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ORIGINAL ARTICLE

Responsive Surface Methodology Optimizes Extraction Conditions of Industrial by-products, *Camellia japonica* Seed Cake

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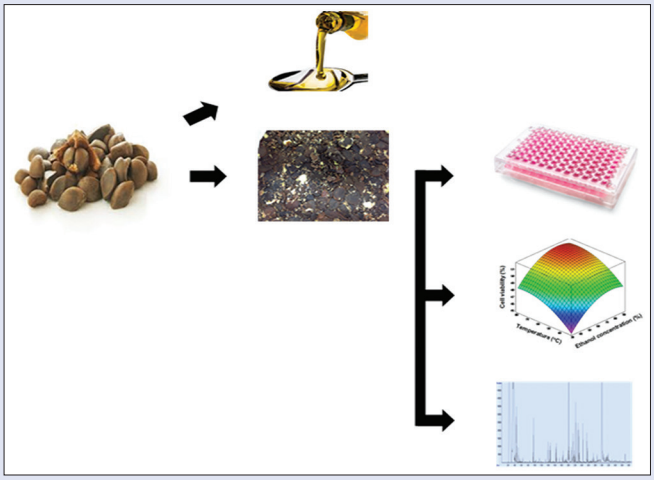
ABSTRACT

Background: The central nervous system is easily damaged by oxidative stress due to high oxygen consumption and poor defensive capacity. Hence, multiple studies have demonstrated that inhibiting oxidative stress-induced damage, through an antioxidant-rich diet, might be a reasonable approach to prevent neurodegenerative disease. **Objective:** In the present study, response surface methodology was utilized to optimize the extraction for neuro-protective constituents of *Camellia japonica* byproducts. **Materials and Methods:** Rat pheochromocytoma cells were used to evaluate protective potential of Camellia japonica byproducts. **Results:** Optimum conditions were 33.84 min, 75.24%, and 75.82°C for time, ethanol concentration and temperature. Further, we demonstrated that major organic acid contents were significantly impacted by the extraction conditions, which may explain varying magnitude of protective potential between fractions. **Conclusions:** Given the paucity of information in regards to defatted *C. japonica* seed cake and their health promoting potential, our results herein provide interesting preliminary data for utilization of this byproduct from oil processing in both academic and industrial applications.

Key words: Agricultural by-products, *Camellia japonica*, organic acids, reactive oxygen species, response surface methodology

SUMMARY

- Neuro-protective potential of *C. japonica* seed cake on cell viability was affected by extraction conditions
- Extraction conditions effectively influenced on active constituents of *C. japonica* seed cake
- Biological activity of *C. japonica* seed cake was optimized by the responsive surface methodology.



Abbreviations used: GC-MS: Gas chromatography-mass spectrometer, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, PC12 cells: Pheochromocytoma, RSM: Response surface methodology.

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INTRODUCTION

It has been shown that oxidative damage is involved in the etiology of various neurodegenerative disorders, including Alzheimer's disease, sclerosis, and stroke.[1] Although reactive oxygen species can be generated during normal cellular respiration and metabolism, unbalanced oxidative stress due to abnormal physiological conditions can result in oxidative damage.[1] In particular, the brain possesses relatively low levels of antioxidant compounds, making it more susceptible to oxidative damage,[2] implying that inhibiting oxidative damage through an antioxidant-rich diet could be a reasonable approach to preventing neurodegenerative disease.

Camellia japonica has been used as an ornamental plant in Asia,[3] and Camellia oil, derived from its seed, is characterized by a high content of oleic acid.[4] However, once defatted, the *C. japonica* seed hull comprises approximately 60% of the seed; thus, the seed hull of *C. japonica* constitutes a major resource for *C. japonica*. Of note, it was reported that *C. japonica* seed hulls possess various biologically active constituents, warranting more studies on the residual by-product of *C. japonica*.[5] In this study, response surface methodology (RSM) was utilized to establish

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the optimal conditions for obtaining *C. japonica* seed hull extracts that represent the highest protective potency against oxidative damage in rat pheochromocytoma (PC12) cells, a neuronal-like cell line, thereby increasing the efficiency of *C. japonica* as a health-promoting plant.

MATERIALS AND METHODS

Sample preparation

For sample extraction, defatted seed cake of *C. japonica* was prepared as we described previously.[6] The levels of each independent variable were determined based on the preliminary results [Table 1].

Cell culture and measurement of cell viability

PC12 cells were cultured and maintained as described elsewhere.[7] Cell survival was assessed by the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay.[7]

Gas chromatography-mass spectrometer analysis

To elucidate candidate active constituents present in *C. japonica*, the fraction exhibited the highest protective potency against oxidative stress was compared with the one with the lowest. Moreover, each fraction was subjected to gas chromatography-mass spectrometer analysis as described elsewhere.[8]

Statistical analysis and response surface methodology design

Specifically, data from the Box-Behnken experimental design were utilized to determine the optimum combination of variables. A fractional 3-level, 3-factor experimental design with 3 replicates at the center point was used to find effects of independent variables on the dependent variables (i.e., cell viability). In the study, independent variables include extraction time (X_1), ethanol concentration (X_2), and extraction temperature (X_3) for *C. japonica* seed cake. Each factor was coded at three levels (−1, 0, and 1). The RSM experimental design is summarized in Table 1. The complete experimental design consisted of 15 points. Data analysis was used to predict the following second-order polynomial model through the response surface regression procedure of the SAS 9.2 (SAS Institute, Cary, NC, USA):

$$Y = \beta 0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_i j X_i X_j$$

where Y is a response; β_0, β_i, β_{ii}, and β_{ij} are constant coefficients; and X_i are uncoded independent valuables. Regression analysis and analysis of variance were used to assess the model. To create response plots, the Maple Software version 7 (Waterloo Maple, Waterloo, ON, Canada) was utilized by holding constant one variable of the second-order polynomial equation. The three-dimensional representation of the response surface is the graphical representation of the regression equation, showing the optimum values of the variables at which response is maximized. The statistical significance between groups was calculated and grouped using one-way ANOVA, followed by the Tukey's test (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

The PC12 cell viability was measured from 15 sets of variable combinations [Table 1] and the data were fitted to the second-order polynomial equation using the response surface regression procedure for all responses investigated, including linear (X_1, X_2, and X_3), interactions (X_1X_2, X_1X_3, and X_2X_3), and quadratic terms (X_1^2, X_2^2, and X_3^2). The quadratic polynomial model is also given in Table 2. The significant interaction between variables was noted in between extraction time and temperature ($P = 0.0177$). The coefficients of determination (r^2) for Y was 0.93 [$P < 0.05$; Table 2], and the analysis of variance indicated that the predicted model was significant at the 5% level. In addition, the lack of fit test which determines the adequacy of the model was not statistically significant, indicating that the model is valid to predict responsible variables (sum of squares = 20.86; $P < 0.05$).

Optimum conditions for extraction and their predicted dependent values are shown in Table 2. Optimum conditions were extraction time of 33.84 min, ethanol concentration of 75.24%, and extraction temperature of 75.82°C. The predicted value for neuronal cell viability at optimized extraction conditions was 52.08%. To validate this, constituents of the *C. japonica* seed cake were extracted using the conditions calculated from the RSM and then applied to PC12 cells with identical experimental conditions. In this, the cell viability was shown to be 52.32 ± 0.89%, which is in excellent agreement with the predicted value from the model [52.08%; Table 2], suggesting that the RSM model demonstrated in the study could be utilized for optimization of *C. japonica* seed cake.

To predict effects of extraction conditions for neuroprotective constituents from *C. japonica* seed cake, the second-order polynomial

Table 1: Experimental design for neuronal cell viability of *C. japonica* by-products

Independent variable		Symbol	Levels		
			-1	0	1
Extraction time (min)		X_1	15	50	85
Ethanol concentration (%)		X_2	30	60	90
Extraction temperature (°C)		X_3	30	55	80
Run	**X_1**	**X_2**	**X_3**	**Variable levels**	**Cell viability (%)**
1	15	30	55	-1 -1 0	46.13
2	85	30	55	+1 -1 0	47.74
3	15	90	55	-1 +1 0	48.95
4	85	90	55	+1 +1 0	47.27
5	15	60	30	-1 0 -1	46.46
6	85	60	30	+1 0 -1	48.24
7	15	60	80	-1 0 +1	51.18
8	85	60	80	+1 0 +1	46.20
9	50	30	30	0 -1 -1	45.63
10	50	90	30	0 +1 -1	49.52
11	50	30	80	0 -1 +1	48.28
12	50	90	80	0 +1 +1	51.78
13	50	60	55	0 0 0	51.75
14	50	60	55	0 0 0	51.26
15	50	60	55	0 0 0	50.98

The levels of each independent variable were determined based on the preliminary experimental results

Table 2: Optimized extraction conditions and predicted RSM mode

Response	Optimum conditions			Predicted value (%)	Experimental value (%)
	Time (min)	Ethanol (%)	Temperature (°C)		
	33.84	75.24	75.82	52.08	52.32±0.89
Cell viability (%)	**Quadratic polynomial model**				**R^2**
	$Y=24.0231+0.3052X_1+0.2887X_2+0.3176X_3-0.0008X_1^2-0.0017X_2^2-0.0001X_3^2-0.0008X_1X_2-0.0018X_1X_3-0.0001X_2X_3$				0.93

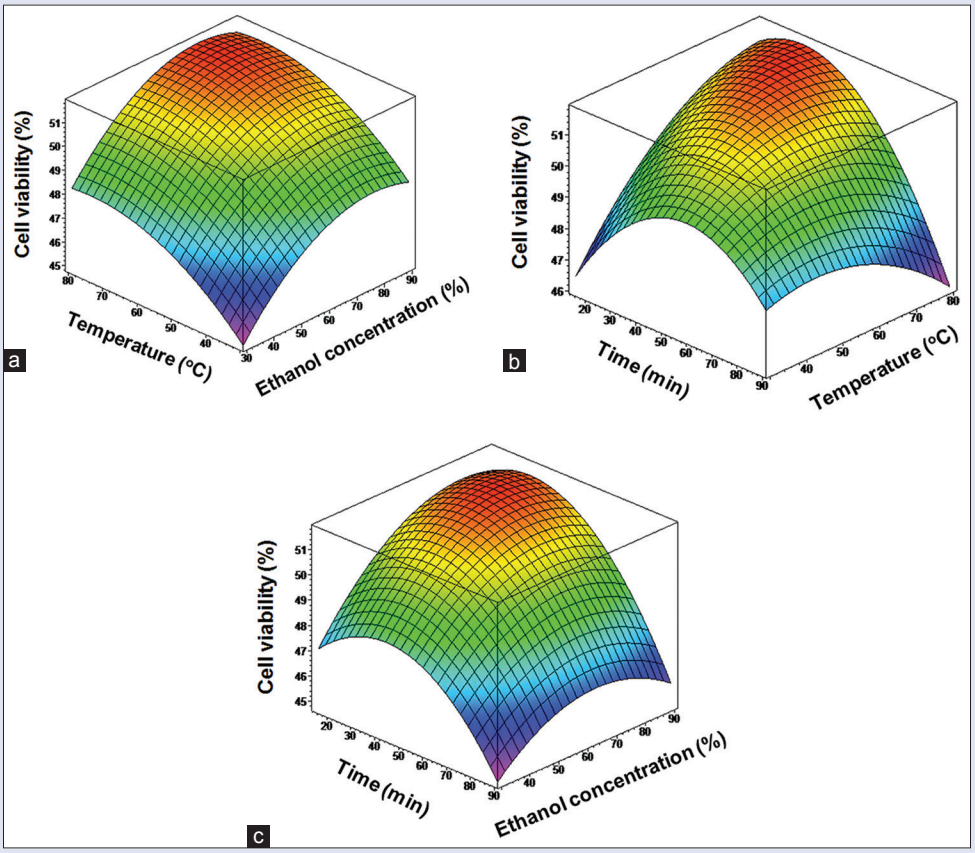

Figure 1: Response surface plot of the effects of extraction time, ethanol concentration, and extraction temperature on cell viability (%) under the fixed optimal conditions of (a) extraction time, 33.84 min; (b) ethanol concentration, 75.24%; (c) temperature, 75.82°C

model was utilized. The response surface plots for the extraction conditions are depicted in Figure 1. Response surface plots allowed visualizing predicted responses and aiding in identification of interactions between tested variables. As shown in Figure 1a, increased extraction temperature and ethanol concentration resulted in increased neuronal cell viability against oxidative stress, up to a threshold level. In particular, the extraction time seems to represent a threshold level in regards to neuronal cell viability, in which the maximum protective potency was observed up to a threshold while it was decreased beyond this limit, suggesting that moderate extraction time yields the greater achievement in cell protection [Figure 1b and c].

In addition, the fractions exhibited either the highest or the lowest protective potency against oxidative stress were compared to elucidate responsible, active constituents in *C. japonica*. Interestingly, it was demonstrated that the fraction #12 with the highest protective potency had significantly higher levels of several organic acids when compared to those of the fraction #9. Specifically, three most abundant organic acids, malic acid, citric acid, and quinic acid, were approximately 1.24-fold, 1.47-fold, and 1.67-fold higher in the fraction #12 (0.21 ± 0.01 mg, 1.62 ± 0.02 mg, and 0.20 ± 0.01 mg of malic acid, citric acid, and quinic acid per g of dried material of the fraction #12). Multiple studies have demonstrated protective capacity of these organic acids and their derivatives against oxidative stress.[9,10] This, also in good agreement with the previous investigation, reported that quinic acid significantly lowered the level of lipid peroxidation products through the induction of antioxidative enzymes.[11] More than one study have addressed that organic acids (e.g., citric acid) may elicit a synergistic potential with plant extract against oxidation due to distinct antioxidative mechanisms (e.g., metal chelating and radical scavenging mechanisms).[12] Thus, it is very possible that fractions high in organic acids represented better protective potency in conjunction with other radical scavenging compounds thereof. Further investigations are warranted with regards to organic acids compositions and their responsible mechanisms in neuronal cell protection against oxidative damages. In addition, bioavailability of active compounds through the blood–brain barrier should also be studied utilizing *in vivo* animal models.

To the best of our knowledge, no study has previously reported on the optimization of extraction conditions for neuroprotective constituents from *C. japonica* seed cake. Ye *et al.* recently isolated sasanqua saponin from *Camellia oleifera* and demonstrated antioxidative potential thereof.[13] However, in this study, we were not able to identify this class of compounds under our experimental conditions, potentially due to differences in species (*C. oleifera* vs. *C. japonica*) as well as extraction conditions.

In general, optimization has been done through looking at effects of one independent variable on a response variable over time to achieve maximum benefits. This procedure, however, is not sufficient for finding associations between multiple independent variables, necessitating alternative multivariate statistical analyses including RSM. RSM has been utilized for modeling situations in which a dependent variable is simultaneously impacted by more than one independent variable.[14] In the present study, the dependent variable was the *in vitro* protective potency of active constituents of *C. japonica* in response to oxidative stress while the independent variables were extraction conditions yielding active constituents from *C. japonica*. Although RSM has been utilized to optimize experimental conditions in biological matrices,[15]

this is the first report regarding the neuronal cell protection of *C. japonica* by-products.

To summarize, we investigated the protective potential of *C. japonica* seed cake in an *in vitro* neuronal model. It was demonstrated that neuroprotective potential of *C. japonica* seed cake on cell viability was affected by the extraction conditions. More importantly, these extraction conditions are effectively influenced on profiles of active constituents, including major organic acids we identified, thereby resulting varying magnitude of protective potential. The results herein might be interesting in several aspects. First, there are limited data available in regards to defatted *C. japonica* seed cake, an industrial by-product from oil processing, yet a few studies investigated beneficial effects of *C. japonica* oil elsewhere.[5] As mentioned, this residual by-product accounts for more than 60% of total weight of seeds, which provides a good rationale for utilization in both academic and industrial applications. Further, we found that the beneficial activity of the *C. japonica* seed cake was optimized by the RSM model. In the subsequent validation, it was demonstrated that the predicted cell viability was in good agreement with the experimental value, indicating that this statistical model can be utilized for optimization of processing conditions. To note, however, aspects of large scale sample processing were not considered in the study, warranting further investigations.

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Conflicts of interest

There are no conflicts of interest.

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