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Optimization of ethanol extraction parameters for polyphenol content and antioxidant activity of *Codonopsis pilosula* root

Phuong P L Tran[2], Phuc N T Le[2], Phu H Le[1*]
[1] Department of Food Technology, School of Biotechnology, International University–Vietnam National University, Ho Chi Minh City, Vietnam
[2] Department of Biotechnology, School of Biotechnology, International University–Vietnam National University, Ho Chi Minh City, Vietnam

Abstract
Traditional folk medicine from herbal plant have been a topic of modern research, and also been proven to contain valuable therapeutic compounds. The current holistic approach towards treatment and well-being maintenance also contributes to the growing use of plant-derived bioactive constituents, opening many economic opportunities for local farmers to profit from cultivating herbal plants. *Codonopsis pilosula* is considered a potential medicinal herb, which is one of the reasons for the need to further study into its medicinal properties and industrialization-friendly methods of extraction. The aim of this study was to determine optimal extraction parameters for total phenolic content (TPC) and antioxidant activity (AOA) from *C. pilosula* via both conventional and modeling approach. The methods used were preliminary optimization of the conventional reflux extraction parameters: i.e. *C. pilosula*'s particle size; ii) ethanol concentration; iii) solvent: material ratio; iv) extraction temperature; v) extraction durartion, followed by a subsequent optimization using response surface methodology and Box-Behnken design (RSM-BBD) approach. Results from the model showed that the optimal values were determined as: 0.25 – 0.5 mm of particle size; 55% of ethanol concentration; 36:1 of solvent: material ratio; 59 ^{o}C of temperature; and 60 min of extraction time, and brought an optimal AOA of 3.449 mg AAE.g^{-1}. Furthermore, 3.45 ± 0.071 mg AAE.g^{-1} of AOA was also obtained from the confirmation experiment applying those optimal conditions. The contribution of this study was the insight of baseline extraction parameters for further scientific research and industrial application in the extraction for higher yield of TPC and AOA from *C. pilosula*.

Keywords: *Codonopsis pilosula*, Đảng sâm, ethanol extraction, polyphenols

Introduction

Codonopsis pilosula, or Đảng sâm in Vietnamese, is an herbaceous species that belongs to the family *Campanulaceae* and is widely regarded as an effective herbal medicine and its dried roots have been used in traditional medicine preparations for hundreds of years. The beneficial biological activities of this plant have been attributed to the polysaccharides and alkaloids, among various constituents found in its extract [1]. However, the current excessive focus on polysaccharides recovery in extraction could skim over the potential of other constituents or combination of them, as it has been reported that the employment of multiple active components could provide better therapeutic effects [2]. Therefore, it remains a necessity for optimization of extraction methods in terms of other components content, or a more general indicator such as antioxidant activity.

Apart from polysaccharides, polyphenols have also been harnessed as plant secondary metabolites for anti-oxidative, anti-inflammatory, anti-mutagenic and anti-carcinogenic properties [3]. They are naturally occurring compounds in plants and plant-based foods with a phenolic ring as basic monomer. The general classification is phenolic acids and phenolic alcohols, flavonoids, and lignans. In human's diet, the most frequent source of antioxidants is from polyphenols, and so they have been a target for research into remedies for various diseases, including cardiovascular, neurogenerative and cancer [4].

This study focused on the optimization of extraction process, with regard to minimizing the cost of production due to an ultimate aim to adapt to large scale production. The chosen solvent was ethanol to ensure safety, as the most common use for herbaceous extracts are medicine, food and cosmetics [5]. A range of parameters were investigated, including material size, solvent concentration, extraction temperature, and material/solvent ratio. The total phenolics content (TPC) and antioxidant activity (AOA) of the extracts were obtained and plotted to: i) determine the possible range where the optimal points were likely to be found, and ii) determine the parameters that most affected the characteristics of the extracts, in order to further optimize them with response surface methodology (RSM) using Box-Behnken Design (BBD).

Materials and methods

Preparation of *C. pilosula*

Dried roots of *C. pilosula* were obtained with undamaged condition from Tu Mơ Rông District (Kon Tum Province), ground and sieved into four different ranges of particles size from [a].

Chemical and reagents

All chemicals and reagents for analysis were purchased from Sigma-Aldrich (Dorset, UK).

Preliminary optimization of extraction parameters for TPC and AOA from *C. pilosula*

Reflux solvent extraction [6] was employed for the preliminary optimization of numerous extraction parameters: [a] *C. pilosula*'s particle size: ≤ 0.25, 0.25 – 0.5,

0.5 – 1.0, and ≥ 1.0 mm; [b] 10, 30, 50, 70, 90 %; [c] solvent:material ratio: 20:1, 30:1, 40:1, 50:1, 60:1; [d] extraction temperature: 40, 50, 60, 70, 80 °C; [e] extraction duration: 30, 60, 90, 120, 150 min. All five parameters were optimized one by one in single factor experiments (in triplicate). And for each parameter, the optimal value exerting the most influence on the TPC and AOA were identified and chosen.

For the method, briefly, for each optimization, 10 g of *C. pilosula* (predetermined particle sizes from [a]) was weighed into a round-bottom flask. Then, an appropriate amount of ethanol (predetermined concentration from [b]) was added accordingly (predetermined solvent:material ratios from [c]). A glass condenser was connected to the flask and the cool water source. A heating mantle with magnetic stirring function was used to heat the solvent (predetermined temperatures from [d]). After the testing extraction durations (predetermined from [e]) had passed, the mixture was vacuum filtered, and the filtrate was dried with rotary evaporation to eliminate ethanol and freeze dried to eliminate water. The final extract was stored in dark vials at 4 °C for further TPC and AOA evaluation.

Optimization of extraction parameters for AOA from *C. pilosula* using response surface methodology and Box-Behnken design (RSM-BBD) approach

After the preliminary optimizations, RSM-BBD approach was employed to evaluate the effect of these factors on AOA [7]. Three parameters (ethanol concentration, solvent: material ratio, and temperature) were chosen to establish the RSM model. Each experiment was conducted independently, randomly, and designed by a possible combination of 3 elements with 3 different levels (–1, 0, +1). Moreover, to identify experimental errors, 6 repetitive experiments at the central location were conducted. The obtained experimental data were then used to fit a second-order function taking AOA as the dependent variable. The ranges and responses were plugged into Design Expert software to calculate experimental combinations of extraction conditions. After the experimental runs, the responses of AOA were expressed as a function of the investigated variables in the form of a quadratic polynomial from which the optimal conditions can be inferred. In addition, a confirmation experiment was also carried out (in triplicate) applying all optimal parameters for accessing AOA.

Data collection

Total polyphenol content (TPC) determination

TPC of *C. pilosula* root's extract (CPRE) was evaluated by Folin-Ciocalteu colorimetric assay [8]. Briefly, the reaction was set up with 0.5 mL of the diluted sample and 2.5 mL of 10% Folin-Ciocalteu reagent in a 15 mL conical tube. The reaction mixture was homogenized via vortexing, then mixed with 2 mL of 7.5% sodium carbonate solution. After an one-hour incubation under dark condition, the absorbance was measured at 765 nm of wavelength using BioTek Epoch 2 Microplate Spectrophotometer (Agilent, USA) and TPC was quantified against the respective gallic acid standard curve.

Antioxidant activity (AOA) determination

AOA of CPRE was determined via its radical scavenging activity with the 2, 2'-azinobis-(3-ethylbenzthiazolin-6-sulfonic acid) (ABTS) colorimetric assay [8]. Briefly, the free radical solution was prepared with 10 mL of 7.4 mM ABTS solution and 10 mL of 2.6 mM potassium persulfate solution. Then, the reaction was set up with 0.5 mL of the diluted extract and 1.5 mL of the pre-made free radical solution in an Eppendorf tube. For the control, absolute ethanol was used to replace extract sample. After a 30-min incubation under dark condition, the absorbance was measured at 734 nm of wavelength and AOA was quantified against the respective vitamin C standard curve.

Data analysis

Data was statistically analyzed by SPSS 27.0 for MacOS. One-way analysis of variance (ANOVA) and Duncan's test were used to determine the significant difference between treatments ($P < 0.05$).

Results and discussion

Preliminary optimization of extraction parameters for TPC and AOA from *C. pilosula*

Optimization of C. pilosula's particle size

The size of the powdered plant root is shown to be a factor affecting the results of the extraction. Figure 1A illustrates the changes of harvested TPC and antioxidant activity indicated by ABTS assay, according to different particle sizes, ranging from smaller than 0.25 mm to over 1.0 mm. Overall, smaller particle sizes tend to yield higher activities ($P < 0.05$), but curiously, the size of smaller than 0.25 mm gave significantly lower ($P < 0.05$) TPC and AOA compared to the next smallest size (0.25 – 0.5 mm).

Smaller particle sizes resulting in more TPC and AOA, but the smallest size, 0.25 mm, performing less effectively, since a variety of studies on solvent extraction of dried materials found that while less than 0.5 mm performs more favorably in extraction, microscopic particle size actually has a negative effect on the yield of targeted compounds. Several reasons have been proposed, including the tendency of tiny particles to float on top instead of being submerged fully into the solvent, or to clump together in contact with moisture. Furthermore, it was also observed that the extra *C. pilosula* root (CPR) powder that was left unused in the experiments, although being stored in a desiccant chamber, showed signs of clumping due to moisture from the air, with the finest particle size showing the worst clumping. This phenomenon is very unfavorable, since the ultimate aim of this and upcoming studies is to adapt CPR extraction to industrial scale, which will inevitably involve longer storage time in a less controlled environment than lab-scale experiments [9]. Therefore, the second smallest size range, 0.25 – 0.5 mm, is chosen as the optimal for CPR extraction.

Optimization of ethanol concentration

As can be shown by figure 1B, both TPC and AOA activity increased greatly when the ethanol concentration rose from 10 to 50% ($P < 0.05$). However, further increase in solvent concentration reversed this upward trend, causing noticeable decrease in both indicators ($P < 0.05$).

Solvent concentration has been hypothesized by the author to be one of the most significant factors in the extraction process, since the target, phenolic compounds, is of polarizing nature and their solubility responds differently to various polarities of solvent [10]. Since water with a dielectric constant of 80.1, is much more polar than ethanol, which has a dielectric constant of 25 [11], mixing the two

solvents in different proportion will result in extraction solution with polarity ranging from more than that of ethanol to less than that of water. There has not been a common ground on which solvent or mixture of solvents at which ratio are more effective in recovering bioactive compounds in different plant materials in literature. However, alcoholic solvents, including ethanol, are still regarded as a fail-safe choice in phenolic compounds extraction from plant materials [12].

Optimization of solvent: material ratio

Figure 1C shows a positive correlation ($P < 0.05$) between solvent: material ratio and TPC and AOA until the 40:1 ratio. This phenomenon is readily predicted, since more solvent being used means more space for plant compounds to get dissolved into. However, the 50:1 ratio showed an adverse effect.

The trend seen in the effects of solvent: material ratio is readily predicted, since more solvent being used means more space for plant compounds to get dissolved into. As the recovery process involved rotary evaporation, although only at a mild temperature of 60 °C, more solvent means that the extract spent a longer time in heat treatment, which could result in more degradation and loss of antioxidant activity [13].

Optimization of extraction temperature

The results from figure 1D show that increasing temperature until 60 °C enhanced the extraction efficiency ($P < 0.05$), although temperature higher than that (70 °C and 80 °C) dampened this effect ($P < 0.05$). This could be the consequence of excess heat affecting the structure, and thus activity of phenolic compounds. Moreover, although TPCs yielded at 60, 70, and 80 °C did not vary a lot, the AOA declined noticeably.

Temperature is intuitively a helpful element in extracting bioactive compounds from natural sources, as evident from the traditional way of preparing medicinal concoction by boiling in water. Therefore, it is not surprising that increasing the temperature enhanced the yields of TPC from CPR. However, this beneficial effect disappeared when temperature was raised to higher than 60 °C, suggesting that there was a point where excessive heat no longer supported the migration of phenolic compounds from plant materials to solvent. This finding is consistent with many studies in plant extraction, where such thresholds that halted TPC yields were found somewhere from 60 °C to 80 °C [14], [15], [16]. Moreover, although TPCs yielded at 60, 70, and 80 °C did not vary a lot, the antioxidant activity declined noticeably. This decrease in antioxidant activity can be attributed to the degradation of compounds of other than phenolic nature but also having antioxidant effects.

Optimization of extraction time

Extraction time was expected to be an influential parameter to the extraction process in common sense. However, the results showed very modest changes in response to changes in extraction time, as indicated by figure 1E ($P < 0.05$). In fact, TPC did not fluctuate much when extending extraction time to higher than 60 minutes, while antioxidant activity even showed slight dips as the time went longer.

The effect of time suggests a more robust release of antioxidant constituents into solvent at the beginning of extraction, which is reasonable due to fresh solvent being added. After 60 minutes, longer heat treatment also showed an unfavorable effect to both TPC and AOA [17].

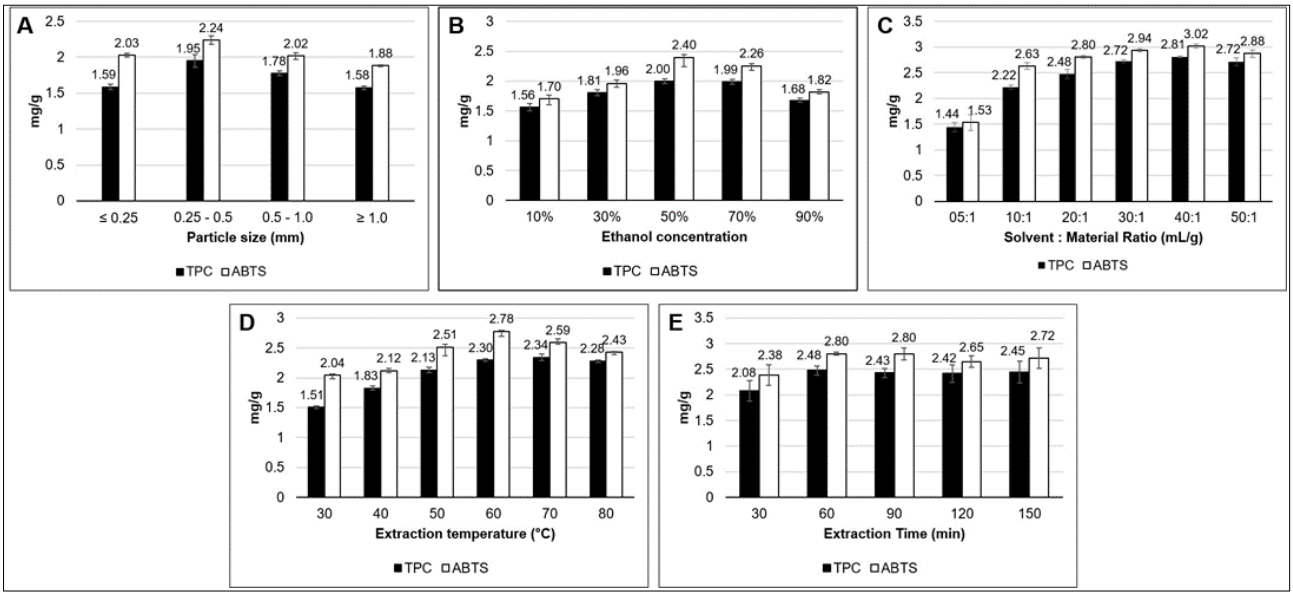

Fig 1: Effect of (A) particle size, (B) solvent concentration, (C) solvent: material ratio, (D) extraction temperature, and (E) extraction time on the total phenolic content (TPC) and antioxidant activity (AOA) of the *Codonopsis pilosula* root's extract (CPRE).

Optimization of extraction parameters for AOA from *C. pilosula* using RSM-BBD

In the previous preliminary experiments, optimal values for various extraction parameters were determined with respect to maximal TPC and AOA obtained from the CPRE. The study then proceeded to carry out a RSM procedure to account for the effect of the interaction of process variables on AOA. Table 1 presents independent factors and their corresponding levels. And the combinations of experimental conditions generated by BBD and their corresponding responses are shown in Table 2. Experimentally, the obtained AOA ranged from 3.07 ± 0.056 to 3.44 ± 0.088 mg AAE.g^{-1} DW. The current results indicated that all 3 evaluated extraction factors had a significant influence on

the AOA. This is also consistent with the general trend of the process of extracting bioactive compounds from plant materials. Accordingly, extraction factors such as temperature, solvent/material ratio, and solvent concentration all affected the extraction of bioactive compounds from materials, including polyphenols. The estimated quadratic function produced by the Design-Expert software is as follows:

"R(mg AAE/g) =3.44+0.1025A+0.0025B+0.005C-0.0375AB-0.0375AC-0.0075BC-0.12625" "A" ^"2" "-0.09125" "B" ^"2" "-0.09125" "C" ^"2"

Analysis of variance test was performed to develop and evaluate the compatibility of the achieved model. The results are presented in Table 3. A model is considered statistically significant when: p-value of the model < 0.0001; adequate precision (AP) used to guide design spaces of greater than 4.0; lack of fit (LOF) values reflecting the fragility of data must be statistically insignificant ($P > 0.05$) and high R^2 values (> 0.8). Considering the above criteria, the quadratic model in this study satisfied all four criteria with p-value < 0.0001; AP = 35.37; LOF = 0.05 and R^2 = 0.92. In addition, a number of other factors were also used to evaluate whether the model is fully compatible with experimental results based on prediction and actual value plots and charts. Accordingly, the data in Figure 6 have shown that the model had a good correlation when the focal points were in a straight line and the distribution of the experimental points was random.

The influence of the 3 factors on the antioxidant activity of the extracts is described by three 3-dimensional surface response graphs (Figure 7): the red region represents the highest activity, while the green area shows lower results. Each chart has an optimal region, which suggests that the surveyed variables interacted with each other and affected the overall antioxidant activity of the extracts. Increasing the value of ethanol concentration, solvent: material ratio, and temperature would lead to an increase in the value of the target function. However, increasing the concentration beyond the optimal value was not beneficial, and this reduced the obtained antioxidant activity. Previously, studies on extraction of antioxidants from herbs have shown that temperature and solvent:material ratio had a great influence on polyphenol content and antioxidant activity of extracts, in which each different material source required different temperature and optimal solvent/extraction ratio [7]. As a consequence, optimization of the obtained function with respect to maximal AOA yielded the following optimal values: A = 55%, B = 36 ($mL.g^{-1}$), and C = 59 °C, corresponding with optimal activity of 3.449 mg $AAE.g^{-1}$. Confirmation experiments were carried out in triplicate using the above optimal conditions, resulting in polyphenol content of 3.45 ± 0.071 mg $AAE.g^{-1}$, which was in accordance with the prediction from the model. In summary, solvent extraction using ethanol and water appears to be a relatively simple approach towards achieving CPRE with reasonable antioxidant activity. Its potential benefits are evident, such as safety to consume in medicinal and cosmetic purposes thanks to the use of food-grade solvents, ease of setting up and adapting to industrial scale, and cost-effectiveness especially after optimization reduces the need for excessive ethanol in solvent and electricity to provide heat during extraction. However, it is essential to acknowledge that there remain various other factors that may impact the yield and effectiveness of CPR extracts that require further investigation.

Table 1: Summary of independent variables with their encoded values and response

Factors	Name	Unit	Coded values		
			-1	0	+1
A	Solvent concentration	%	40	50	60
B	Solvent : Material ratio	mL/g	30	40	50
C	Temperature	°C	50	60	70
Response	Name	Unit			
R	Antioxidant activity	mg AAE/g			

Table 2: Design table with values and the responses

Std	Run	A (%)	B (mL/g)	C (oC)	R (mg AAE/g)
5	1	-1	0	-1	3.07
9	2	0	-1	-1	3.26
15	3	0	0	0	3.44
7	4	-1	0	+1	3.17
1	5	-1	-1	0	3.07
14	6	0	0	0	3.44
4	7	+1	+1	0	3.30
12	8	0	+1	+1	3.24
10	9	0	+1	-1	3.26
11	10	0	-1	+1	3.27
13	11	0	0	0	3.44
6	12	+1	0	-1	3.35
2	13	+1	-1	0	3.35
16	14	0	0	0	3.44
3	15	-1	+1	0	3.17
17	16	0	0	0	3.44
8	17	+1	0	+1	3.30

Table 3: ANOVA for the quadratic model

Source	Sum of squares	df	Mean square	F-value	p-value
Model	0.2486	9	0.0276	154.67	< 0.0001
A	0.0841	1	0.0841	470.68	< 0.0001
B	0.0001	1	0.0001	0.2800	0.6131
C	0.0002	1	0.0002	1.12	0.3251
AB	0.0056	1	0.0056	31.50	0.0008
AC	0.0056	1	0.0056	31.50	0.0008
BC	0.0002	1	0.0002	1.26	0.2987
A2	0.0671	1	0.0671	375.83	< 0.0001
B2	0.0351	1	0.0351	196.33	< 0.0001
C2	0.0351	1	0.0351	196.33	< 0.0001
Residual	0.0013	7	0.0002		
Lack of fit	0.0013	3	0.0004		
Pure error	0.0000	4	0.0000		
Predicted R^2		0.9199			
Adjusted R^2		0.9886			

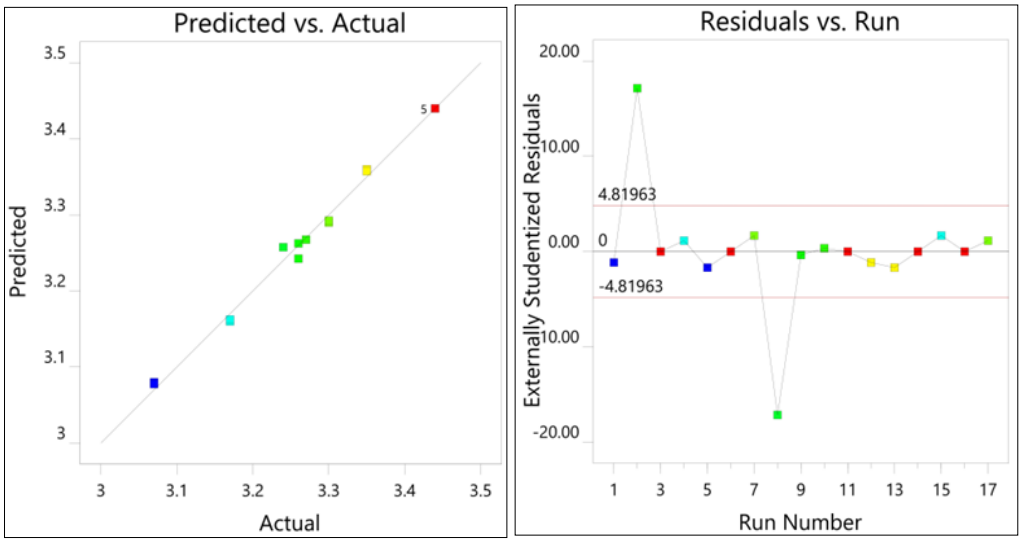

Fig 2: Predicted vs. experimental value graph and random distribution of 17 experiments.

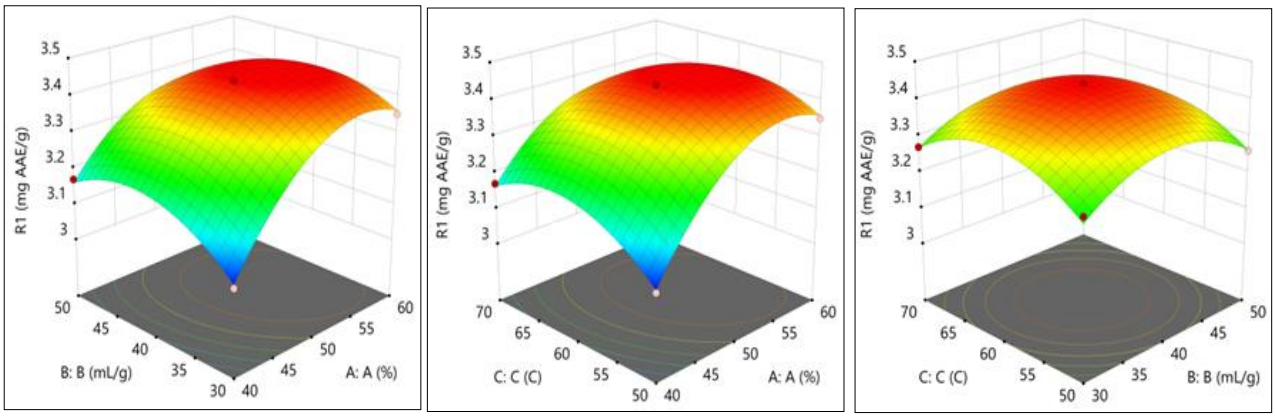

Fig 3: Three-dimensional surface interpretation of the effects of the three parameters (ethanol concentration, solvent: material ratio, and temperature) had on *Codonopsis pilosula* root extract (CPRE) antioxidant activity

Conclusion

In conclusion, the optimal extraction parameters (particle size, solvent concentration, solvent: material ratio, extraction temperature and duration) for improvements in the yield of TPC and AOA were successfully established via both the conventional approach and RSM-BBD modeling approach. For future development, possible methods of processing samples before extraction should be considered, such as the use of enzymes to break down plant cell walls and aid in release of desired substance. In addition, further studies in plant materials collecting and processing, including harvesting time, drying methods, storage, and assisting methods before extraction, are necessary to develop a comprehensive and effective manufacturing line from raw plants to products.

List of abbreviations

CPR: *Codonopsis pilosula* root
CPRE: *Codonopsis pilosula* root's extract
TPC: total phenolic content
AOA: antioxidant activity
ABTS: 2,2'-azinobis-(3-ethylbenzthiazolin-6-sulfonic acid)
AP: adequate precision
LOF: lack of fit

Competing interests

The authors declare no conflicts of interest.

Author contributions

Phu H. Le conceived the idea, provided support, and critically revised the manuscript. Phuong P. L. Tran carried out all experiments and wrote the manuscript. Phuc N.T. Le structured the contents and revised the manuscript. All authors read and approved the final manuscript.

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