# Evaluation of Antioxidant Activity and Chemical Profile of *Cudrania tricuspidata* Tree Pruning Extract Obtained by Optimized Microwave-Assisted Extraction

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The residue from pruning *Cudrania tricuspidata* trees is considered a rich source of energy and bioactive compounds. Recovering these compounds from *C. tricuspidata* tree pruning could help mitigate potential economic and environmental concerns. The primary aim of this study was to investigate the impact of extraction temperature, duration, and liquid-to-residue ratio on the antioxidant activity, total phenolic content, and total flavonoid content of *C. tricuspidata* tree pruning, using the response surface methodology. The results indicated that the microwave-assisted extraction temperature or microwave-assisted extraction time was the most crucial variable in the extraction process (Significance at p-value < 0.001 - 0.05 for antioxidant activity and chemical profile). It is worth mentioning that the optimal extraction conditions for achieving maximum 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation induction activity, total polyphenol content, and total flavonoid content were distinct from one another, necessitating specific optimization for each targeted characteristic. The optimized extraction conditions yielded 85.8% DPPH radical scavenging activity, 95.4% ABTS radical cation induction activity, 2.72 mg/g total polyphenol content, and 2.53 mg/g total flavonoid content. These results highlight the potential of *C. tricuspidata* as a valuable dietary source of phenolic antioxidants for human health.

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## INTRODUCTION

The presence of free radicals and reactive oxygen species is a matter of concern due to their extensively documented adverse effects on human health and their involvement in food quality deterioration. When cells use oxygen to generate energy, free radicals are created as a consequence of ATP (adenosine triphosphate) production by the mitochondria in the body. These by-products are generally reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) that result from the cellular redox process. Oxidative stress has been linked to over a hundred human diseases, characterized by an imbalance between prooxidants and antioxidants, resulting from an accelerated oxidation rate (Barrett *et al.* 2010; Ribon *et al.* 2016; Rathod *et al.* 2021).

Tree pruning residue is widely recognized as a biomass source and a significant repository of biologically active compounds with medicinal value. Typically, tree pruning

residue is rich in dietary fiber, making it an excellent source of antioxidants due to the accumulation of various secondary metabolites, including phenolic compounds (Haminiuk *et al.* 2012). Phenolics constitute a class of antioxidants commonly encountered in tree pruning residue, exhibiting redox properties that enable them to function as reducing agents, hydrogen donors, free radical scavengers, and singlet oxygen quenchers (Abacan *et al.* 2017).

*Cudrania tricuspidata* (*C. tricuspidata*) is a deciduous broad-leaved tree characterized by thorns that belong to the Moraceae family. It is widely distributed across East Asia, encompassing regions such as Korea, China, and Japan (Xin *et al.* 2017). *Cudrania tricuspidata* blooms in June and ripens between September and October. The bark of *C. tricuspidata* is grayish brown and has thorns whose length range from 0.5 to 3.5 cm. In addition, leaves of *C. tricuspidata* are split into three or have flat edges and are egg-shaped, both of which are found on a single tree (Lee *et al.* 2023). The characteristic of the *C. tricuspidata* is that it has a round head shape with fruits and flowers. The *C. tricuspidata* fruit is as hard as walnuts. It starts out as green, but as it ripens to red from September to October, the bitter taste disappears, and sweetness is increased (Jang *et al.* 2022). This tree is recognized by various names, including curing, mandarin melon berry, silkworm thorn, and arugula (in Japanese) (Ko *et al.* 2021), and this species is a fruit tree. *C. tricuspidata* tree pruning residue is a lignocellulosic material widely generated by the agriculture industries, as a consequence of the fruit market. Conversely, this waste represents a pollution hazard if discharged into the environment (Jeong *et al.* 2014). In contrast, *C. tricuspidata* tree pruning is economically underutilized. However, it has attracted a lot of attention due to its interesting properties and its possible use as a raw material to obtain value-added compounds (Xin *et al.* 2017; Lee *et al.* 2023). In fact, this by-product can be considered a rich energy source and bioactive compounds, and the recovery of these compounds from tree pruning could avoid the possible economic and environmental concerns (Song *et al.* 2022). In this context, some authors have proposed different alternative applications for valorizing this residue (Narayani and Srivastava 2017).

Various methods have been developed to extract bioactive components from natural products, including innovative technologies such as ultrasound-assisted extraction (Chen *et al.* 2018), enzyme-assisted extraction (Li *et al.* 2006), microwave-assisted extraction (Liu *et al.* 2010), and maceration (Coelho *et al.* 2019). Among these, microwave-assisted extraction is considered a green and efficient method (Pan *et al.* 2003). Compared to traditional extraction methods, microwave-assisted extraction offers benefits such as shorter extraction times and lower temperatures, which result in less degradation of heat-sensitive compounds (Xiao *et al.* 2012). Additionally, it has been shown that microwave-assisted extraction is compatible with water as a solvent, reducing the need for organic solvents (Flórez *et al.* 2015). Microwave-assisted extraction can also extract more medium- and non-polar organic compounds from plant materials (Yu *et al.* 2007).

During the microwave-assisted extraction process, several factors can affect the extraction efficiency, including extraction time and power, temperature, solvent composition, and solvent/material ratio (Zhao *et al.* 2018). These factors not only have direct effects on the extraction efficiency but also interact with one another. Therefore, it is important to evaluate the interaction effects of these factors.

The effectiveness of an extraction process can be influenced by numerous factors, including, but not limited to, the type and concentration of the solvent, extraction temperature, pH, storage duration, solvent-to-solid ratio, and particle size of the sample such as pruning residue (Mokrani and Madani 2016; Soliman *et al.* 2022; Alsalamah *et al.*

2023). Response surface methodology (RSM) is a potent mathematical and statistical tool for assessing the adequacy of empirical models, elucidating the relationships between response variables and independent factors, and exploring interactions among these factors. Furthermore, RSM facilitates the optimization of these factors that may impact process outcomes (Veza *et al.* 2023). The RSM is an invaluable approach for optimizing chemical and biochemical processes, surpassing the conventional one-factor-at-a-time method.

The research on the antioxidant activity of *C. tricuspidata* tree pruning in South Korea is currently limited, with only a few prior studies reporting on optimizing extraction conditions for its antioxidant potential. The present study focuses specifically on extraction time and temperature within the realm of various extraction conditions. This study is to employ the RSM approach to optimize both extraction time and temperature, to maximize the yield of antioxidant activity and total phenolic content from *C. tricuspidata* tree pruning.

## EXPERIMENTAL

### Sample and Extraction Process

The pruning residue from *C. tricuspidata* trees (South Korea National Agricultural Products Quality Management Agency No. 23 Certification Authority; Certification number: No. 16303207) was obtained from the Affiliated Experimental Forest, Gyeongsang National University, South Korea (Latitude: 35.2792585190325; Longitude: 127.829799010458). The tree was identified by Hee-Gon Kang of the Academic Forest. The samples were oven-dried at 45 °C for 24 h. The dried samples were finely ground into a powder using a miller equipped with a 2 mm mesh sieve

Samples were refrigerated at 4 °C until used for extraction. The powdered *C. tricuspidata* tree pruning (5 g) was used for microwave-assisted extraction with 50 mL of distilled water. The extraction process occurred under specific temperature and time conditions (Table 1), facilitated by a microwave. These temperature and time parameters were determined through an experimental design generated by RSM software (Statease Inc., Design Expert; version 13, Minneapolis, MN, USA). Following the initial microwave-assisted extraction, a secondary microwave-assisted extraction was conducted under identical conditions. Subsequently, both microwave-assisted extracts were combined and subjected to centrifugation at 4000 rpm for 10 min. The resulting supernatant was filtered through a Whatman No. 2 filter paper to obtain an extract, which was then used for antioxidant activity analysis.

**Table 1.** Coded Levels of Independent Variables Used in the RSM Design

| Symbols | Independent Variables of Extraction | Coded Levels | | |
|---|---|---|---|---|
| | | -1 | 0 | 1 |
| $X_1$ | Temperature (°C) | 30 | 40 | 50 |
| $X_2$ | Time (min) | 30 | 60 | 90 |
| $X_3$ | Liquid to Residue ratio (w/v) | 10 | 20 | 30 |

## DPPH Radical Scavenging Activity

The assessment of radical scavenging activity by antioxidants in the extract followed a method originally described by Ferreira *et al.* (2007) with slight modifications. To prepare the DPPH• solution, 5.9 mg of DPPH• was dissolved in 100 mL of ethanol. Then, precisely 3.8 mL of the DPPH• ethanolic solution was combined with 0.2 mL of *C. tricuspidata* tree pruning extract. The resultant mixture was vigorously shaken for 1.0 min and then allowed to rest at room temperature in the absence of light for 30 min. The absorbance was measured at 517 nm using a SpectraMax 190 spectrophotometer (Molecular Devices, LLC, USA), with all measurements conducted in triplicate. The calculation of radical scavenging activity was performed according to Eq. 1 as follows:

$$\text{DPPH radical scavenging activity (\%)} = (1- ABS_{sample}/ABS_{control}) \times 100 \quad (1)$$

## ABTS Radical Cation Inhibition Activity

The ABTS radical cation inhibition activity assessment of the *C. tricuspidata* tree pruning extract followed the method outlined by Lo and Cheung (2005), albeit with some modifications. To create the ABTS•+ reagent, 5 mL of a 7 mM ABTS•+ solution was mixed with 88 µL of 140 mM potassium persulfate ($K_2S_2O_8$). This mixture was transferred to an amber bottle and kept in darkness at room temperature for 12 h to ensure complete radical generation. After this 12 h incubation period, the absorbance of the ABTS•+ reagent was adjusted to 0.70 at 734 nm using 95% ethanol.

For the assay, 1.0 mL of the prepared ABTS•+ reagent was combined with 10 µL of the extract. Following this addition, the mixture was allowed to stand at room temperature for 6 min. Absorbance readings were taken at 734 nm using a SpectraMax 190 spectrophotometer, with all measurements conducted in triplicate. The calculation of radical inhibition activity was determined using Eq. 2 shown below:

$$\text{ABTS radical scavenging activity (\%)} = (1-ABS_{sample}/ABS_{control}) \times 100 \quad (2)$$

## Total Phenolic Content (TPC) Analysis

The TPC analysis was performed using the Folin–Ciocalteu method, as described by Blainski *et al.* (2013) and Zhao and Hall (2008), with slight adaptations. In this procedure, 1.0 mL of the sample was mixed with an equal volume of Folin–Ciocalteu's solution. After an incubation period of 3 min, 1.0 mL of a 7.5% sodium carbonate solution was added to the mixture, which was then adjusted to a final volume of 10 mL with deionized water. The mixture was subsequently left to stand at room temperature in a dark environment for 90 min. The determination of TPC involved measuring absorbance at 725 nm using a SpectraMax 190, with deionized water as the blank reference.

To create the calibration curve, gallic acid concentrations ranging from 50 to 1000 µg/mL were used ($R^2 = 0.99$). The analysis of gallic acid followed the same procedure as described above. The results of the analysis were expressed as mg gallic acid eq./g of the sample. All experiments were meticulously conducted in triplicate.

## Total Flavonoid Content (TFC) Analysis

To determine the total flavonoid content (TFC) in each extract, the authors employed the aluminum chloride colorimetry method as originally outlined by Kalita *et al.* (2013), with slight adjustments. In summary, the extract sample was diluted with methanol until a concentration of 100 µg/mL was achieved. To construct the calibration curve,

quercetin was serially diluted in methanol over a range of 0 to 100 µg/mL.

For the assay, 2.0 mL of either the diluted extract or quercetin solution was combined with 0.1 mL of a 10% (w/v) aluminum chloride solution and 0.1 mL of a 0.1 mM potassium acetate solution. This mixture was allowed to stand at room temperature for 30 min. Subsequently, the maximum absorbance of the mixture was measured at 415 nm using a UV–VIS spectrophotometer (SpectraMax 190, Molecular Devices, LLC, USA). The results were expressed as mg quercetin eq./g of the sample (mg Quercetin/g; $y = 0.007x + 0.0221$ ($R^2 = 0.9981$)). All experiments were conducted in triplicate.

## Experimental Design and Statistical Analysis

Before initiating the RSM study, the experimental ranges for independent variables were selected using a one-at-a-time variable approach—specifically, extraction time and temperature—utilizing total polyphenol, total flavonoid, and condensed tannin contents as pivotal determinants. This methodology corresponds to prior research conducted by Yim *et al.* (2012).

Subsequently, RSM was employed to determine the optimal levels of extraction time, temperature, and the liquid-to-residue ratio (w/v) while utilizing water as the extraction medium. These optimizations were performed concerning four response variables: DPPH• scavenging and ABTS•+ inhibition capacities, total phenolic content (TPC), and total flavonoid content (TFC) in the extracts obtained from *C. tricuspidata* tree pruning. For the RSM approach, the two factors—extraction temperature ($X_1$) and time ($X_2$)—along with the liquid-to-residue ratio ($X_3$)—were coded into five levels (-1, 0, 1). Both the coded and uncoded independent variables utilized in the RSM design are outlined in Table 1. The ranges for extraction time and temperature, including the central points, were determined based on preliminary experimental findings.

The experiments were designed following a Box-Behnken Design (BBD), comprising a 17 factorial design incorporating four factorial points, four axial points, and six central points (Table 2). This statistical experimental design approach is being increasingly used in different stages of fermentation optimization because it effectively tests multiple process variables with fewer experimental trials compared to the conventional "one-factor-at-a-time" method. The optimal ranges were determined based on prior experience. These experiments were executed randomly, and the resulting data were analyzed using multiple regressions employing the least-squares method. The response function (Y) was decomposed into linear, quadratic, and interactive components.

A reliable method for assessing the quality of the fitted model is through the application of analysis of variance (ANOVA). The fundamental concept behind ANOVA is to compare the variation attributed to the treatment (alteration in the combination of variable levels) with the variation arising from inherent random errors in measuring the generated responses. This comparison enables the assessment of the significance of the regression utilized to predict responses, considering the sources of experimental variance. The significance of each coefficient was determined by the p-value of the F-test ($p < 0.05$). Generally, higher Fisher's F-test values and lower p-values indicate the relative significance of each term. The quality of the fit of the polynomial model equations was expressed by the coefficients of determination ($R^2$).

**Table 2.** Experimental Design and Responses of the Dependent Variables to Extraction Conditions

| No. | Independent Variables | | | Dependent Variables (Responses) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Temp.[a], $X_1$ (°C) | Time, $X_2$ (min) | R/L [b], $X_3$ (w/v) | $Y_1$[c] | | $Y_2$ | | $Y_3$ | | $Y_4$ | |
| | | | | Act.[d] | Pred.[e] | Expt. | Pred. | Expt. | Pred. | Expt. | Pred. |
| 1 | 40 | 30 | 30 | 49.15[f] | 51.84 | 78.28 | 75.09 | 1.18 | 0.99 | 0.40 | 0.29 |
| 2 | 40 | 60 | 20 | 85.53 | 84.09 | 93.50 | 93.82 | 2.70 | 2.71 | 2.70 | 2.37 |
| 3 | 40 | 90 | 10 | 77.22 | 74.53 | 85.99 | 89.19 | 1.36 | 1.56 | 1.61 | 1.72 |
| 4 | 40 | 60 | 20 | 85.22 | 84.09 | 95.13 | 93.82 | 2.72 | 2.71 | 1.03 | 2.37 |
| 5 | 40 | 60 | 20 | 81.26 | 84.09 | 91.76 | 93.82 | 2.72 | 2.71 | 2.72 | 2.37 |
| 6 | 40 | 30 | 10 | 59.22 | 57.04 | 82.03 | 80.68 | 1.18 | 1.05 | 1.39 | 1.07 |
| 7 | 50 | 60 | 10 | 59.11 | 58.83 | 88.82 | 89.31 | 1.32 | 1.26 | 1.32 | 1.45 |
| 8 | 40 | 60 | 20 | 84.32 | 84.09 | 95.02 | 93.82 | 2.71 | 2.71 | 2.71 | 2.37 |
| 9 | 30 | 60 | 10 | 40.20 | 45.36 | 81.37 | 79.03 | 0.65 | 0.64 | 0.74 | 0.80 |
| 10 | 30 | 90 | 20 | 45.70 | 43.24 | 82.01 | 81.15 | 1.10 | 0.92 | 1.10 | 0.92 |
| 11 | 40 | 90 | 30 | 48.26 | 50.45 | 89.90 | 91.25 | 1.61 | 1.75 | 0.46 | 0.77 |
| 12 | 50 | 60 | 30 | 44.66 | 39.50 | 87.28 | 89.62 | 1.32 | 1.33 | 0.52 | 0.45 |
| 13 | 40 | 60 | 20 | 84.12 | 84.09 | 93.69 | 93.82 | 2.72 | 2.71 | 2.72 | 2.37 |
| 14 | 50 | 90 | 20 | 43.92 | 46.90 | 88.44 | 84.75 | 2.32 | 2.18 | 2.35 | 2.10 |
| 15 | 50 | 30 | 20 | 41.50 | 43.97 | 80.31 | 81.17 | 0.72 | 0.91 | 0.69 | 0.87 |
| 16 | 30 | 60 | 30 | 35.15 | 35.43 | 75.68 | 75.19 | 0.65 | 0.71 | 0.21 | 0.07 |
| 17 | 30 | 30 | 20 | 33.06 | 30.08 | 56.37 | 60.06 | 0.79 | 0.93 | 0.78 | 1.02 |

[a] Temperature
[b] Residue/Liquid ratio
[c] $Y_1$: DPPH radical scavenging (%); $Y_2$: ABTS radical scavenging (%); $Y_3$: total polyphenol content (GAE mg/g); $Y_4$: total flavonoid content (QE mg/g)
[d] Actual
[e] Predicted
[f] expressed as means

## RESULTS AND DISCUSSION

### Fitting the Models

The summaries of DPPH• scavenging activity ($Y_1$), ABTS•+ inhibition ($Y_2$), total polyphenol content ($Y_3$), and total flavonoid content ($Y_4$) are presented in Table 2. The experimental data were fitted to second-order polynomial equations, and then the regression coefficients were calculated. The significance of these coefficients within the models was evaluated using analysis of variance (ANOVA), and the results are showcased in Table 3.

To assess the adequacy of the model in fitting the experimental data, a lack-of-fit test was performed. Remarkably, the ANOVA results for the lack-of-fit tests on all response variables were insignificant ($p > 0.05$), suggesting that the models adequately represented the experimental data. It is important to note that larger regression coefficients in models with significant p-values indicate more pronounced effects on the corresponding response variables (Xu *et al.* 2022). For the models concerning DPPH• scavenging activity and ABTS response, square root transformations were applied. This adjustment aimed to achieve a more accurate fit for these models based on robust statistical considerations. The transformations were conducted with a constant of zero to normalize the standard distribution of data (Dijkstra and Henseler 2015). The analysis results reveal that both temperature and time exhibited significant linear and quadratic effects on all responses

(except for total flavonoid content). Conversely, the liquid-to-residue ratio displayed a significant linear effect solely on DPPH• scavenging activity ($p < 0.01$). Moreover, the coefficients of multiple determinations ($R^2$) for DPPH• scavenging activity, ABTS•+ inhibition, TPC, and TFC were calculated as $R^2 = 0.9809$, $R^2 = 0.9508$, $R^2 = 0.9791$, and $R^2 = 0.7986$, respectively. These values indicate that the second-order polynomial models effectively represented the respective experimental data.

**Table 3.** Regression Coefficients of the Predicted Second-Order Polynomial Models for Antioxidant Activities, Total Polyphenol Content, and Total Flavonoid Content

| | Regression Coefficients[a] | | | |
|---|---|---|---|---|
| | $Y_1$ | $Y_2$ | $Y_3$ | $Y_4$ |
| Intercept | 84.09 | 93.82 | 2.71 | 2.38 |
| $X_1$: Extraction temperature | 4.39* | 6.18*** | 0.3104** | 0.2579 |
| $X_2$: Extraction time | 4.02* | 6.17*** | 0.3160** | 0.2838 |
| $X_3$: Extraction liquid to residues | -7.32** | -0.8847 | 0.0313 | -0.4335 |
| $X_1 X_2$ | -2.56 | -4.38* | 0.3222** | 0.3317 |
| $X_1 X_3$ | -2.35 | 1.04 | 0.0000 | -0.0668 |
| $X_2 X_3$ | -4.72 | 1.91 | 0.0627 | -0.0416 |
| $X_1^2$ | -28.36*** | -8.90*** | -0.9147*** | -0.7078 |
| $X_2^2$ | -14.68*** | -8.14*** | -0.5643*** | -0.4379 |
| $X_3^2$ | -10.95*** | -1.63 | -0.8120*** | -0.9715 |
| Mean | 58.68 | 85.04 | 1.63 | 1.38 |
| Standard deviation | 4.12 | 3.20 | 0.1791 | 0.6223 |
| $R^2$ | 0.9809 | 0.9508 | 0.9791 | 0.7986 |
| Adjusted $R^2$ | 0.9563 | 0.8875 | 0.9521 | 0.5395 |
| Coefficient of variation | 7.02 | 3.76 | 10.96 | 45.12 |
| F-value (model) | 39.89 | 15.02 | 36.36 | 3.08 |
| F-value (lack of fit) | 12.51 | 11.35 | 2466.04 | 0.2657 |
| *p*-value (model) | <0.0001 | 0.0009 | <0.0001 | 0.0059 |
| *p*-value (lack of fit) | 0.2168 | 0.3200 | 0.1801 | 0.8475 |

[a]$Y_1$: DPPH radical scavenging; $Y_2$: ABTS radical cation inhibition activity; $Y_3$: total polyphenol content; $Y_4$: total flavonoid content
*< 0.05; <0.01; <0.001

## Response Surface Analysis of DPPH• Scavenging Activity

The RSM of the experimental data outlined in Table 2 indicates that extraction temperature ($p < 0.05$), extraction time ($p < 0.05$), and liquid-to-residue ratio ($p < 0.01$) each exerted quadratic effects on DPPH• scavenging activity, demonstrating robust regression coefficients. Equation 3 precisely defines the relationship between DPPH• scavenging activity and the extraction parameters as follows:

$$-541.32626 + 24.11079A + 2.74716B + 5.53105C - 0.008521AB - 0.023500AC - 0.015739BC - 0.283637A^2 - 0.016312B^2 - 0.109463C^2 \quad (3)$$

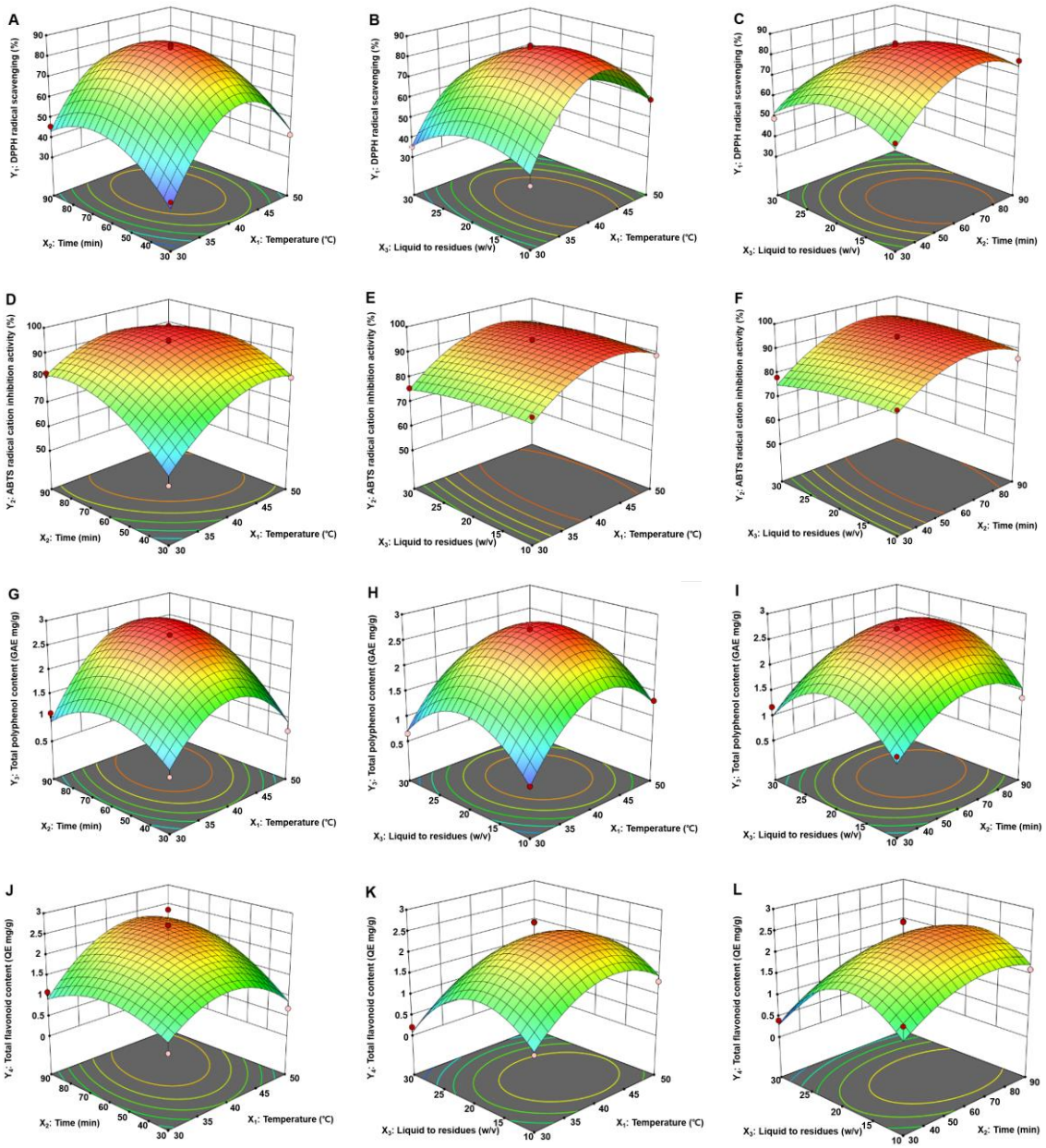


**Fig. 1.** Response surface plot of the DPPH radical scavenging (A to C), ABTS radical cation inhibition activity (D to F), total polyphenol content (G to I), total flavonoid content (J to L) as a function of extraction condition. A, D, G, J: as extraction temperature and time condition; B, E, H, K: as extraction temperature and liquid to residues; C, F, I, L: as extraction time and liquid to residues

Extraction temperature ($p < 0.05$) and extraction time ($p < 0.05$) showed significant positive linear effects. Additionally, both extraction time and temperature exhibited negative quadratic effects on DPPH• scavenging activity ($p < 0.01$), while no significant interaction effect was observed between extraction time and temperature (Table 3). Concerning the liquid-to-residue ratio, it displayed a significant negative linear effect and exerted a negative quadratic effect on DPPH• scavenging activity ($p < 0.001$), with no significant interaction effect observed with extraction temperature or time.

Figure 1 shows the response surface plot illustrating the impact of extraction time, temperature, and liquid-to-residue ratio on DPPH• scavenging activity. Especially, DPPH• scavenging activity showed an increasing trend as extraction time increased from 30 to 60 min. However, the activity was kept constant at over 60 min and showed a slight decrease with further increases of time. Similarly, DPPH• scavenging activity showed a decrease at 40 ℃. Therefore, to achieve a high DPPH• scavenging activity, extraction temperature and extraction time played an important role. Generally, the highest DPPH• scavenging activity could be attained through extraction at low temperatures (30 ℃ to 40 ℃) for a duration spanning 30 to 40 min. Yang *et al.* (2009) reported that at lower temperatures, the mass transfer rate is reduced, requiring more time for crocin in the raw materials to dissolve into the solution.

According to the RSM, the maximal predicted DPPH• scavenging activity was 85.8% under optimal extraction conditions, which included an extraction time of 69.0 min, an extraction temperature of 40.7 ℃, and a liquid-to-residue ratio of 16.6. Lee *et al.* (2007) showed 5.18% and 4.16% DPPH antioxidant activity in the stem bark and root of *C. tricuspidata*, respectively. Therefore, the extraction method proposed in this study is judged to be a better way to increase the DPPH antioxidant activity of *C. tricuspidata*.

## Response Surface Analysis of ABTS•+ Inhibition Activity

The RSM applied to the experimental data, as presented in Table 2, indicates that both extraction temperature ($p < 0.001$) and extraction time ($p < 0.001$) demonstrated a linear effect on ABTS•+ inhibition activity, supported by robust regression coefficients. The analysis reveals that both extraction temperature ($p < 0.001$) and extraction time ($p < 0.001$) exerted a quadratic effect on ABTS•+ inhibition activity, also substantiated by strong regression coefficients. The relationship between the extraction parameters and ABTS•+ inhibition activity is mathematically expressed in Eq. 4 as follows:

$$-141.97295 + 8.40520A + 1.74652B - 0.234458C - 0.014591AB + 0.010391AC + 0.006380BC - 0.088998A^2 - 0.009041B^2 - 0.016311C^2 \quad (4)$$

Extraction temperature and time both demonstrated significant linear positive effects ($p < 0.001$) on ABTS•+ inhibition, while also exhibiting significant linear negative effects ($p < 0.001$) on this activity. Furthermore, a significant interaction effect between extraction time and temperature was observed (Table 3; $p < 0.05$). The response surface plot in Fig. 1 illustrates the impact of extraction time and temperature on ABTS•+ inhibition. The ABTS•+ inhibition activity increased from 240 to 340 min and then it was kept constant. It was evident that as the extraction temperature rose from 30 to 40 ℃, the inhibition activity substantially increased (Fig. 1).

Xu *et al.* (2008) suggested that points representing possible combinations of the lowest factor levels within the optimal zone would be preferable over other combinations for practical cost-saving. Thus, it was considered important to achieve the highest ABTS•+ inhibition under moderate conditions of time (ranging from 30 to 60 min) and temperature (30 to 40 ℃). According to the predictive model, the optimal extraction conditions for maximal ABTS•+ inhibition activity were estimated as an extraction time of 70.7 min, a temperature of 43.1 ℃, and a liquid-to-residue ratio of 23.9, resulting in a maximal ABTS•+ inhibition activity of 95.4%. Jeong *et al.* (2009) reported that leaves, stems, roots, and fruits of *C. tricuspidata* all exhibited ABTS activity of 10% or less. Therefore, the

present work has demonstrated that microwave-assisted extraction is effective in increasing the antioxidant activity of ABTS in *C. tricuspidata*.

## Response Surface Analysis of Total Phenolic Content

The response surface analysis presented in Table 2 indicates that the relationship between TPC and extraction parameters is quadratic, supported by a strong regression coefficient ($R^2 = 0.9791$). Equation 5 below represents this relationship:

$$-16.53601 + 0.698353A + 0.038642B + 0.315394C + 0.001074AB + 1.55738E - 18AC + 0.000209BC - 0.009147A^2 - 0.000627B^2 - 0.008120C^2 \quad (5)$$

Extraction temperature and time both displayed significant linear positive effects ($p < 0.01$) on total phenolic content. Conversely, extraction temperature and time exhibited significant linear negative effects ($p < 0.001$) on this content. Additionally, a significant interaction effect between extraction time and temperature was observed (Table 3; $p < 0.01$). Figure 1 illustrates the response surface plot, detailing the impact of extraction time and temperature on TPC. The TPC was observed to increase at lower extraction temperatures and with moderate extraction times. There was a linear decrease in TPC from 40 min to 50 min. A similar trend was reported by Moyo *et al.* (2003), who stated that the more linear the response, the smaller the magnitude of interactions that occur. Increased extraction temperature led to an increase in TPC from 30 °C to 40 °C; beyond 40 °C, further increases in temperature resulted in a decrease in TPC. In summary, TPC increased with shorter extraction times (ranging from 30 to 60 min) and moderate extraction temperatures (30 to 40 °C). According to the predictive model derived from response surface analysis, the maximum TPC was estimated as 2.72 mg gallic acid/g, with optimal extraction conditions including an extraction time of 80.3 min, an extraction temperature of 43.0 °C, and a liquid-to-residue ratio of 22.2. Jeong *et al.* (2009) analyzed the phenolic content using the same gallic acid as in our study and found 73, 57, 70, and 56 mg/g in leaves, stems, roots, and fruits of *C. tricuspidata*, respectively. This was a higher level of phenolic content than in our study, which may be a result of the different natural habitats and cultivation environments of *C. tricuspidata*.

## Response Surface Analysis of Total Flavonoid Content

The RSA (Table 2) demonstrated a relatively low regression coefficient ($R^2 = 0.7986$) and Eq. 6 shows the relationship between TFC and extraction parameters of extraction time and temperature:

$$-13.36639 + 0.539041A + 0.026401B + 0.380289C + 0.001106AB - 0.000668AC - 0.000139BC - 0.007078A2 - 0.000487B2 - 0.009715C2 \quad (6)$$

There was no significant interaction effect for both linear and quadratic negative effects (Table 3). The response surface plot (Fig. 1) illustrates the impact of extraction time and temperature on TFC. It is evident that TFC increased with an increase in extraction time from 30 to 60 min but decreased beyond 60 min as the extraction temperature rose. Similarly, increasing the extraction temperature from 30 °C to 40 °C promoted a higher TFC level. However, from 30 to 40 °C, the TFC level experienced a slight decline with an increase in extraction time.

Extraction time and temperature are critical parameters to optimize to minimize the energy cost of the process. The results indicate that the highest TFC yield was achieved

with a moderate extraction time of approximately 300 to 320 min and a moderate temperature range of 30 to 40 °C. An increase in working temperature favors extraction by enhancing the solubility of the solute and the diffusion coefficient. However, beyond a certain point, phenolic compounds may become denatured, leading to reduced compound stability due to chemical and enzymatic degradation or losses *via* thermal decomposition. This mechanism has been the primary cause of the reduction in polyphenol content in onions, as reported by Kiassos *et al.* (2009). Additionally, phenols can react with other plant components, hindering their extraction. In this result, extraction time becomes significant, as longer extraction periods may result in more extensive polyphenol losses.

It is reported that the presence of phenolic compounds in edible wild mushrooms is a major contributor to their antioxidant activity (Barros *et al.* 2007). Phenolic compounds such as turpentine, found in *Pinus nigra* and associated with antioxidant activity, are believed to play a crucial role in lipid stabilization (Gülçin *et al.* 2004). Moreover, significant correlations have been reported between total phenolics and free radical scavenging activity in buckwheat (Sun and Ho 2005) and medicinal plant extracts (Djeridane *et al.* 2006).

Consequently, in this study, the optimal extraction time and temperature for maximizing the yield of TFC in *C. tricuspidata* tree pruning were determined. The predicted optimal extraction conditions include an extraction time of 73.47 min, a temperature of 43.0 °C, and a liquid-to-residue ratio of 17.6. According to the RSM, the maximum predicted TFC value was 2.53 mg quercetin eq./g.

## Verification of Predictive Model

A verification step was undertaken to ensure that the predicted results were not biased toward practical values, with the objective of achieving the maximum yield using the deduced optimal conditions. Table 4 presents the suitability of the model equation for predicting the maximum responses, which was verified using optimal extraction conditions for each respective response. Four separate verification experiments were conducted to assess DPPH• scavenging activity ($Y_1$, %), ABTS•+ inhibition ($Y_2$, %), TPC ($Y_3$, mg gallic acid eq./g), and TFC ($Y_4$, mg quercetin eq./g) under the respective optimal extraction conditions within the experimental range.

**Table 4.** Experimental Data of the Verification of Predicted Extraction Parameters

| Dependent Variables[a] | Extraction Temperature (°C) | Extraction time (min) | Extraction Liquid to Residues (w/v) | Predicted Value | Experimental Value | Difference % (CV) |
|---|---|---|---|---|---|---|
| $Y_1$ (%) | 40.69 | 68.98 | 16.63 | 85.85 | 84.38 | 0.22 |
| $Y_2$ (%) | 43.11 | 70.66 | 23.88 | 95.36 | 65.41 | 0.83 |
| $Y_3$ (mg/g) | 43.00 | 80.30 | 22.20 | 2.72 | 2.88 | 0.06 |
| $Y_4$ (mg/g) | 42.99 | 73.47 | 17.57 | 2.53 | 2.59 | 0.54 |

[a]$Y_1$: DPPH radical scavenging; $Y_2$: ABTS radical cation inhibition activity; $Y_3$: total polyphenol content; $Y_4$: total flavonoid content

The experimental values obtained were as follows: 84.38% ($Y_1$), 65.41% ($Y_2$), 2.88 mg gallic acid/g ($Y_3$), and 2.59 mg quercetin/g ($Y_4$). These values closely matched the predictions made by the regression models, with coefficient of variation (CV) values ranging from 0.06% to 0.83%.

## CONCLUSIONS

1. The observation and prediction of the effects of extraction conditions on antioxidant capacities (including 2,2-diphenyl-1-picrylhydrazyl (DPPH•) scavenging activity and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+) inhibition activity), total phenolic content (TPC), and total flavonoid content (TFC) from *C. tricuspidata* tree pruning were effectively conducted using response surface methodology (RSM).
2. The optimal extraction conditions for each of the four responses were efficiently determined and predicted through a graphical optimization method combined with RSM's desirability function.
3. The experimental values obtained based on the optimized extraction parameters closely matched the predicted values.
4. This study provided valuable insights into optimizing the extraction conditions (including temperature, time, and liquid-to-residue ratio) to achieve the maximum yield of antioxidant activity from *C. tricuspidata* tree pruning.
5. The highest antioxidant activities were achieved under the following optimized conditions: DPPH• scavenging activity: 40.7 ℃, 69.0 min, and 16.6 liquid-to-residue ratio; ABTS•+ inhibition activity: 43.1 ℃, 70.7 min, and 23.9 liquid-to-residue ratio; Total polyphenol content (TPC): 43.0 ℃, 80.3 min, and 22.2 liquid-to-residue ratio; Total flavonoid content (TFC): 43.0 ℃, 73.5 min, and 17.6 liquid-to-residue ratio.
6. Further research, such as the purification and identification of antioxidative compounds present in *C. tricuspidata* tree pruning extracted under these optimal conditions, is currently underway to identify the specific compounds contributing to the antioxidative capacity.

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### Data Availability

All datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Authors' Contributions

Si Young Ha, Ji Young Jung, Jung Myoung Lee, and Jae-Kyung Yang contributed equally to this work.

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