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In Vitro Evaluation of Antioxidant Activity of *Lycium barbarum* Hot Water Extract and Optimization of Production Using Response Surface Methodology

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Abstract : This study is concerned with the optimization of the manufacturing process of a hot water extract containing antioxidant activity from *Lycium barbarum*, traditionally known to have various physiological activities. For the establishment of the optimization process, the central composite design of response surface methodology(RSM) was used. Thirteen extraction processes were performed by encoding the independent variables, extraction temperature (65.9°C－94.1°C) and extraction time (2.59 hr－5.41 hr). As a result of the experiment, the optimal manufacturing conditions for the extract were 340.0 mg/100 g of GAE at an extraction temperature of 94.1°C and an extraction time of 5 hr. The maximum yield of flavonoids was 22.44 mg/100 g of HES at an extraction temperature of 94.1°C and an extraction time of 4 hr. The conditions for producing the extract with the maximum antioxidant capacity (DPPH 92.12%) were 90°C and 4.5 hr extraction time. Therefore, the optimal manufacturing process conditions for extracts containing total phenol content, flavonoid content, and DPPH radical scavenging activity, which are dependent variables, were extraction temperature of 90-95°C and extraction time of 4 hr, which were not significantly different from the actual values. Therefore, *Lycium barbarum* extract rich in total phenol and flavonoid content related to antioxidant function is expected to be used as a functional food and cosmetic material.

Key words: Lycium barbarum, RSM, Flavonoids, DPPH radical, Phenolic compounds.

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1. Introduction

Natural physiologically active compounds contained in plants and animals exhibit excellent biological activity such as antioxidants, anticancer, and other functions due to their various structures and functions. Among them, antioxidant activity is mainly contributed by carotenoids, flavonoids, ascorbic acid and its derivatives, and polyphenols [1]. *Lycium barbarum* or goji berry, known to contain large amounts of antioxidant active substances, is a plant in the family Solanaceae [2,3] traditionally grown in China, Tibet, and other parts of Asia. Asia(71.2%), followed by Africa(15.8%), the United States(8.3%), Oceania(3.4%), and Europe (1.3%)[4].

For thousands of years, goji fruit has been used as a herbal medicine in Asian countries [5]. Based on its rich nutritional value and medical characteristics such as antioxidant, antibacterial, immunomodulatory, and anti-inflammatory effects, goji fruit has been used as an anti-aging, stabilizer and thirst quenching treatment[6]. As a private drug, *L. Barbarum* fruit has been used by local residents to provide blood nutrition, treat early-onset diabetes, tuberculosis, dizziness and chronic cough, and protect eye health[7].

Recently, *L. Barbarum* fruit has a sweet taste and is widely used as a daily food and health supplement[8-10]. Most of the growth is consumed in cultivation areas around the world, and most of the rest are consumed either dry or processed like juice, herbal tea, yogurt products, granola, powder, and pills [10-12]. GoChi, a leading beverage company related to Gu Jia, showed a great effect as an antioxidant-related product that is sensational among consumers by giving a neurological and psychological "well-being" feeling as well as improving human stomach[13].

Polysaccharides, proteins, and phenyl-propanoids are included in the flesh of goji, which is known to account for 5-8% of dry goji [14]. The principle of producing the ball-based extract is to destroy and decompose the cell wall under mild conditions without changing the properties of the substances contained[15]. The extraction methods used so far have been developed based on these principles, such as water extraction, enzyme auxiliary extraction, microwave auxiliary extraction, ultrasonic auxiliary extraction, and supercritical fluid extraction[15]. In particular, the extraction method using ultrasonic waves, the extraction method using enzymes, the extraction method using microwaves, and the supercritical fluid extraction method are recently applied to the production of extracts [16-19]. However, most food and functional food companies use traditional hydrothermal extraction methods. The yield of the extract by the hydrothermal extraction method is greatly affected by the extraction time, temperature, and the ratio of water to raw material[20].

This study was investigated to confirm the optimization of the extract process using response surface methodology. The dependant variables were the total phenol content, total flavonoid amount, and DPPH scavenging ability. The independent variables was the extraction temperature and time, respectively.

2. Materials and methods

2.1. Reagents and materials

The dried *Lycium barbarum* used in this experiment was purchased at the local farm market located at Cheongyang, Chungcheongnam-do. Folin-Ciocalteu reagent (FCR), aluminium chloride, DPPH, gallic acid, and hesperidine were purchased from Sigma Aldrich(Seoul, Korea).

2.2. Preparation of *Lycium barbarum* extraction

In order to powder the dried material, the crude grinding was carried out using a grinder. The crude pulverized *Lycium barbarum*

powder was selected using a 50 mesh sieve. The selected powder was collected and stored in a container until it was used in the experiment. In order to prepare the extract, the powder was put into an extraction reactor and placed in an extraction reaction container. The ratio of the powder to the extraction solvent (distilled water) was 1 to 10. The extraction process was performed following the Fig. 1.

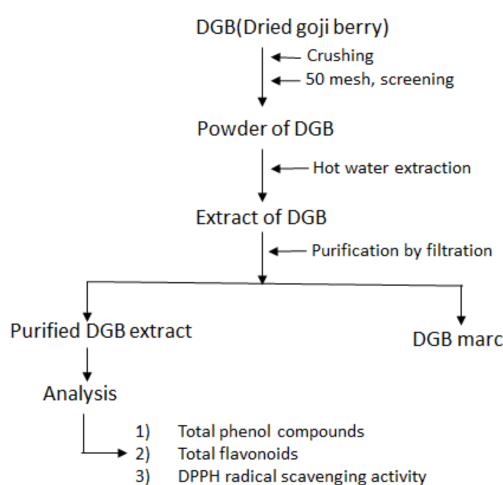

Fig. 1. Flow chart of manufacturing process to produce the extract from dried goji berry (*Lycium barbarum*) using hot water extraction device.

2.3. Surface response methodology

The surface response methodology experiment plan consisted of a total of 13 treatment combinations, including five the center run under the conditions of 80℃ and two independent variables such as extraction temperature and extraction time. The central composite design applied to this experiment is carried out according to three important procedures: first, the experiment was conducted statistically according to the designed experiment, second, the coefficients of the model were obtained, and third, the suitability of the model was determined. In order to facilitate statistical calculation, independent variables were standardized as follows and used. The two variables were set to X_1(°C) and X_2(hr), respectively. The values of standardization can be obtained by the following formula and the value is set to Z.

$$Z = (X - X^0)/\Delta X$$

Xo is the center value of the standardized value and X is the standardized value. The $\triangle X$ is the size of the value increasing or decreasing by one unit. The analysis of the experimental results was used as a surface response analysis method, and multiple regression equations representing the optimal process conditions are as follows.

$$Y = B_0 + \sum_{i=1}^{k} B_i X_i + \sum_{i=j=1}^{k} B_{ij} X_i X_j$$

Here, Y is a predicted response, and if there are two variables as in this experiment, the k value becomes 2, and ultimately it is expressed in the following equation.

$$Y = B_0 + B_1X_1 + B_2X_2 + B_{11}X_1^2 + B_{22}X_2^2 + B_{12}X_1X_2$$

Design Expert (Courtesy: Stat-ease Inc., Statistics Made Easy, Minneapolis, USA) was used for statistical analysis of the results. The values of the independent variables were selected from the results obtained in the preliminary experiment, and X_1(°C) was set to 65.9℃(-1.41), 70℃(-1), 80℃(0), 90℃(+1), 94.1℃(+1.41), and X_2(hr) was 2.59 (-1.41), 3(-1), 4(0), 5(+1), and 5.41(+1.41) (Table 1).

2.4. Analysis of total phenolic compound content

The quantification of phenolic compounds was analyzed by the Paulin-Ciacalte agent (FCR) coloring method. The gallic acid(Sigma Aldrich Co., USA) was as a standard substance. The reference material was produced by mixing 1 mL of a gallic acid (20, 40, 60, 80,

Table 1. Levels of independent variables such as the extraction temperature and time in central composite design

X_i	Independent variables	Level				
		-1.41	-1	0	+1	+1.41
X_1	Temperature (℃)	65.9	70	80	90	94.1
X_2	Time (hr.)	2.59	3	4	5	5.41

100 mg/L) solution with 9 mL DIW (deionized water). For the quantitative reaction, a 100 μL sample solution was mixed with 1.5 mL Na_2CO_3(20 g/100 mL), 500 μL FCR, and 6 mL DIW, followed by a 2 hr reaction at room temperature and the absorbance was measured at 765 nm. The total phenolic compound content was evaluated as the amount of gallic acid (GAE) as dry basis(g).

2.5. Analysis of total flavonoid content

The total flavonoid content was measured using aluminum chloride colorimetric method, and the standard material was prepared by mixing 1 mL of 20, 40, 60, 80, and 100 mg/L solution with hesperidin(Sigma Aldrich Co., USA) with 9 mL DIW. A 0.5 mL sample solution, 1.5 mL 95% methanol, 0.1 mL 10% aluminum chloride(Sigma Aldrich Co., USA), 0.1 mL 1 M NaOH, and 2.8 mL DIW were mixed, followed by a 30 min reaction at room temperature and absorbance was measured at 415 nm. The total flavonoid content was evaluated as the amount of hesperidin per g as dry basis(g).

2.6. DPPH radical scavenging activity

The DPPH radicals are very stable free radicals. The antioxidant action was evaluated by the degree of elimination of this radical. After the extracted sample was dissolved in distilled water at a constant concentration (10%), 2 mL of a solution containing the sample and 0.5 mL of a DPPH(0.2 mM) solution were mixed. The mixture was stored in a dark chamber at room temperature for 30 minutes and then the absorbance value was measured at 517 nm using a UV-1601 spectrophotometer (Shimadzu Co., Australia). Ascorbic acid was used as a control tool and measured under the same conditions as the sample. The value of DPPH-radical scavenging activity was calculated by the following equation.

$$\text{DPPH-radical scavenging activity (\%)} = [(B-A)/B] \times 100$$

A: Absorbance when adding samples.
B: Absorbance when no sample is added.

3. Results and discussion

3.1. Extraction experimental results of response surface methodolgy

According to the central composite method, 13 extraction processes were performed by the extraction temperature (65.9℃-94.1℃) and extraction time (2.59 hr- 5.41 hr), which are the independent variables. The the total phenol content, flavonoid content, and DPPH radical scavenging activity were shown in Table 2.

3.2. Validation of response surface methodology and the optimization of total phenol extraction

The validation of the overall model, linear, quadratic, cross-product, and coefficient of determination were evaluated(Table 3). This model showed that the effect of extraction temperature(X_1) and extraction time (X_2) on total phenols, flavonoids, and DPPH radical

Table 2. Experimental values of total phenolic compounds, flavonoids and DPPH scavenging activity.

Process No.	Temperature (℃)	Time (hr.)	Total phenolic compounds (mg/GAE100g)	Total flavonoids (mg/HES100g)	DPPH (%)
1	70	3	89.2	5.3	30.1
2	90	3	189.2	10.5	67.2
3	70	5	111.5	7.6	32.5
4	90	5	324.4	21.6	75.2
5	65.9	4	126.0	6.8	34.3
6	94.1	4	345.6	22.6	88.4
7	80	2.59	78.4	5.5	25.2
8	80	5.41	228.5	14.5	74.2
9	80	4	254.1	15.4	74.5
10	80	4	234.2	14.2	85.8
11	80	4	224.6	14.9	86.8
12	80	4	214.8	13.5	78.9
13	80	4	258.9	15.5	83.8

Table 3. Analysis of variance(ANOVA) for fitted second-order polynomial model and lack of fit

Source	DF	Sum of squares		
		The phenolic compounds	total flavonoides	DPPH
Model	5	83,563.12	370.16	8,288.13
Residual	7	2,316.85	6.46	163.76
Lack of Fit	3	890.91	3.60	69.38
Pure Error	4	1,425.95	2.86	94.39
Cor Total	12	85,879.97	376.62	8,451.9
Prob ＞ F		＜ 0.0001	＜ 0.0001	＜ 0.0001

scavenging activity showed the significant results of $P<0.0001$ and $P<0.002$ respectively. The optimal response model for the extraction of the total phenol content was $Y= 237.32 + 77.93X_1+46.22X_2-45.95X_2^2$ (R^2 = 0.95). The lack of fit test was $P<0.5414$, showing that the regression model derived in this experiment was valid(Table 4).

The optimal response model for the extraction of the total falvonoids content was $Y=14.7+5.21X_1+3.27X_2-0.27X_1^2-2.62X_2^2+2.23X_1X_2$($R^2$ = 0.97). The lack of fit test was $P<0.5414$, showing that the regression model derived in this experiment was valid. The optimal response model for the extraction of the DPPH radical scavenging activity was $Y=69.48+22.68X_1+15.58X_2-4.76X_1^2-15.54X_2^2+11.28X_1X_2$ (R^2 = 0.96). The lack of fit test was $P<0.5414$, showing that the regression model derived in this experiment was valid(Table 4).

Table 4. Polynomial equations calculated by response surface program.

Response	Second oder polynomial equations	R^2
Total phenolic compounds (mg/GAE100g)	$Y= 237.32 + 77.93X_1+46.22X_2-45.95X_2^2$	0.95
Total flavonoids (mg/g)	$Y= 14.7+5.21X_1+3.27X_2-0.27X_1^2-2.62X_2^2+2.23X_1X_2$	0.97
DPPH (%)	$Y= 69.48+22.68X_1+15.58X_2-4.76X_1^2-15.54X_2^2+11.28X_1X_2$	0.96

X_1: Temperature (℃) and X_2: Time (hr)

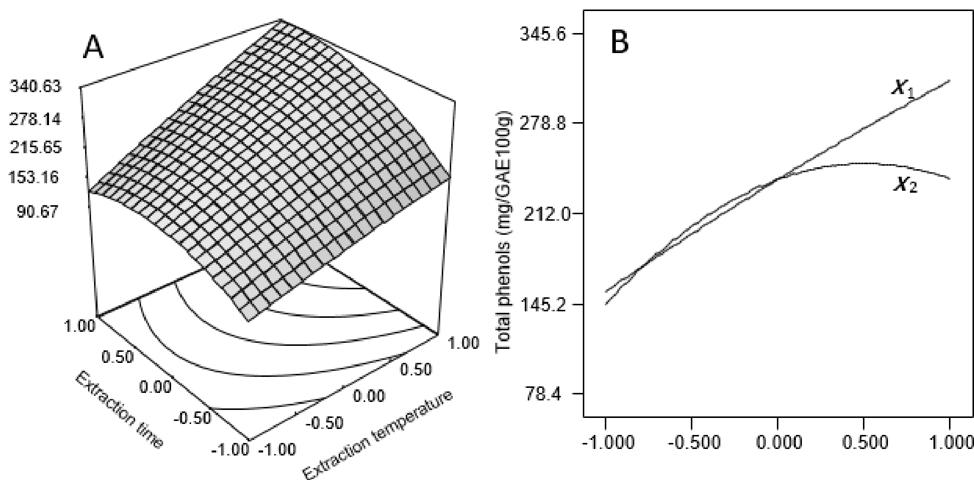

Fig. 2. 3-D response surface plots for the levels of total phenolic compound of the extract of *Lycium barbarum* depending on extraction temperature and time (A). Prrtubation plot of the levels of total phenolic compound the extract of *Lycium barbarum* depending on extraction temperature and time (B).

3.3. The effect of extraction time and temperature on total phenol compound extraction

The total phenol content according to the two independent variables was shown at Fig. 2A as 3-D response surface plots. Pertubation plot was shown at of total phenolic compound the extract of *Lycium barbarum* depending on extraction temperature and time was shown at Fig. 2B. The results explained that the total phenol content of the extract was affected by the temperature and time of extraction according to the change of the independent variable. Within the applied extraction process conditions, the extraction temperature increased, and the total phenol content increased almost linearly. In contrast, the extraction time increased, the total phenol content decreased over a certain extraction time. The total phenol content was 340.0 mg/GAE100g at the

extraction temperature of 94.1℃ and the extraction time of 5 hr within the experimental range conditions. The total amount of phenol compound varies depending on raw material and the extraction method. The other research reported that the total amount of phenol was 31.6 mgGAE/100 g[21], 1,413 mgGAE/100 g[22], 268.5 mg/100 g[23], and 97.23 mg/100 g[24], respectively.

3.4. The effect of extraction time and temperature on total flavonoids extraction

In order to confirm the randomness of the total flavonoid content according to the interaction between the two independent variables, the correlation between each variable was shown as a response surface curve (Fig. 3A). In addition, the extraction temperature and time, which are dependent variables for the production of flavonoid-containing extracts, which are independent variables, are shown in a one-variable optimization curve (Fig 3B). It was confirmed that the flavonoid content of the extract was affected by the extraction temperature and time, as shown in the dispersion analysis. It was found that the effect of the independent variable on the change in the total flavonoid content was received at the extraction time rather than the extraction temperature. As the extraction temperature is increased, the flavonoid content of the extract increases linearly, and the rate of increase decreases due to the interaction of extraction time. As the extraction temperature and extraction time were raised, the amount of flavonoid yield of the extract increased to 4 hr, and then gradually began to decrease. In the experimental process, the maximum flavonoid yield was 22.44 mg/HES100g. The total amount of flavonoids varies depending on the type of ball material used as a raw material and the extraction method. Past researchers reported that the total amount of flavonoids was 28.3 mg CAE/100 gdw [21], 74.5 mg QE/100 gdw [25].

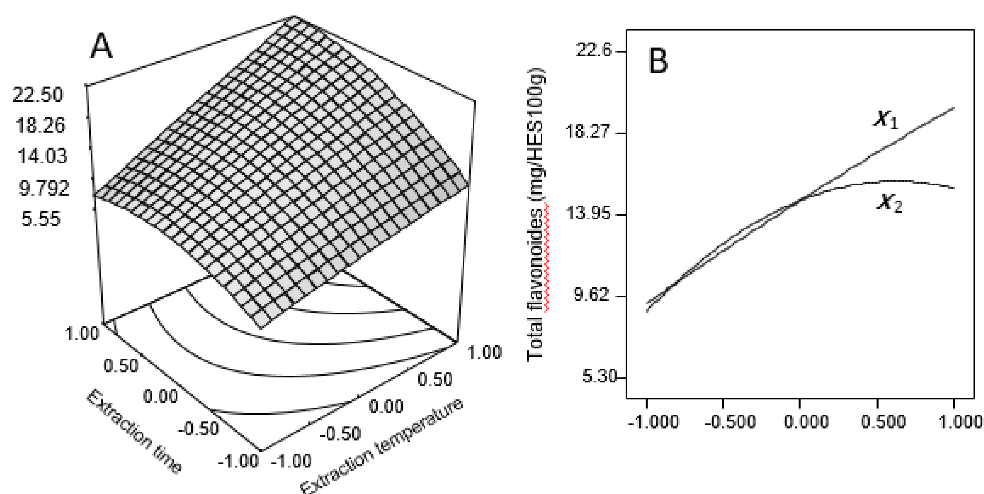

Fig. 3. 3-D response surface plots for the levels of total flavonoid compound of the extract of *Lycium barbarum* depending on extraction temperature and time (A). Perturbation plot of the levels of total flavonoid compound the extract of *Lycium barbarum* depending on extraction temperature and time (B).

3.4. The effect of extraction time and temperature on DPPH radical scavenging activity

The correlation between each variable was shown as a reaction surface curve to check the variance of the overall antioxidant performance of the extract, which is a dependent variable according to the interaction between the extraction temperature and extraction time, which are process conditions for the production of the old extract. (Fig. 4A). And then one independent variable is shown as a variable optimization curve that affects the dependent variable (Figure 4B). As shown in the dispersion analysis, it was confirmed that the antioxidant ability of the ball-based extract was affected by the extraction temperature and time. As the extraction temperature and extraction time increase, extracts containing high antioxidant properties are produced, but they decrease at extraction temperature of 90℃ and extraction time of 4.5 hr or higher, which is the condition of producing extracts containing maximum antioxidant properties (92.12%). Radical erasure ability varies depending on the type of ball material used as a raw material and the extraction method.

4. Conclusion

This study was conducted to manufacture extracts from gujija, traditionally known to have various effects, and to provide them as raw materials in various fields such as functional foods and cosmetics. Therefore, the establishment of an optimal extraction process containing the maximum functional ingredients is an essential step. In this study, the traditional extraction method, the spherical hydrothermal extraction method, was established. The surface reaction analysis method (central synthesis method) was designed with the extraction temperature and extraction time as independent variables, and the total phenol content, flavonoid content, and DPPH radical erasure ability of the

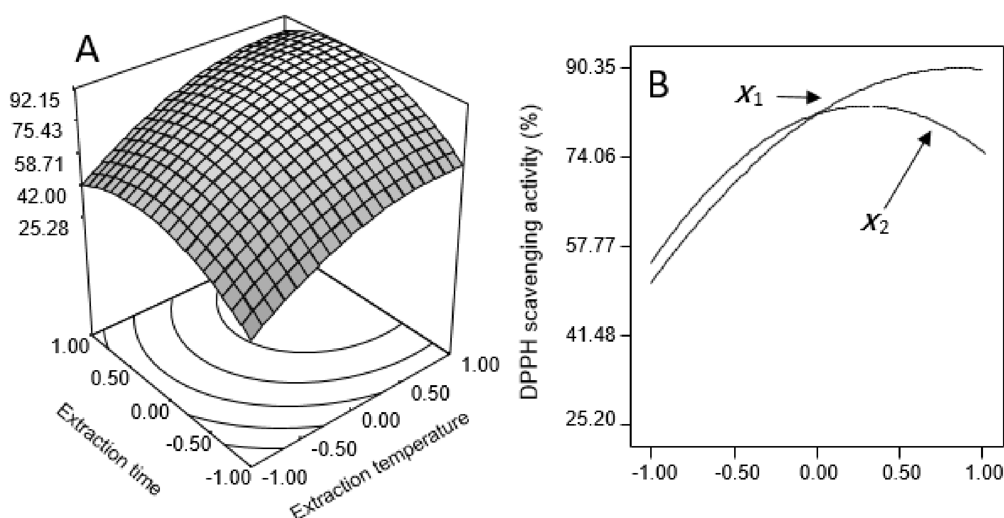

Fig. 4. 3-D response surface plots for the levels of DPPH radical scavenging activity the extract of *Lycium barbarum* depending on extraction temperature and time (A). Perturbation plot of the levels of DPPH radical scavenging activityof the extract of *Lycium barbarum* depending on extraction temperature and time (B).

extract as dependent variables. The suitability of the model was confirmed through regression equation and variance analysis that resulted in the relationship between the experimental and predicted values. The optimal manufacturing conditions for extracts containing total phenol content were 340.0 mg/GAE100g at the extraction temperature of 94.1℃ and the extraction time of 5 hr. In the experimental process, it was confirmed that the maximum yield of flavonoids could be obtained at an extraction temperature of 94.1℃ and an extraction time of 4 hr. It was found to decrease at an extraction temperature of 90℃ and an extraction time of 4.5 hr or higher, which are conditions for producing extracts containing maximum retroactive oxidation ability (92.12%). Therefore, the optimal manufacturing process conditions for extracts containing the dependent variables total phenol content, flavonoid content, and DPPH radical erasure ability were not significantly different from the actual values at 90-95℃ and extraction time of 4 hr. Therefore, it is expected that the oral extract rich in total phenols and flavonoids related to antioxidant functions can be used as functional food and cosmetics materials.

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