, and ethanol concentration of 90%. A mean value of 0.55 ± 0.3 mg was gained from the actual experiment, which was found to be significantly in agreement with predicted value ($P > 0.05$). Hence, the RSM could

Table 3: Regression coefficients and their significances in the second-order polynomial regression equations for narcissin content

	Coefficient	SE	*t*	*P*
Intercept	0.530	0.018		
X_1	−0.049	0.011	19.855	0.007
X_2	0.036	0.011	11.111	0.021
X_3	0.016	0.011	2.017	0.215
X_1X_2	0.020	0.015	1.679	0.252
X_1X_3	−0.033	0.015	4.642	0.084
X_2X_3	−0.003	0.015	0.044	0.842
X_1^2	−0.032	0.016	3.866	0.106
X_2^2	−0.032	0.016	3.989	0.102
X_3^2	−0.022	0.016	1.848	0.232

SE: Standard error

Table 4: Predicted and experimental values of the response variables under the optimum conditions

Parameters	Optimum values	
	Predicted values[a]	Experimental values[b]
Extraction time (h)	6.02	6
Solvent-to-material ratio (mL/g)	8.16	8
Ethanol concentration (%)	93.48	90
Narcissin (mg)	0.57	0.55±0.3%

[a]Predicted using ridge analysis of response surface quadratic model, [b]Mean±standard deviation of triplicate determinations from different experiments

Table 5: The *K* values (partition coefficient) of narcissin in different solvents systems

Solvent system	Ratio (v/v)	*K*[a]
n-hexane: water	1:1	0.04
Chloroform: ethyl acetate: water: acetic acid	1.5:3:3:1	2.52
Chloroform: ethyl acetate: water: acetic acid	1.5:3:3:0.5	2.75
n-hexane: ethyl acetate: methanol: water	1.5:5:5:0.5	2.61
n-hexane: ethyl acetate: methanol: water	1.5:5:5:1.2	2.11
n-hexane: ethyl acetate: methanol: water	1.5:5:5:1.5	1.56

[a]Upper phase area/lower phase area

be applied effectively to the prediction of narcissin extraction from OFIF.

Selection of high-speed countercurrent chromatography solvent system

The selection of the two-phase solvent system is one of the most important steps in performing HSCCC. Suitable two-phase solvent system provides an optimum range of partition coefficient ($0.5 \leq K \leq 2.0$) for the targeted compounds. In general, small K values (<0.5) always result in a poor peak resolution, while large K values (>2) lead to excessive sample band broadening as well as a long separation procedure.[32] Several two-phase solvent systems were tested; their K values are listed in Table 5. Based on K value, a two-phase solvent system composed of n-hexane: ethyl acetate: methanol: water (1.5:5:5:1.5, v/v) was selected for the HSCCC separation.

High-speed countercurrent chromatography separation

Narcissin was successfully separated by HSCCC [Figure 1a and b]. The purity of isolated narcissin was >95% as evaluated by HPLC-UV at 254 nm [Figure 1c]. The structure was confirmed by ESI-MS, ^{1}H, and ^{13}C NMR spectra by comparing previous literature [Figure 1d].[33,34]

CONCLUSION

Narcissin is well known for their various biological activities. To recover narcissin from OFIF, an efficient method was developed by combination of RSM and HSCCC. First, optimization of extraction conditions was studied by the RSM to enrich narcissin extracts. The results indicated that the optimal extraction condition was extraction time, 6.02 h; solvent-to-material ratio, 8.16 mL/g; and ethanol concentration, 93.48%. Then, a rapid and efficient method for the isolation of narcissin from the rich narcissin extracts was developed using HSCCC. The two-phase solvent system used for HSCCC separation was composed of n-hexane-ethyl acetate-methanol-water (1.5:5:5:1.5, v/v/v/v). The isolation was accomplished within 60 min and the purity of narcissin was over 98%.

Acknowledgement

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Nil.

Conflicts of interest

There are no conflicts of interest.

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