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오미자의 모유두세포 증식 활성성분과 반응표면분석을 이용한 추출조건의 최적화

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Dermal Papilla Cells Proliferation Constituent of *Schisandra chinensis* Fruits and Optimization Using Response Surface Methodology

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요 약: 본 연구에서는 오미자 열매로부터 모유두세포 증식에 활성을 나타내는 gomisin N을 정제하고 gomisin N의 함량이 높은 추출물을 얻기 위한 최적의 추출조건을 확인하였다. 추출물 및 분획물에 대하여 모유두세포 증식 활성 실험을 진행한 결과, *n*-hexane 분획물 1 μg/mL 처리군에서 약 29%의 증식 활성을 나타내었다. *n*-Hexane 분획물의 활성성분을 규명하기 위하여 column chromatography를 진행하였으며 2 개의 화합물 gomisin N (1)과 schizandrin (2)를 분리 동정하였다. 분리된 화합물에 대한 모유두세포 증식 활성 실험 결과, gomisin N에서 최대 20%이상의 증식 활성을 나타내었다. 따라서 오미자 열매로부터 최대치의 gomisin N을 얻을 수 있는 최적추출조건을 반응표면분석(RSM)을 사용하여 확인하였다. 최적추출조건을 설정하기 위해 에탄올의 비율, 추출시간, 추출온도를 독립변수로 하여 실험한 결과, 0.95 이상의 결정계수와 0.05 이하의 *p*-value를 나타내어 모델의 적합성이 확인 되었다. Gomisin N의 최대함량을 위한 최적추출조건은 에탄올 비율 83.8%, 추출온도 80 ℃, 추출시간 8.7 h으로 확인되었고, 최적추출조건에서 gomisin N의 함량은 378,300 ppm으로 예측되었다. 최적추출조건에 대한 실제 검증에서 평균 376,884 ppm으로 예측된 값과 유사한 결과를 확인 할 수 있었다.

Abstract: In the present study, we have refined gomisin N, which represents activity in the proliferation of dermal papilla cells (HFDPCs) from the fruit of *Schisandra chinensis (S. chinensis)*, and have identified optimal extraction conditions for obtaining extracts with high content of gomisin N. The activity of the extracts and fractions was evaluated, and the results indicated approximately 29% proliferation activity in the group treated with 1 μg/mL of *n*-hexane fraction. Column chromatography was used to assess the active ingredient in the *n*-hexane fraction, and two compounds, namely gomisin N(1) and schisandrin(2), were isolated and identified. When the HFDPCs proliferation activity was tested for the isolated compounds, gomisin N exhibited ≥ 20% proliferation activity. Thus, via response surface methodology (RSM), the optimum extraction conditions to obtain the maximum level of gomisin N from the fruit of S. chinensis were determined, where ethanol proportion, extraction time, and extraction temperature were used as the independent variables. The results revealed coefficient of determination ≥ 0.95 and *p*-value ≤ 0.05, which confirmed the fit of the model.

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The optimum extraction conditions to achieve the maximum content of gomisin N were as follows: ethanol proportion 83.8%, extraction temperature 80 ℃, and extraction time 8.7 h. The content of gomisin N using these conditions was predicted as 378,300 ppm, and a mean value close to the predicted value (376,884 ppm) was obtained while validating the aforementioned conditions.

Keywords: *Schisandra chinensis, gomisin N, schizandrin, dermal papilla cells proliferation, RSM*

1. Introduction

The extraction yield and bioactivity are influenced by various factors such as extraction solvent, extraction time, extraction temperature, ratio of raw material versus solvent, and pH of the solvent. Thus, optimization of the extraction conditions is essential while developing a functional cosmetic ingredient based on a natural substance to ensure the maximum extraction yield and the highest level of bioactivity[1,2].

The response surface methodology (RSM) evaluates the optimum conditions using a set of predicted values obtained by analyzing the results of limited experiments. Multiple variables can be optimized using RSM as it allows simultaneous analyses of numerous variables to determine the optimum conditions within a short time period and in reasonable ways[3,4].

The fruit of *Schisandra chinensis* (*S. chinensis*) is widely used as an ingredient in Korean medicine. It is also known as Omija, which means a balance of five different flavors: sweetness, sourness, bitterness, spiciness, and saltiness. According to previous studies, *S. chinensis* fruit extracts possess various pharmacological compounds that can be effective in treating skin irritation, cough, breathing difficulty, heart disease, rheumatoid arthritis, and other disorders[5-8]. These fruit extracts comprise several different lignan-based compounds such as gomisin N, gomisin A, and schisandrin[9,10].

In general, hair protects the scalp and maintains body temperature. In humans, the hair is formed in the hair follicle, and the number is estimated at approximately 100,000 - 150,000. Each hair undergoes a different cycle that comprises phases of growth, regression, and rest until it falls out. Such cycle is repeated over a period of 3–6 years and on average, 50 - 100 hair naturally fall out daily[11,12]. The hair cycle is altered by internal and external factors including hormones, stress, disease, environmental pollution, and cigarette smoking, wherein the altered cycle may enter the reduced growth phase, early regression phase, or prolonged rest phase. Alopecia is an abnormal increase in hair loss when the growth phase is hindered and the hair count in regression or rest phase increases. This condition may be suspected if the daily number of hair that falls out is $\geq$ 100[13,14].

To date, minoxidil application and finasteride administration have been effective in preventing alopecia or promoting hair growth; however, they should be continuously used or else the effects wear off. For minoxidil, the reported side effects include sticky sensation upon use and skin irritation, whereas for finasteride, reduced stamina and sexual dysfunction such as impotence were reported, to restrict their continuous use[12].

The present study was conducted to identify a candidate material that helps prevent alopecia by isolating and purifying a single substance from *S. chinensis* based on the effects of dermal papilla cell (HFDPCs) proliferation. The extraction conditions were optimized to maximize the advantages in product development.

This study aimed to find a natural material that promotes HFDPCs proliferation to alleviate hair loss. Among the various materials screened, the fruit of *S. chinensis* was the most effective. Therefore, substances promoting HFDPCs proliferation were separated and purified from *S. chinensis* fruit extract, and the extraction process was optimized using RSM for efficient extraction.

2. Materials and Methods

2.1. *S. chinensis* Fruit Extraction and the Isolation of Compounds

S. chinensis fruits were purchased from Mungyeong Market,

located in Mungyeong, North Gyeongbuk province (Korea). The fruits were dried before use. The dried *S. chinensis* fruits (2 kg) were extracted at room temperature using 95% EtOH (10 L). The EtOH extract was enriched using a rotary evaporator (Basis Hei-VAP, Heidolph, Germany) and a vacuum pump (Rotavac valve control, Heidolph, Germany). The enriched extract (603 g) was suspended in 20% EtOH (3.0 L), and then fractionated according to the order of solvent polarity. As a result, *n*-hexane (67 g), CH_2Cl_2 (62 g), EtOAc (81 g), BuOH (98 g), and water (104 g) fractions were obtained.

Silica gel column chromatography was performed for the *n*-hexane fraction (60.6 g) among the obtained fractions, and 10 fractions were produced using *n*-hexane/EtOAC (20 : 1 - 1 : 2). Among the 10 fractions (HF1 - HF10), Sephadex LH-20 column chromatography (CH_2Cl_2/MeOH (1 : 1)) was used for HF2, and following the recrystallization using EtOAC, Compound 1 (gomisin N, 310 mg) was obtained. Compound 2 (gomisin A, 680 mg) was obtained by using Sephadex LH-20 column (CH_2Cl_2/MeOH (1 : 1)) for HF4.

The structure of the two isolated compounds was determined using NMR.

2.2. Proliferation of Human Hair Follicle Dermal Papilla Cells

S. chinensis fruit extract (SCE), *n*-hexane fraction (SCH) and the compounds isolated *S. chinensis* fruit were evaluated proliferation of human hair follicle dermal papilla cells (HFDPCs) (Promo cell, Germany). The HFDPCs were treated with SCE, SCH, and isolated compounds. The cell proliferation was determined with MTT assay.

The HFDPCs were cultured in DMEM supplemented with 1% FBS (100 unit/mL, 100 μg/mL, respectively) at 37 ℃ in a humidified atmosphere under 5% CO_2. HFDPCs (1.0×10^4 cells/mL) were seeded into 96 well plates, cultured for 24 h under 1% serum conditions, and treated with vehicle (DMSO diluted 1 : 500 in DMEM containing 1% FBS), or with the SCE (3, 10, 30, 100 μg/mL), SCH (1, 3, 10 μg/mL) and isolated compounds (0.3, 1, 3, 5 μg/mL) for 48 h. After incubation, 0.1 mg (20 μL of a 5 mg/mL solution) of MTT was added to each well, and the cells were incubated at 37 ℃ for 1 - 2 h. The plates were centrifuged and the media was carefully aspirated. DMSO 100 μL was added to each well to dissolve the formazan crystals and the absorbance of the plate at 550 nm was read immediately on a microplate reader. All experiments were performed in triplicate and the mean absorbance values were calculated. The results were expressed as the percentage of vehicle treated groups.

2.3. High Performance Liquid Chromatography Conditions for the Isolated Compounds

The contents of compound 1 and compound 2 isolated from *S. chinensis* fruit extracts were quantified using HPLC (2695 Separations Module, Waters, USA). The column was Mightysil RP-18GP 5 μm 4.6 × 250 mm (Kanto Chemical, Japan), while the waters 2998 PDA detector was used. The detection wavelength was 254 nm, for which 10 μL sample was injected and the column temperature was 35 ℃. The mobile phase A was acetonitrile and B was distilled water, and the analysis was conducted based on 70% A and 30% B, whereas the flow rate was maintained at 1.0 mL/min for 25 min.

2.4. Response Surface Methodology (RSM)

The RSM used in this study was the Box-Behnken design (BBD), a model used to determine the secondary regression equation and optimum conditions by setting three ranges for three variables. The extraction solvent, time, and temperature were used as variables to optimize the extraction conditions. The range of the three independent variables were encoded into three stages as −1, 0, 1 (min, median, max), and the extraction was carried out based on the conditions determined by BBD. Each response can be expressed as the following quadratic polynomials[15-17].

$$Y_n=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{12}X_1X_2+\beta_{23}X_2X_3+\beta_{13}X_1X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2$$

β_1, β_2, and β_3 are the linear coefficients for the respective variables; β_{12}, β_{23}, and β_{13} are the coefficients indicating the correlation among the variables; β_{11}, β_{22}, and β_{33} are the binary coefficients for the respective variables[18,19].

2.5. Statistical Analysis

For statistical optimization of RSM, MINITAB (Minitab Inc., USA) was used. To confirm the fit of the model, the analysis of variance (ANOVA) was applied, and significance was set to p-value $\leq$ 0.05[15,20].

3. Results and Discussion

3.1. Dermal Papilla Cell Proliferation Activity of *S. chinensis* Extract

HFDPC proliferation activity of five *S. chinensis* fruit extracts was investigated. Among these, SCE and SCH possessed HFDPCs proliferation activity; other fractions did not show any significant effects.

The effects of SCE and SCH on HFDPCs proliferation are as illustrated in Figure 2. HFDPCs were treated with SCE and SCH at following concentrations: 3, 10, 30, 100 μg/mL and 1, 3, 10 μg/mL, respectively. The group treated with 100 μg/mL SCE revealed approximately 15% increase in HFDPCs proliferation compared to that observed in the control group (Figure 1A). In the case of SCH, an increase in HFDPCs proliferation compared to the control was found across all concentrations (Figure 1B). The 1 μg/mL group, in particular, revealed highest rate of proliferation at approximately 29%.

3.2. Gomisin N Structure Identification

For the *n*-hexane fraction of *S. chinensis* fruit extract with an excellent HFDPCs proliferation activity, silica column chromatography and Sephadex LH-20 column chromatography were performed to isolate two compounds. The structures of the isolated compounds were determined using ^{1}H- and ^{13}C-NMR. Compound 1 was obtained as white yellow amorphous powder. By using ^{1}H- and ^{13}C- NMR, four methoxy groups, two methyl groups, one deoxymethylene group [δ H 5.94 (2H,s), δ C 101], and two lignan-based compounds with a single aromatic benzene proton were predicted. From literature review, the compound was identified as gomisin N[9]. Compound 2 was yellow amorphous powder. By using ^{1}H- and ^{13}C- NMR, six methoxy groups were detected, and the shift of H-8 proton was δ 1.88 (1H, m). From literature review, the compound was identified as schisandrin[10].

Compound 1 (gomisin N) : ^{1}H-NMR (700 MHz, $CDCl_3$) δ : 0.74 (3H, d, J=7.2 Hz, CH_3-17), 0.97 (3H, d, J=7.0 Hz, CH_3-18), 1.85 (2H, m, H-7), 1.80 (2H, m, H-8), 2.02 (1H, br d, J=13.2 Hz, H-6), 2.28 (1H, dd, J=13.2, 9.6Hz, H-9), 2.55 (2H, m, H-9), 2.58 (2H, m, H-6), 3.57, 3.84, 3.92 (3H, s, OCH_3 ×4), 5.94 (2H, s, OCH_2O), 6.47 (1H, s, H-11), 6.58 (1H, s, H-4); ^{13}C-NMR (175MHz, $CDCl_3$) δ : 151.5 (C-1), 140.3 (C-2), 152.0 (C-3), 111.0 (C-4), 134.8 (C-5), 39.3 (C-6), 33.3 (C-7), 40.6 (C-8), 35.9 (C-9), 136.9 (C-10), 103.1 (C-11), 148.4(C-12), 134.2 (C-13), 140.9 (C-14), 121.1 (C-15), 123.8 (C-16), 21.6 (CH_3-17), 12.2 (CH_3-18), 101.0 (OCH_2O), 61.0, 60.4, 59.8, 56.0 (OCH_3 × 4)

Compound 2 (schisandrin) : ^{1}H-NMR (700 MHz, $CDCl_3$) δ : 0.81 (3H, s, CH_3-17), 1.24 (1H, s, CH_3-18), 1.88 (1H, m, H-8), 2.35 (1H, dd, J=14.2, 7.2 Hz, H-9), 2.36 (1H, d,

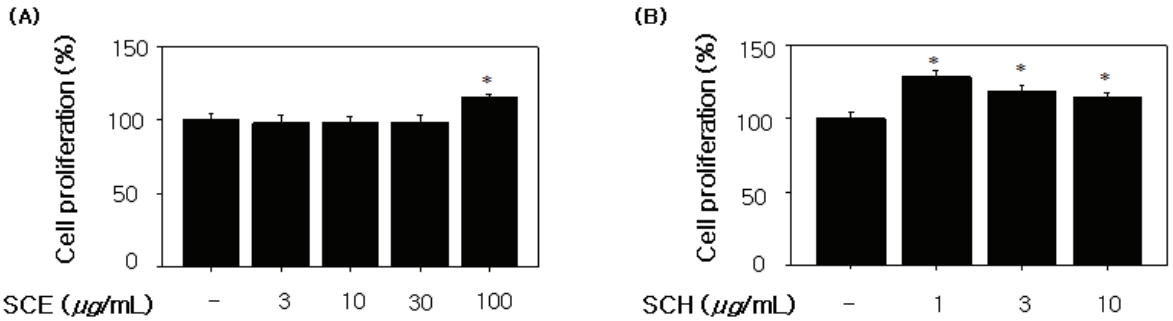

Figure 1. Effect of *S. chinensis* fruit extract (SCE) and its *n*-hexane fraction (SCH) on human hair follicle dermal papilla cells proliferation. Significance was determined compared to untreated cells (*p < 0.05). All data are expressed as mean ±SD of three separate experiments performed in triplicate.

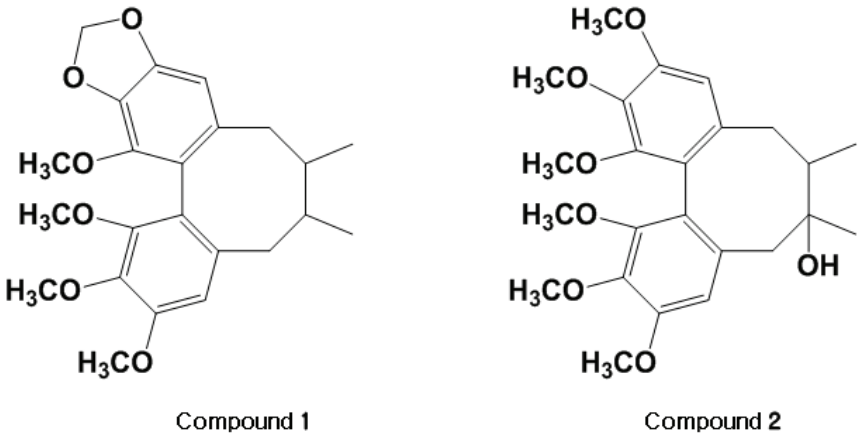

Figure 2. Structures of compounds isolated from *S. chinensis* fruits.

J=13.5 Hz, H-6), 2.62 (1H, dd, *J*=14.4, 1.6 Hz,, H-9), 2.64 (1H, d, *J*=13.2 Hz, H-6), 3.55 (3H, s, OCH_3-1), 3.57 (3H, s, OCH_3-2), 3.87 (3H, s, OCH_3-12), 3.88 (3H, s, OCH_3-13), 3.90 (3H, s, OCH_3-14), 6.54 (1H,s, H-11), 6.61 (1H, s, H-4); ^{13}C-NMR (175 MHz, $CDCl_3$) δ : 152.0 (C-1), 140.2 (C-2), 152.3 (C-3), 111.0 (C-4), 131.9 (C-5), 41.3 (C-6), 72.1 (C-7), 42.0 (C-8), 34.8 (C-9), 134.2 (C-10), 110.0 (C-11), 152.3 (C-12), 140.1 (C-13), 151.7 (C-14), 123.0 (C-15), 124.3 (C-16), 15.9 (C-17), 30.0 (C-18), 60.7 (OCH_3-1, 14), 61.1 (OCH_3-2, 13), 56.3 (OCH_3-3, 12)

3.3. Dermal Papilla Cell Proliferation Activity of Gomisin N

Furthermore, the effects of gomisin N and schisandrin, the compounds isolated from *S. chinensis* fruit, are depicted in Figure 3. HFDPCs were treated with 0.3, 1, 3, 5 μg/mL of gomisin N and schisandrin. Gomisin N increased HFDPCs proliferation as high as $\geq$ 20% compared to the control at the concentration of 3 μg/mL or higher (Figure 3A). Schisandrin did not affect the HFDPCs proliferation (Figure 3B).

3.4. Optimization of Gomisin N Extraction Conditions Using RSM

Gomisin N had a remarkable effect on HFDPCs proliferation. Thus, to efficiently extract gomisin N from the fruit of *S. chinensis*, an experiment optimizing the extraction conditions was conducted.

A single factor experiment was performed considering the following four factors: extraction solvent, time, temperature, and solvent proportion per extraction sample; however, the solvent proportion did not have any remarkable effect on the extraction. The three factors: extraction solvent (X_1), extraction

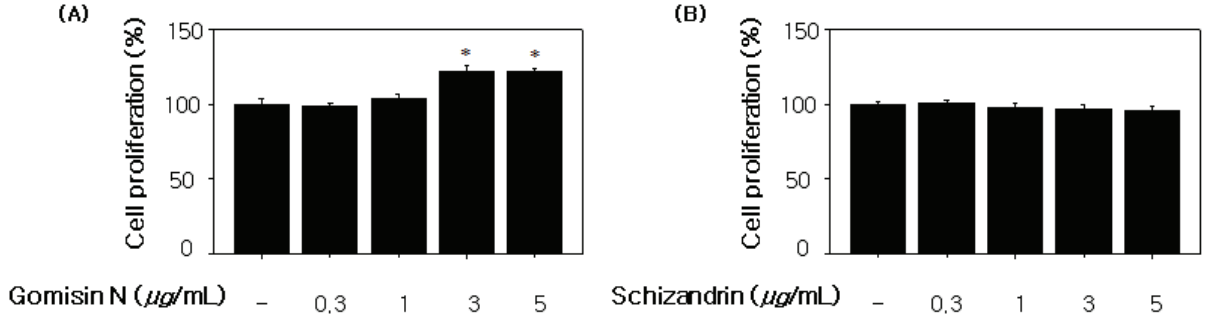

Figure 3. Effect of compounds 1 and 2 on human hair follicle dermal papilla cells proliferation. Significance was determined compared to untreated cells (*$p < 0.05$). All data are expressed as mean ±SD of three separate experiments performed in triplicate.

Table 1. Independent Variables Levels Used for BBD

Variables	Level		
	-1	0	1
X_1 : EtOH ratio (%)	50	70	90
X_2 : extraction time (hr)	3	6	9
X_3 : extraction temperature (℃)	40	60	80

Table 2. A Box-Behnken Design for Independent Variables and Their Responses

Run	Coded variables			Actual variables			Observed values
	X_1	X_2	X_3	EtOH ratio (%)	Time (h)	Temperature (℃)	Gomisin N (ppm)
1	1	1	0	90	9	60	371,145
2	1	-1	0	90	3	60	370,179
3	0	1	-1	70	9	40	354,843
4	0	0	0	70	6	60	367,032
5	0	0	0	70	6	60	364,485
6	0	-1	-1	70	3	40	358,735
7	1	0	1	90	6	80	377,239
8	-1	0	1	50	6	80	337,426
9	-1	-1	0	50	3	60	333,257
10	-1	1	0	50	9	60	317,645
11	1	0	-1	90	6	40	371,227
12	0	-1	1	70	3	80	368,665
13	0	0	0	70	6	60	369,583
14	-1	0	-1	50	6	40	316,153
15	0	1	1	70	9	80	370,612

temperature (X_2), and extraction time (X_3), were selected as the variables for determining the optimum extraction conditions. To identify the correlations among these three variables and the content of gomisin N, the 3-level-3-factor Box-Behnken design (BBD) was used. Table 1 summarizes the encoded experimental range for each variable. The dependent variable influenced by the independent variables is the gomisin N content (Y_1), which was measured and applied in the regression analysis. The extraction conditions obtained using the BBD and the respective effects on the dependent variables, are presented in Table 2.

3.4.1. Optimum Extraction Conditions to Maximize Gomisin N Content

The gomisin N content is expressed in ppm of the content in the *S. chinensis* fruit concentrate.

For the values obtained using the BBD, a secondary regression equation was produced via multivariate regression analysis to reveal the relationship between each dependent and independent variable.

$$Y_1 \text{ (gomisin N)} = 367033 + 23164X_1 - 2074X_2 + 6623X_3 + 4145X_1X_2 - 3815X_1X_3 + 1460X_2X_3 - 15840X_1^2 - 3137X_2^2 - 682X_3^2$$

Each coefficient was determined by the *t*-value and *p*-value, where *p*-value ≤ 0.050 defines significance. The *t*-value increases and *p*-value decreases for more important factors that influence the results. As listed in Table 3, EtOH concentration has the highest impact on gomisin N content; moreover, the squared value of EtOH concentration and extraction temperature markedly influence gomisin N content. Analyzing

Table 3. Regression Coefficients and Their Significances in the Second-order Polynomial Regression Equation for Gomisin N

	Coefficient	Standard error	*t*-value	*p*-value
Intercept	367033	1548.5	237.032	0.000
X_1	23164	948.2	24.428	0.000
X_2	-2074	948.2	-2.187	0.080
X_3	6623	948.2	6.985	0.001
X_1^2	-15840	1395.8	-11.348	0.000
X_2^2	-3137	1395.8	-2.248	0.074
X_3^2	-682	1395.8	-0.489	0.646
X_1X_2	4145	1341.0	3.091	0.027
X_1X_3	-3815	1341.0	-2.845	0.036
X_2X_3	1460	1341.0	1.089	0.326

Table 4. ANOVA for Response Surface Regression Equation

	Sum of square	Degree of freedom	Mean square	*F*-value	*p*-value
Model	5754951635	9	7105.56	88.90	0.000
Residual error	35965682	5	79.72		
Lack-of-fit	22970878	3	85.26	1.18	0.490
Pure error	12994805	2	71.40		
Total	5790917318	14			
R^2 = 0.994, adjusted R^2 = 0.983					

the effect of gomisin N on each independent variable revealed that the order of increasing coefficient was as follows: EtOH proportion X_1 (24.428), extraction temperature X_2 (6.985), and extraction time X_3 (−2.187), which indicated that the main effects were presented in the order of X_1, X_3, and X_2.

The ANOVA results for the regression model indicate the significance and reliability. An increase in the *F*-value and a decrease in the *p*-value indicate higher influence of the given factor, and $R^2 \geq 0.950$ confirms reliability. Table 4 presents the ANOVA results in this study. The *p*-value was < 0.050 and R^2 (0.994) was > 0.950 to verify the reliability.

The 3D graph and contour plot of the regression model are presented in Figure 4. The graph in Figure 4A illustrates the effects of EtOH proportion and extraction time on the gomisin N content, when the extraction temperature is fixed at level 0 (60 ℃). The graph indicates an increase in gomisin N content with higher EtOH proportion. Furthermore, although gomisin N content increases with the increasing extraction time, the effect was negligible in comparison to the effect of EtOH proportion. Figure 4B illustrates the effects of extraction time and temperature on gomisin N content, when EtOH proportion is fixed at level 0 (70%). The graph presents an increase in gomisin N content with increasing extraction time and temperature. The graph in Figure 4C depicts the effects of EtOH proportion and extraction temperature on gomisin N content. While Figure 3A depicts an increase in gomisin N content with increasing EtOH proportion, Figure 4C illustrates that the increase in EtOH proportion led to an increase in gomisin N content only when the extraction was performed at or above a specific temperature due to the interaction between EtOH proportion and extraction temperature. The results collectively indicated that the interaction among the independent variables had a remarkable impact on the increased gomisin N content.

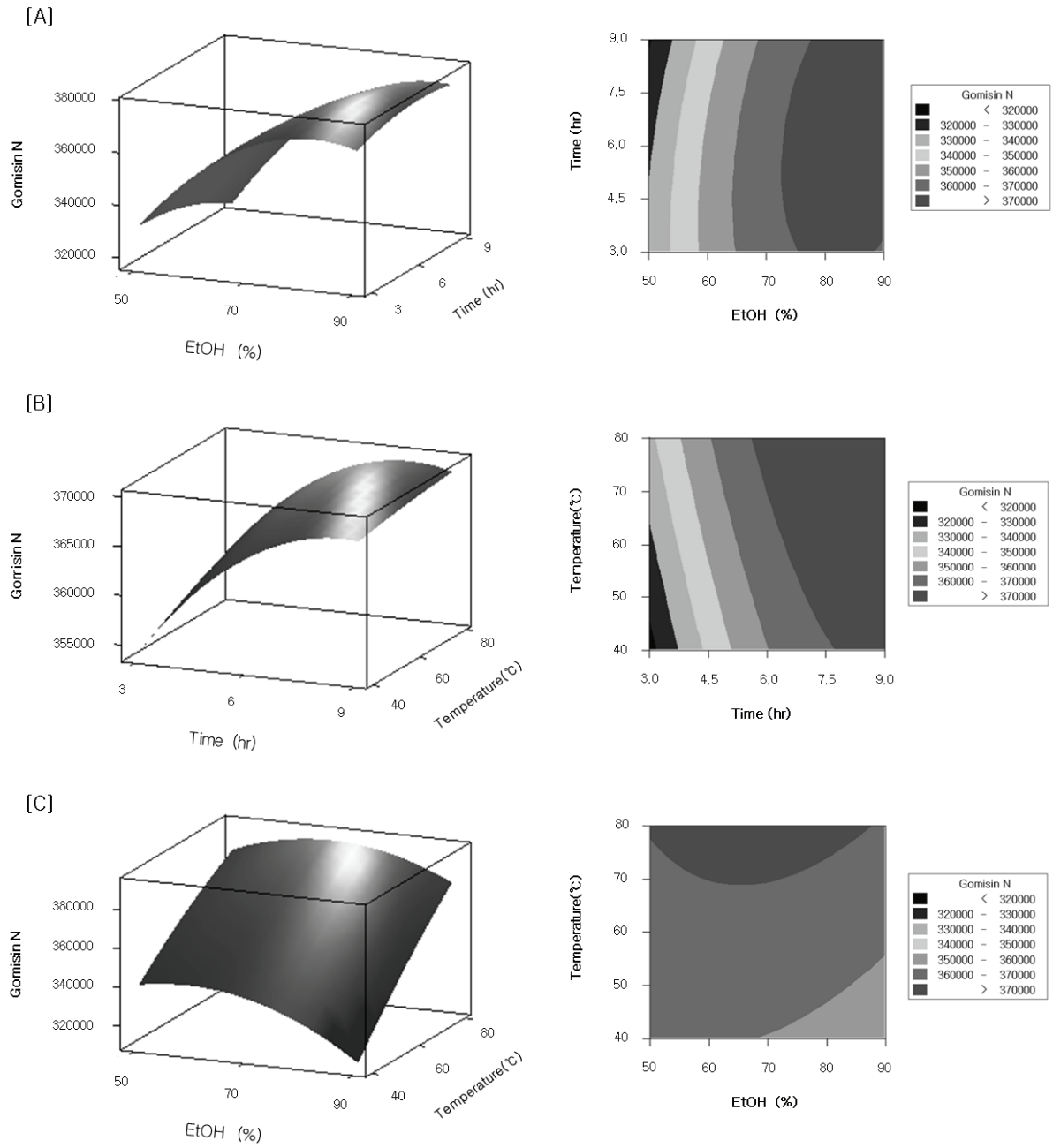

Figure 4. Response surface plots and contour plots for the effect of extraction variables on gomisin N.

3.4.2. Prediction of Optimum Extraction Conditions

Based on the RSM results, the optimum conditions for maximizing gomisin N content were determined, and the predicted values are summarized in Table 5. The prediction revealed that the optimum extraction conditions to achieve the maximum gomisin N content require application of 83.8% EtOH during extraction at 80 ℃ for 8.7 h, which would lead to 378,300 ppm of gomisin N. The experiment was repeated thrice in the predicted optimum extraction conditions, and the results revealed a mean content of 373,884 ppm gomisin N, while the reproducibility was verified based on 95% confidence interval.

Table 5. Predicted and Observed Values of Gomisin N and Extraction Yield under Optimized Condition

Optimized extraction condition		
EtOH ratio (%)	Extraction time (h)	Extraction temperature(℃)
83.8	8.7	80

Gomisin N	
Predicted value (ppm)	Observed value (ppm)
378,300	376,884

4. Conclusion

In the present study, gomisin N, an active ingredient with HFDPCs proliferation activity in the fruit of *S. chinensis* was isolated and purified, and the optimum extraction conditions to maximize the extracted content of gomisin N were determined. To examine the effects of *S. chinensis* fruit extract and fractions on HFDPCs, the cells were treated with varying concentrations. The results indicated that the group treated with 100 μg/mL of *S. chinensis* fruit extract led to approximately 15% activity of HFDPCs proliferation and the group treated with 1 μg/mL of *n*-hexane fraction led to approximately 29% activity. To identify the active ingredient in *n*-hexane fraction, column chromatography was performed to isolate and identify two compounds, namely gomisin N and schisandrin. The analysis of HFDPCs proliferation activity of the two isolated compounds revealed that, while compound 1 exhibited $\geq$ 20% HFDPCs proliferation activity, compound 2 presented no proliferation activity. To determine the optimum conditions for extracting the highest content of gomisin N from the fruit of *S. chinensis* using RSM, the independent variables used to design the experiment based on the Box-Behnken design (BBD) were EtOH proportion (50 − 90%), extraction time (3 - 9 h), and extraction temperature (40 - 80 ℃). The optimum extraction conditions based on the RSM were 83.8% EtOH proportion, 80 ℃ extraction temperature, and 8.7 h extraction time. The predicted content of gomisin N for applying the optimum extraction conditions was 378,300 ppm, which was close to 373,884 ppm, the mean value obtained while validating the optimum extraction conditions.

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