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Research Note

Optimization of Mixing Ratio to Improve Antioxidant Activity of Aged Garlic with Pine Needle Using Response Surface Methodology

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ABSTRACT: Aged garlic has been reported to possess beneficial pharmacological activities, including anti-stress and anti-fatigue properties, and to exert protective effects on the cardiovascular system and liver. Pine needles are widely used in folk medicine and as food additives owing to their pharmacological properties such as anti-aging and anti-inflammatory effects. It has long been known that combining certain phytochemicals with other phenols or organic acids can produce synergistic effects. Therefore, the purpose of this study was to develop an optimal formula of aged garlic with added pine needle powder for improved antioxidant activity using the statistical technique of response surface methodology. The antioxidant activities of aged garlic mixed with pine needle powder were confirmed by measuring oxygen radical absorbance capacity and total polyphenol content. An optimized antioxidant formula was identified that contained 5.08 g aged garlic and 1.97 g pine needle powder. The antioxidant activities of the mixture prepared using this optimal formula were significantly higher than the predicted values according to an additive model. Hence, this study confirms that the addition of pine needle powder to aged garlic can improve its antioxidant activity. This study demonstrated an optimal mixing ratio to produce an aged garlic product with improved functionality through the addition of pine needle powder that could be successfully employed by the food industry to prepare functional foods.

Keywords: aged garlic, pine needle, response surface methodology, ORAC value, synergistic effect

INTRODUCTION

Currently, many garlic products are used in alternative medicine, including fresh garlic extract, garlic oil, aged garlic, and various organosulfur compounds derived from garlic (1). Aged garlic is produced by aging fresh garlic at a high temperature for at least 1 month. During the aging of garlic, non-enzymatic browning produces changes in physicochemical parameters including color, flavor, texture, and the contents of macro- and micronutrients (2). Aged garlic contains water-soluble allyl amino acid derivatives that account for most of its organosulfur content, stable lipid-soluble allyl sulfides, flavonoids, saponins, and essential macro- and micronutrients (3). Further, pharmacological studies have found that aged garlic and its components possess antioxidative, antiaging, cardiovascular, and hepatoprotective effects (4).

Pine (*Pinus densiflora*) needles have been used in Asia to prepare folk medicines to treat conditions such as hypertension (5). Pine needle extracts were reported to show protective effects against oxidative DNA damage and apoptosis induced by hydroxyl radicals (6).

Studies involving the evaluation of synergistic effects of plant combinations are emerging and seem to highlight the potential for the mixtures to have enhanced functional properties when compared with the effects of each plant individually (7). Synergy assessment has become a key area in phytomedicine research in recent years to find a scientific rationale for the centuries-old, often observed therapeutic superiority of multidrug combinations over single constituents in traditional medicine (8).

Response surface methodology (RSM) is a statistical tool for performing, improving, and optimizing the independent factors that influence responses in a given set of experiments. RSM defines not only the effects of independent variables, but also their interaction effects (9). When multiple factors and interactions affect the desired responses for a given process, RSM is an effective technique for evaluating the process parameters with the fewest experiments (10,11).

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The objective of this study was to investigate the optimal mixing ratio of aged garlic and pine needle powders for maximizing the functional properties of the resultant mixtures, including their total polyphenol content (TPC) and oxygen radical absorbance capacity (ORAC), using RSM.

MATERIALS AND METHODS

Samples and sample preparation

For the preparation of aged garlic, raw garlic cloves were put into an incubator within *Chamaecyparis obtusa* boxes and then fermented for more than 600 h in the temperature range of 25°C to 75°C. The aged garlic was dried to a moisture content of 40%, ground to pass through a 6-mm sieve, and then cooled to 50°C. It was then gelatinized at 80°C for 2 h, dried at 60°C, and ground again to pass through a 3-mm sieve. This procedure was repeated with grinding to pass through a 1-mm sieve. Finally, the mixture was dried to a final moisture content of approximately 3%. The pine needle powder was purchased at a local market (Garunara, Gyeonggi, Korea).

Measurement of TPC

TPC was determined according to the method of Park et al. (12). Briefly, each sample was mixed with 2 mL of 1 N Folin-Ciocalteu reagent and incubated at 25°C for 3 min. Then, 2 mL of 10% Na_2CO_3 was added, and the mixture was incubated at 25°C for 1 h. The absorbance at 690 nm was measured with a microplate reader (Tecan, Männedorf, Switzerland). TPC was expressed as gallic acid equivalents (GAE).

ORAC assay

According to the method of Kim et al. (13), ORAC assays were performed using a GENios multi-functional plate reader (Infinite® F500; Tecan) with fluorescent filters (excitation wavelength 485 nm and emission wavelength 535 nm). In the final assay mixture, fluorescein (40 nM) was used as a target of free radical attack with 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH) (20 mM) as a peroxyl radical generator in peroxyl radical-scavenging capacity (ORAC ROO·) assays. Trolox (1 μM) was used as a control and prepared fresh daily. The analyzer was programmed to record the fluorescence signal every 2 min after AAPH was added. All fluorescence measurements were expressed relative to the initial reading. Final results were calculated based on the difference in the area under the fluorescence decay curve between the blank and each sample. ORAC ROO· values were expressed as μmol of Trolox equivalents (TE). One ORAC unit was equivalent to the net antioxidant effect provided by 1 μM Trolox.

Experimental design and statistical analysis

The tested mixing ratios of aged garlic and pine needle powders are given in Table 1. Aged garlic powder (X_1) and pine needle powder (X_2) were chosen as independent variables. The range and center point values of these two independent variables, as presented in Table 1, are based on the results of preliminary experiments. The TPC and ORAC values were selected as responses for the combination of the independent variables given in Table 2. Two experiments for each ratio were carried out, and the mean values are reported as the observed responses. The mixtures of aged garlic and pine needle powders (total weight 5 g) were extracted with ethanol (100 mL) for 72 h, and the extracts were filtered through Whatman No. 1 filter paper (Whatman, Maidstone, UK). The extraction solvents were removed with an evaporator (Eyela N-1000, Tokyo Rikakikai Co., Tokyo, Japan), and the dry residue was re-dissolved in dimethyl sulfoxide to a concentration of 50 mg/mL and kept at 20°C until use.

The experimental runs were randomized to minimize the effects of unexpected variability in the observed responses.

The variables were coded according to the equation:

$$x=(X_i-X_0)/\Delta X \qquad \text{(Eq. 1)}$$

where x is the coded value, X_i is the corresponding actual value, X_0 is the actual value in the center of the domain, and ΔX is the increment of X_i corresponding to a variation of 1 unit of x.

The mathematical model corresponding to the compo-

Table 1. Response of dependent variables to mixing ratio for independent variables

Treatment no.	Actual independent variables		Dependent variables	
	Pine needle	Aged-garlic	ORAC (μM TE)	TPC (mg GAE /100 g)
1	0.3	3.0	1.73	1,869.2
2	1.7	3.0	2.14	1,450.8
3	1.0	5.4	1.71	1,394.1
4	1.0	4.0	1.80	1,572.8
5	1.0	4.0	1.83	1,616.9
6	2.0	4.0	2.21	1,847.6
7	0.0	4.0	1.49	1,507.5
8	1.0	4.0	1.83	1,620.0
9	1.0	2.6	2.01	1,720.3
10	1.0	4.0	1.86	1,644.0
11	0.3	5.0	1.47	1,874.9
12	1.0	4.0	1.73	1,385.0
13	1.7	5.0	1.97	1,722.4

ORAC, oxygen radical absorbance capacity; TE, Trolox equivalents; TPC, total polyphenol content; GAE, gallic acid equivalents.

Table 2. Coefficients and R^2 values of variables in the predictive model for dependent variables

	Coefficient	
	ORAC	TPC
Intercept		
β_0	1.81	1,567.74*
Linear		
β_1[1)]	0.24103*	−11.24
β_2	−0.10678*	−23.00
Quardratic		
β_{11}	0.01313	82.89
β_{22}	0.01812	22.72
Interaction		
β_{12}	0.02250	66.47
Model (F-value)	61.46*	0.36
R^2	0.98	0.21
Lack of fit (*P*)	0.758	0.046*

ORAC, oxygen radical absorbance capacity; TPC, total polyphenol contents.
*Significant at $P<0.05$.
[1)]β_1, pine needle powder; β_2, aged-garlic powder.

site design is:

$$Y=\beta_0+\sum_{i=1}^{3}\beta_i X_i+\sum_{i=1}^{3}\beta_{ii}X_i^2+\sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij}X_iX_j \qquad \text{(Eq. 2)}$$

where *Y* is the dependent variable (ORAC or TPC), β_0 is the model constant, and β_i, β_{ii}, and β_{ij} are the model coefficients, which represent the linear, quadratic, and interaction effects of the variables, respectively. Analysis of the experimental design data and calculation of the predicted responses were carried out using Minitab 14 software (Minitab, State College, PA, USA). Additional confirmatory experiments were subsequently conducted to verify the validity of the experimental design. The parameters measured were expressed as mean±standard deviation, and the expected and actual values were compared using the unpaired Student's *t*-test. A *P* value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

In this study, the ORAC and TPC values of aged garlic powder combined with pine needle powder at various ratios were modeled using RSM. The ORAC assay, which closely reflects the antioxidant capacity of a test substance in biological systems (12), is a method for assessing total antioxidant activity. Polyphenols and other antioxidant compounds may contribute to the health-promoting effects of fruits, vegetables, whole grains, and nuts. Revealing an association between the amount of polyphenols and their potential health benefits requires an accurate assessment of the content of these compounds in plant foods. Significant positive correlations have been reported between ORAC and TPC, indicating that a high antioxidant activity could be attributed to a high content of total polyphenols (14-17). For this reason, two dependent variables, namely ORAC and TPC, were chosen in this experimental design. In a study by Quispe-Fuentes et al. (18), only the ORAC value was chosen as a dependent variable for the optimization of antioxidant activity from Maqui berries.

The experimental data for all responses using different mixing ratios are presented in Table 1. Aged garlic in the range of 2.6∼5.4 g was mixed with pine needle powder in the range of 0∼2.0 g. Values of the dependent variables were within the range of 1.47∼2.21 μM TE for the ORAC value and the range of 1,385.0∼1,874.9 mg GAE/100 g for the TPC. The experimental data were used to calculate the coefficients of the second-order polynomial equation. The regression coefficient and significance of the coefficients of the models are presented in Table 2. The coefficient of determination (R^2) indicates the degree to which a model fits the experimental data (19), and the calculated R^2 values are also presented in Table 2. A linear relationship with a good regression coefficient between ORAC and the mixing ratio was found; this relationship is shown in Eq. 3.

$$\text{ORAC}=1.81+0.24103\,X_1-0.10678\,X_2 \qquad \text{(Eq. 3)}$$

The correlation coefficient of the regression equation was 0.98, indicating a good fit between the experimental data and the regression model for the ORAC values, with a non-significant lack of fit (Table 2). Hence, it can be concluded that the proposed model approximates the response surfaces and is suitable for predicting any values of the parameters within the experimental domain. The addition of increasing concentrations of pine needle powder showed a significant positive linear relationship with ORAC values. The addition of aged garlic had a linear negative influence, and higher contents of aged garlic resulted in lower ORAC values. The quadratic and interaction effects had no significant influence. The response surfaces of the ORAC values are shown in Fig. 1.

The correlation coefficient (0.21) of the TPC model was lower than that of the ORAC model, and the lack-of-fit value was significant (Table 2). Therefore, this model could not be used for the prediction of TPC. A quadratic relationship with a poor regression coefficient (R^2=0.21) was found between the TPC values and the mixing ratio with a significant lack of fit. Moreover, the concentrations of aged garlic and pine needle powders showed no significant effect on TPC. The response surfaces of the TPC are shown in Fig. 1.

Response optimization using RSM helped to determine

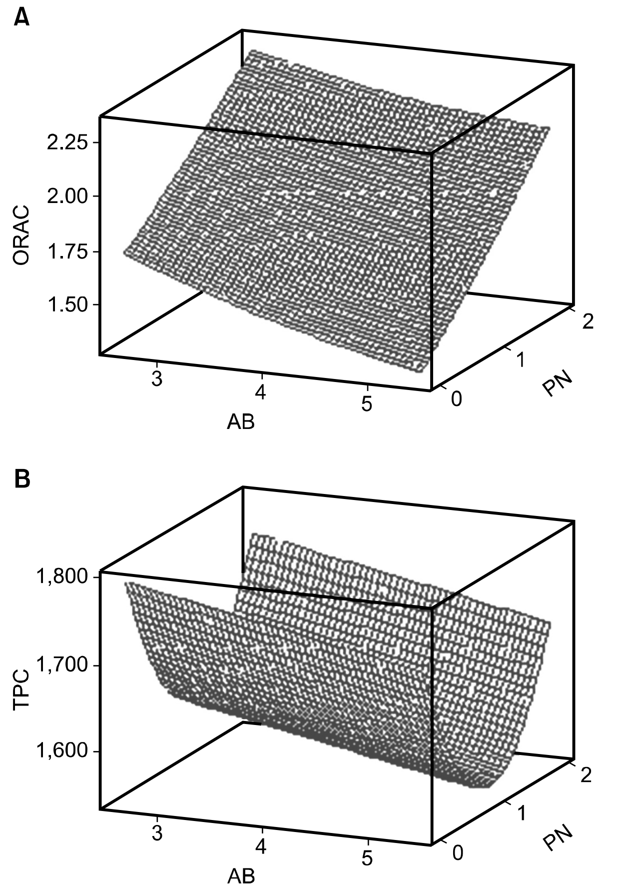

Fig. 1. Response surface plots for the oxygen radical absorbance capacity (ORAC) value (A) and total polyphenol contents (TPC) (B) in aged-garlic powder (AB) added with pine needle powder (PN).

the optimal combination of independent variables that maximized the ORAC value. The optimal mixing ratio for producing a high ORAC value was an aged garlic powder content of 5.08 g and a pine needle powder content of 1.97 g. Using this mixing ratio, ORAC and TPC values of 2 μM TE and 1,798.7 mg GAE/100 g were predicted, respectively. The predictive capacities of the developed models were tested and validated using this optimal mixing ratio and actual ORAC and TPC values of 6.4 μM TE and 2017.8 mg GAE/100 g were obtained, respectively. The actual TPC and ORAC values of the mixture prepared using the optimal formula of aged garlic with pine needle powder were significantly higher than the predicted values ($P<0.05$).

In conclusion, RSM proved to be a useful tool for establishing the optimum mixing ratio of aged garlic (5.08 g) and pine needle powder (1.97 g) for maximizing the antioxidant activity of the resultant mixture. The results obtained using the optimized mixing ratio exhibited a high antioxidant activity. From these results, it can be concluded that the predictive model described herein produced a well-fitted mixing ratio. The outcomes of this study will contribute to the development of other optimized mixtures of herbal powder to obtain desired functional properties.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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