Optimal Processing Conditions of *Boswellia carteri* **Birdw. Using Response Surface Methodology**

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ABSTRACT

Background: Boswellia carteri Bridw. is being widely used for its anti-inflammatory properties, as well as for wound healing, antimicrobial, and immunomodulatory properties, and boswellic acids (BAs) are considered to be the main active constituents. Objectives: To investigate optimal conditions of stir-baking process for the resin of B. carteri with vinegar of using response surface methodology (RSM). Materials and Methods: The concentration of acetic acid, heating temperature, and heating time were set as influential factors, and the yields of chemical compounds were the response values which were optimally designed by a Box-Behnken design. The amounts of 11-keto- β -boswellic acid (KBA) and α -boswellic acid (aBA) in *B. carteri* resin were quantified using high-performance liquid chromatography analysis. **Results:** Maximum amounts of KBA and α BA in B. carteri resin were obtained using 6% acetic acid for 10 min at 90°C in preliminary test. Two factor interactions, such as acetic acid concentrationheating temperature and heating temperature-heating time, were significantly observed by multiple regression analysis. Optimal processing conditions from RSM were 5.83% for acetic acid concentration, 9.56 min for heating time, and 89.87°C for heating temperature. Under the modified conditions, the experimental value of the response was 11.25 mg/g, which was similar to the predicted value. Conclusions: The results suggest that the optimal conditions for the stir-baking process of B. carteri resin were determined by RSM, which was reliable and applicable to practical processing of herbal medicine.

Key words: *Boswellia carteri,* high-performance liquid chromatography, optimization, processing conditions, response surface methodology

SUMMARY

• The resin of Boswellia carteri was macerated in aqueous acetic acid and heated using an oven for stir baking process

- The interaction between heating temperature and heating time was the most significant
- Optimal conditions for processing *B. carteri* resin were determined as 5.83% acetic acid, 9.56 min for heating time, and 89.87°C for heating temperature.



Abbreviations used: BAs: Boswellic acids; KBA: 11 keto β boswellic acid; α BA: α boswellic acid; BBD: Box–Behnken design; RSM: Response surface method; HPLC: High performance liquid chromatography; LOD: Limits of determination; LOQ: Limits of quantification; RSD: Relative standard deviation; ANOVA: Analysis of variance.

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INTRODUCTION

Olibanum is an oleogum resin produced by *Boswellia carteri* Birdw. and other *Boswellia* genus. Its medicinal properties lie in the treatment of pain in the epigastrium, various kinds of injuries, and bruises. It is also used for blood stasis syndrome in gynecology diseases, such as dysmenorrhea.^[11] The resin of *B. carteri* is a complex mixture composed of essential oils (mono- and sesquiterpenes), alcohol-soluble resins (di- and triterpenes), and water-soluble gums. Especially, the triterpenes, α -boswellic acid (α BA) and β -boswellic acids (β BA) and their derivatives, constitute the main pharmaceutical activity.^[2,3] These compounds in *B. carteri* resin show anti-inflammatory effects because of the inhibitory activity on 5-lipoxygenase by the boswellic acids (BAs).^[4-6] Recently, incensole acetate and its derivatives were reported to be responsible for its anti-inflammatory effects.^[7] Furthermore, the BAs show immunomodulatory, cytotoxic, and anticancer activity.^[8-10] Although some herbal medicines gathered from their habitats are used in natural form or after only drying, most of them need to undergo processing before use. Only after that the herbal medicines can present curative effects adequately and be used safely. Stir-baking, a fire-processing method, is used primarily to process olibanum with vinegar, as an assistant liquid material. In conventional processing, the herbal medicine is blended with brewed vinegar evenly at the ratio

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of 10:1 or 10:1.5. In sequence, olibanum is parched until its surface becomes glossy and then cooled. The purpose of processing olibanum as mentioned in numerous ancient texts is to remove impurities such as the tree barks and grains of sand, and to facilitate decoction, the preparation can be easily crushed because of its viscosity.^[11,12] By this method, the smell and taste of drugs can be altered to facilitate their administration. Moreover, vinegar-baked olibanum has the enhanced therapeutic effects of relieving pains and promoting the circulation of blood.^[1]

A previous study reported a comparison of the concentration of acetyl-11-keto- β -boswellic acid (%) between olibanum and processed olibanum, but the result was not statistically significant.^[13] Even though *B. carteri* resin extract showed the pharmacological effectiveness against inflammatory diseases in many studies,^[4,5] there has been insufficient scientific evaluation of the changes in the chemical composition during processing, which is essential for its effective and safe usage.

The aims of this study were to establish the analytical methods of marker compounds in the methanol extract of *B. carteri* resin and to find optimal processing conditions of *B. carteri* resin with vinegar, adjusting several variables such as acetic acid concentration, heating temperature, and heating time using a Box–Behnken design (BBD) with a response surface method (RSM) for statistical analysis.

MATERIALS AND METHODS

Material and reagents

The crude drugs of *B. carteri* were purchased from Kwangmyungdang Medicinal Herbs (Ulsan, Korea) and authenticated by Prof. Ju (Department of Herbology, Woosuk University, Republic of Korea). A voucher specimen (2014-WS-BC) has been deposited in the Department of Herbology of Woosuk University.

Two marker compounds, 11-keto- β -boswellic acid (KBA) and α BA, with the purity of both compounds >95%, were obtained from Fluka (Buchs, Switzerland). Their chemical structures are shown in Figure 1. High-performance liquid chromatography (HPLC) grade acetonitrile and water were purchased from J.T.Baker Inc. (Phillipsburg, NJ, USA), and HPLC grade methanol was purchased from Merck Chemical Co. (Darmstadt, Germany). Acetic acid was obtained from Fluka (Buchs, Switzerland).

Standardized procedure of stir-baking with acetic acid

The resin of *B. carteri* was macerated for 10 min in aqueous acetic acid, and then, samples were dried at room temperature for 24 h. The dried resins were heated using a vacuum oven (OV-12, Jeio Tech Co., Daejeon, Korea), while adjusting factors such as heating temperature and heating time. To find the optimal conditions for processing of *B. carteri*



Figure 1: Chemical structures of the two marker compounds in the methanol extract of *Boswellia carteri* resin. 1, 11-keto- β -boswellic acid; 2, α -boswellic acid

resin, preliminary single-factor tests were performed to determine the required range of acetic acid concentration (X_1 , 3%–12%), heating temperature (X_2 , 60°C–150°C), and heating time (X_3 , 5–20 min).

Sample extraction for high-performance liquid chromatography analysis

The processed resins were powdered and a 200 mg of powdered resin was extracted with 2 mL methanol using ultrasonication for 50 min at 50°C. The methanol extract was centrifuged at 10,000 rpm for 20 min. The supernatants were concentrated *in vacuo* to dryness at 45°C for 24 h. The concentrated extract was dissolved in methanol at the concentration of 10 mg/mL and filtered through a 0.20 μ m syringe filter before injecting into the HPLC.

High-performance liquid chromatography analytical conditions

The methanol extracts of *B. carteri* resin were analyzed using an Agilent 1200 (Agilent Technologies, Palo Alto, CA, USA) equipped with an autosampler, degasser, quaternary solvent pump, and diode array detector. Separation was performed on an Eclipse XDB C₈ column (150 mm × 4.6 mm, 5 µm; Agilent) at 35°C. The mobile phase consisted of 0.1% trifluoroacetic acid in water (TFA; A) and acetonitrile (B). The following gradient (B%) was applied to the elution of marker compounds: 75%–75% (B) over 0–2 min, 75%–88% (B) over 2–12 min, held for 3 min, and then re-equilibrated to 75% until the end of analysis. The flow rate was set to 1.0 mL/min and the detection wavelength was determined at 250 nm for KBA and 280 nm for α BA.

Method validation

Linearity, limit of detection, and limit of quantification

The stock solutions were prepared in methanol by dissolving accurately weighed standard compounds at concentrations of $1000 \,\mu$ g/mL. Working solutions produced by diluting the stock solutions were used to construct calibration curves. The diluted concentrations of marker compounds were plotted against the peak areas and calibration curves were used to evaluate the linearity.

The blank samples were injected three times and the area of the noise peak was calculated. Limit of detection (LOD) and limit of quantification (LOQ) were determined as follows: LOD = $3.3 \times$ standard deviation (SD)/slope of regression; LOQ = $10 \times$ SD/slope of regression.

Precision, recovery, and reproducibility

The precision was evaluated for intra-day (n = 3) and inter-day (n = 3) by analyzing standard mixture solutions containing low and high concentrations of marker compounds. The values were calculated as the relative SD: RSD (%) = (SD/mean) × 100.

A recovery test was evaluated to determine the accuracy of the method. Experiments were carried out by adding two known amounts of marker compounds (low and high) to samples. The recovery was represented as follows: Recovery (%) = ([detected concentration – initial concentration)/ spiked concentration) \times 100.

The reproducibility was evaluated by calculating the RSD values for the retention times and the absolute areas of marker compounds (n = 5).

Experimental design and statistical analysis

A three-level-three-factor BBD was employed to determine the optimal processing conditions for *B. carteri* resin, with quantification of the two marker compounds.

Obtained experimental data from the BBD were fitted to a second-order polynomial model and the regression coefficients were calculated using the equation:

$$Y = \beta_{0} + \sum_{j=1}^{k} \beta_{j} X_{j} + \sum_{j=1}^{k} \beta_{jj} X_{j}^{2} + \sum_{i < j} \beta_{ij} X_{i} Y_{j}$$

where *Y* is the estimated response, $\beta_{0'} \beta_{j'} \beta_{jj'}$ and β_{ij} are the regression coefficients for intercept, linearity, square, and interaction terms, respectively. X_i and X_j are the independent variables, which were coded as -1, 0, and 1.

The fitness of the second-order polynomial model was investigated through the term of lack-of-fit and coefficient of determination (r^2). *F* and *P* values were calculated using an ANOVA test and the significance of the regression coefficients determined at *P* < 0.05 or 0.01 was evaluated. Three-dimensional (3-D) response surface plots and contour plots were used to represent the interaction and influence of the three variables on the yield of the two marker compounds. The open-source software R (version 3.1.1; The R Foundation for Statistical Computing) was employed to generate the experimental design, perform statistical analysis, and evaluate the regression model.

RESULTS

System suitability

Under the developed HPLC methods developed above, the two marker compounds showed apparent separation and any interference was not found on the chromatograms at their maximum absorption



Figure 2: Chromatograms of a standard mixture (a) and methanol extracts of *Boswellia carteri* resin (b and c). 1, 11-keto- β -boswellic acid; 2, α -boswellic acid

Table 1: System suitability

| Compound | k | α | Rs | n | S |
|----------|------|------|-------|--------|------|
| KBA | 2.88 | 2.52 | 40.28 | 30,952 | 0.98 |
| αBA | 7.26 | | | 64,401 | 1.07 |

k: Capacity factor; α: Relative retention; *n*: Theoretical plate number; KBA: 11-keto-β-boswellic acid; αBA: α-boswellic acid; S: Symmetry; Rs: Resolution wavelengths [Figure 2]. Capacity factor (*k*), relative retention (α), resolution (*Rs*), theoretical plate number (*N*), and symmetry (*S*) were evaluated using the peaks of the two marker compounds. The range of the capacity factor was 2 < *k* <7, the relative retention was 2< α <3, the resolution was >40, the theoretical plate number was 30,000< *N* <70,000, and symmetry was 0.9< *S* <1.1, which indicate that the two marker peaks were separated, and tailing or peak fronting was not observed [Table 1].

Linear regression, limit of detection, and limit of quantification

The linearity, which was represented as the correlation coefficient (r^2) of KBA and α BA, was 0.9999 and 0.9998, respectively. The values of the LODs and LOQs of the two compounds were 0.24 and 0.46 µg/mL and 0.74 and 1.38 µg/mL, respectively [Table 2].

Precision, recovery, and reproducibility

The intra-day and inter-day precision of the two marker compounds was calculated as RSD values and their values were $\leq 2.0\%$ in two concentration levels [Table 3]. The recoveries of the two compounds were 95.36%–105.98%, with RSD values of < 3.0% [Table 4]. The reproducibility of the two marker compounds was also calculated as RSD values and their values were < 0.1% for the retention time and < 2.0% for the absolute area (n = 5).

Regression analysis and model fitting

The required ranges of acetic acid concentration (X_1 , 3%–9%), heating temperature (X_2 , 60°C–120°C), and heating time (X_3 , 5–15 min) were determined for BBD from the results of preliminary single-factor tests [Figure 3].

The regression coefficients of the predicted quadratic polynomial model were calculated by the coded values and responses from the BBD of the experiment and the significance of each coefficient was determined by the *P* value. A significant interaction between two variables was observed: the concentration of acetic acid (X_1) and heating temperature (X_2) (P < 0.05); and heating temperature (X_2) and heating time (X_3) (P < 0.001). However, the linear terms (X_1, X_2, X_3), quadratic terms (X_1, X_2, X_3, X_3), and two-factor interaction (X_1, X_3) were not significantly influential on the regression model (P > 0.05) [Table 5].

The regression coefficient of the model was applied to determine the predicted response values and was calculated by the following second-order polynomial equation: Y (response) = 11.930379 + 0.042851 X_1 + 0.188227 X_2 + 0.027972 X_3 + 0.460208 X_1X_2 + 0.289783 X_1X_3 + 1.840442 X_2X_3 + 0.134214 X_1X_1 - 0.022731 X_2X_2 + 0.020012 X_3X_3 , where Y is the yield of the two compounds (mg/g) and the coded variables, X_1 , X_2 , and X_3 , represent the concentration of acetic acid, heating temperature, and heating time, respectively.

An ANOVA was performed for the fitted quadratic polynomial model for the yield of the two compounds, showing that two-factor interaction was significant with F > 49 and P < 0.001, respectively [Table 6]. The coefficients of determination for multiple R^2 and adjusted R^2 were 0.9618 and 0.9045, respectively, with a significant lack-of-fit at P < 0.05.

| Table 2: Linear equation | s, coefficients of determination | (<i>r</i> ²), limit c | of detection | , and limit of | quantification | for the bioactive | compounds |
|--------------------------|----------------------------------|------------------------------------|--------------|----------------|----------------|-------------------|-----------|
|--------------------------|----------------------------------|------------------------------------|--------------|----------------|----------------|-------------------|-----------|

| Compound | Linear equation | r ² | Linear range (µg/mL) | LOD (µg/mL) | LOQ (µg/mL) |
|----------|------------------|-----------------------|----------------------|-------------|-------------|
| KBA | y=13.576×-10.289 | 4.69-300.00 | 0.9999 | 0.24 | 0.74 |
| αBA | y=0.4606×-0.5358 | 3.13-200.00 | 0.9998 | 0.46 | 1.38 |

y: Peak area (mAU); x: Concentration of the compound (μ g/mL); LOD: Limit of detection; LOQ: Limit of quantification; KBA: 11-keto- β -boswellic acid; α BA: α -boswellic acid



Figure 3: The results of a preliminary test within the selected ranges of independent variables. (a) Concentration of acetic acid. (b) Heating temperature. (c) Heating time

Table 3: Intra- and inter-day precision of the marker compounds

| Compound | Spiked concentration | Intra- | day (n =3) | | Inter-day (<i>n</i> =3) | | | |
|----------|----------------------|-----------------------|----------------------|--------|--------------------------|---------|--------------|--|
| | (µg/mL) | Detected | RSD (%) Accuracy (%) | | Detected | RSD (%) | Accuracy (%) | |
| | | concentration (µg/mL) | | | concentration (µg/mL) | | | |
| KBA | 15 | 15.05 | 0.14 | 100.17 | 15.09 | 0.32 | 100.32 | |
| | 30 | 29.97 | 0.03 | 99.96 | 29.96 | 0.08 | 99.92 | |
| αBA | 5 | 4.97 | 2.00 | 99.99 | 4.98 | 1.82 | 99.98 | |
| | 10 | 10.01 | 0.50 | 100.02 | 10.01 | 0.46 | 100.02 | |

RSD: Relative standard deviation (%)=(SD/mean)×100. SD: Standard deviation; KBA: 11-keto-β-boswellic acid; αBA: α-boswellic acid

Table 4: Recovery of the marker compounds

| Compound | Initial concentration (μg/mL) | Spiked concentration (μ g/mL) | Detected concentration (μ g/mL) | Recovery (%) | RSD (%) |
|----------|-------------------------------|------------------------------------|--------------------------------------|--------------|---------|
| KBA | 38.68 | 15 | 54.58 | 105.98 | 1.87 |
| | | 30 | 70.14 | 104.85 | 0.69 |
| αBA | 15.54 | 5 | 20.30 | 95.36 | 2.64 |
| | | 10 | 25.27 | 97.33 | 2.85 |

RSD: Relative standard deviation (%)=(SD/mean)×100. SD: Standard deviation; KBA: 11-keto-β-boswellic acid; αBA: α-boswellic acid

Table 5: Regression coefficients of the predicted quadratic polynomial model

| Variables | Estimate | SE | t | Р |
|---------------|-----------|---------|---------|---------|
| X_1 | 0.042851 | 0.11182 | 0.3832 | 0.715 |
| X_2 | 0.188227 | 0.11182 | 1.6833 | 0.143 |
| X_3 | 0.027972 | 0.11182 | 0.2502 | 0.811 |
| $X_1:X_2$ | 0.460208 | 0.15814 | 2.9102 | 0.027* |
| $X_1:X_3$ | 0.289783 | 0.15814 | 1.8325 | 0.117 |
| $X_{2}:X_{3}$ | 1.840442 | 0.15814 | 11.6383 | 0.000** |
| $X_1:X_1$ | 0.134214 | 0.15814 | 0.8487 | 0.429 |
| $X_2:X_2$ | -0.022731 | 0.15814 | -0.1437 | 0.890 |
| $X_{3}:X_{3}$ | 0.020012 | 0.15814 | 0.1265 | 0.903 |

P value significant at *<0.05 or **<0.01. X_1 : Acetic acid concentration (%); X_2 : Temperature (°C); X_1 : Time (min); SE: Standard error

Analysis of response surface

The interaction between variables and their influences on the response, the yields of the two marker compounds, was graphically visualized via a 3-D response plots and two-dimensional (2-D) contour plots, where the response values from the BBD obtained from regression analysis above were applied.

The predicted values of response were calculated by the second-order polynomial equation above using the coded values

of X_1 , X_2 , and X_3 , respectively [Table 7]. The coded values at two margins, 1.0:-1.0 and -1.0:1.0, produced less yield of the two marker compounds compared with those at the margins of -1.0: -1.0 and 1.0:1.0 in all 3-D surface plots and 2-D contour plots and the response surface derived from each margin formed curves, which was most apparent in the plot of X_2 : X_3 interaction at the zero level of X_1 [Figure 4].

Optimization and verification of the stir-baking processing of *Boswellia carteri* resin by response surface methodology

The conditions that produced the optimal response of the stir-baking processing of *B. carteri* resin were determined as coded values: -0.05725 of X_1 (acetic acid concentration; %); -0.00427 of X_2 (heating temperature; °C); and -0.08806 of X_3 (heating time; min). The optimized coded values were calculated to actual values, namely 5.83% of acetic acid concentration, 89.87°C of heating temperature, and 9.56 min of heating time, respectively, which produced 11.93 mg/g of predicted response, determined by the second-order polynomial equation above. The modified conditions represented 11.25 mg/g, which was very close to the actual response [Table 8]. These results confirm that the model for the stir-baking processing was able to predict the experimental conditions.

 Table 6: Analysis of variance for the fitted quadratic polynomial model for the extraction of compounds

| | df | SS | MS | F | Р |
|-----------------------|----|--------|--------|---------|----------|
| FO (X_1, X_2, X_3) | 3 | 0.3044 | 0.1015 | 1.0143 | 0.449 |
| TWI (X_1, X_2, X_3) | 3 | 14.732 | 4.9107 | 49.0924 | 0.000*** |
| PQ (X_1, X_2, X_3) | 3 | 0.0757 | 0.0252 | 0.2523 | 0.857 |
| Residual | 6 | 0.6002 | - | - | - |
| Lack of fit | 3 | 0.5608 | 0.1869 | 14.2518 | 0.028* |
| Pure error | 3 | 0.0394 | 0.0131 | - | - |

P value significant at *<0.05 and ***<0.001, respectively. df: Degree of freedom; SS: Sum of square; MS: Mean square; FO: First order; TWI: Two-factor interactions; PQ: Pure quadratic; X_1 : Acetic acid concentration (%); X_2 : Temperature (°C); X_2 : Time (min)

 Table 7: Experimental ranges and values of the independent variable in the

 Box-Behnken design for processing conditions

| Run | n Coded variables levels | | | Yield of compounds (mg/g) | | |
|-------|--------------------------|-----------------------|-----------------------|---------------------------|-----------------|--|
| order | X ₁ | X ₂ | X ₃ | Actual value | Predicted value | |
| 1 | 0 (6) | 0 (90) | 0 (10) | 11.93 | 11.88 | |
| 2 | -1(3) | -1(60) | 0 (10) | 12.27 | 12.64 | |
| 3 | 0 (6) | -1(60) | 1 (15) | 9.93 | 9.76 | |
| 4 | 0 (6) | 1 (120) | 1 (15) | 13.98 | 14.19 | |
| 5 | 1 (9) | -1(60) | 0 (10) | 11.44 | 11.44 | |
| 6 | 0 (6) | 0 (90) | 0 (10) | 11.93 | 11.94 | |
| 7 | 0 (6) | 0 (90) | 0 (10) | 11.93 | 12.08 | |
| 8 | 0 (6) | -1(60) | -1(5) | 13.55 | 13.34 | |
| 9 | -1(3) | 0 (90) | 1 (15) | 11.78 | 11.57 | |
| 10 | 1 (9) | 1 (120) | 0 (10) | 12.73 | 12.36 | |
| 11 | -1(3) | 0 (90) | -1(5) | 12.30 | 12.14 | |
| 12 | 0 (6) | 0 (90) | 0(10) | 11.93 | 11.81 | |
| 13 | 1 (9) | 0 (90) | -1(5) | 11.81 | 12.02 | |
| 14 | 1 (9) | 0 (90) | 1 (15) | 12.45 | 12.61 | |
| 15 | -1(3) | 1 (120) | 0 (10) | 11.73 | 11.73 | |
| 16 | 0 (6) | 1 (120) | -1(5) | 10.25 | 10.41 | |

 X_1 : Acetic acid concentration (%); X_2 : Temperature (°C); X_3 : Time (min)

 Table 8: Optimum conditions and the predicted and experimental values of the response at the optimum conditions

| Condition | X ₁ | X ₂ | X ₃ | Yield of compounds (mg/g) |
|-------------------|-----------------------|-----------------------|-----------------------|------------------------------|
| Optimal condition | 5.83 | 89.87 | 9.56 | 11.93 |
| Modified optimal | 6.00 | 90.00 | 10.00 | 11.25 |
| condition | | | | |

 X_1 : Acetic acid concentration (%); X_2 : Temperature (°C); X_3 : Time (min)

DISCUSSION

Optimization of chromatographic conditions

The mobile phase, modifier, and ultraviolet (UV) wavelength of the diode array detection were chosen to be crucial factors of HPLC analysis. A C_8 column was selected to separate the peak of KBA from adjacent peaks because a C_{18} column was not able to separate overlapping peaks. The mobile phase consisting of water (solvent A) and acetonitrile (solvent B) was used for the analysis. Various modifiers, such as 0.5% acetic acid, 0.1% phosphoric acid, 0.1% TFA, and 0.1% formic acid, were tested to obtain distinct separation between the peaks of the two marker compounds and adjacent peaks. Resolution and peak shapes of the two marker compounds were achieved when using 0.1% TFA as a modifier. The UV wavelengths of maximum absorption were chosen to determine the optimal absorption wavelength for each

marker compound, namely KBA for 250 nm and α BA for 280 nm, as previously reported.^[6,14,15]

Fitting of the regression model

To predict quadratic polynomial models for the stir-baking processing of *B. carteri* resin, multiple regression coefficients were calculated using least squares methodology. ANOVA represents the adequacy of the selected variables of polynomial models. The model coefficient of multiple R^2 indicates that the predicted model could explain 96.18% of the results and only 3.82% of the total variance was not explained by the model; adjusted R^2 also represents the same explanation as above, at a level of significance (P < 0.02). These results demonstrate that this multiple regression model is adequate and can explain most of the variability for variables because a larger *t*-value and smaller *P* value show the significance of the corresponding coefficient.^[16]

In ANOVA, the parameter for two-factor interaction was highly significant for all variables (P < 0.001), while linear and quadratic parameters were not significant (P > 0.05). Hence, the interactions between variables were the primary determining terms that might affect significantly on the response with insignificant linear and quadratic terms.^[17] However, there is significant lack-of-fit in this model (P < 0.05). The nonsignificant value of lack-of-fit (P > 0.05) revealed that the quadratic model is statistically significant for the response, and therefore, it can be used for further analysis.^[18] Evidence of lack-of-fit may come from a violation of one or more of three characteristics: (1) the fitted model's residual variation is small; (2) displays no systematic tendency; and (3) follows the variability postulated by the model.^[19]

Box–Behnken design and response surface methodology

RSM has been used to determine the optimum conditions of independent variables that produce the maximum value of response, showing visual interaction of two independent variables via 3-D response plots and 2-D contour plots.^[20] BBD is a class of second-order designs based on three-level incomplete factorial designs, and because they do not contain combinations for which all factors are simultaneously at their highest or lowest levels, they are considered useful in avoiding experiments conducted under extreme conditions, for which undesirable results may occur.^[21] RSM combined with BBD has been successfully applied to optimizing the extraction conditions - such as extraction temperature, extraction time, or ratio of raw materials to solvents - of chemical compounds or polysaccharides from herbal medicines using various extraction methods.^[22-25] A convex response plot and an elliptical contour plot usually indicate that the interaction between the variables is significant, while a circular contour plot means negligible interaction.[26]

However, the response plots and contour plots in this study did not show typical convex and elliptical shapes; instead, they showed the curves focused in the middle values. These results indicate that the variables that produce the optimum value of response, not the maximum value, can be determined by the response and contour plots, because the response values from both contrary margins were gathered in one point. In the present study, the interaction between acetic acid concentration (%) and heating temperature (°C) showed weak influential interaction on the yield of the two marker compounds, at a level of significance (P < 0.05), while the heating temperature (°C) and heating time (min) strongly interacted and significantly affected the yield of the two marker compounds (P < 0.001). Therefore, the two factors of heating temperature and heating time are thought to be key factors in determining optimal stir-baking processing of the *B. carteri* resin with acetic acid.



Figure 4: Response surface plots and contour plots of the interactions between acetic acid concentration (X₁, %), temperature (X₂, °C), and time (X₃, min)

Strengths and limitations of this study

In the present study, analytical conditions for the marker compounds in *B. carteri* resin extract were established with validated methods. Using statistical tools, optimized conditions for stir-baking processing of *B. carteri* resin with vinegar was performed, while adjusting three influential factors – acetic acid concentration, heating temperature, and heating time – with the aim of providing objective processing methods for the herbal medicine. However, two chemical marker compounds cannot guarantee the entire chemical characteristics and therapeutic effect of *B. carteri* resin. Moreover, there may be other influential variables, such as extraction solvent and time of maceration in aqueous acetic acid, and these should be considered in further study.

CONCLUSIONS

Established analytical methods were applied to quantify two marker compounds in a *B. carteri* resin extract. Two factor interactions – acetic acid concentration–heating temperature and heating temperature–heating time – were significant; of these, the interaction between heating temperature and heating time was the most significant. Optimal conditions for stir baking process of *B. carteri* resin were determined as 5.83% acetic acid, 89.87°C for heating temperature, and 9.56 min for heating time, which were modified to 6.00%, 90.00°C, and 10 min, respectively.

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Conflicts of interest

There are no conflicts of interest.

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