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Optimization of Caffeic Acid Extraction from *Dendropanax morbifera* Leaves Using Response Surface Methodology and Determination of Polyphenols and Antioxidant Properties

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Abstract: The aim of this study was to optimize the extraction method for caffeic acid from the leaves of *Dendropanax morbifera* using response surface methodology (RSM), and subsequently determine the polyphenolic content and antioxidant capacity of the *D. morbifera* leaves extracts. The extraction process considered operating variables such as solvent concentration, extraction temperature, and extraction time, which were optimized via Box–Behnken experimental design. In addition, the antioxidant capacity was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The optimal extraction conditions of 41.23% (*v/v*) methanol concentration, at 88.61 °C, and 1.86 h produced a maximum caffeic acid (CA) yield of 20.35 mg/g. Additionally, total polyphenols, total tannins, and total flavonoids contents in the leaves extracts of *D. morbifera* were 32.48 ± 2.05 mg GAE (gallic acid equivalents)/g, 17.61 ± 2.61 mg GAE/g, and 9.14 ± 0.17 mg QE (quercetin equivalents)/g, respectively. The results showed that methanol extracts exhibited an IC50 of approximately 14.3 mg AAE (ascorbic acid equivalent)/g. The results suggested that the extracts of *D. morbifera* leaves contain potential antioxidant activity, and could be a good source of functional food and used in medicinal applications.

Keywords: antioxidant; caffeic acid; *Dendropanax morbifera*; extraction; polyphenols; response surface methodology

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1. Introduction

Dendropanax morbifera, a special tree species in South Korea that belongs to the family *Araliaceae* [1], has been used in folk medicine to treat migraine and dysmenorrhea, and remove wind-dampness [2]. Recently, several studies on the medical effects of *D. morbifera*, such as hypertension [3,4], anti-oxidation [5,6], anti-cancer [1], memory enhancement [1,7], and skin whitening [8] have been reported.

The antioxidants have been used in food, cosmetics, and pharmaceutics; among them, phenolic acids are major plant compounds that possess antioxidant capacity [9]. Caffeic acid (CA) and chlorogenic acid are representative phenolic acids in *D. morbifera* [10]. Previous studies have revealed the antioxidant capacity [11–13], antiviral [14], anti-inflammation [15], anti-cancer [16,17], and neuroprotective [12,18] effects of CA. Chlorogenic acid exhibits antioxidant and DNA-protective activities [19], along with anti-inflammation [20], anti-cancer [21], antibacterial, and anti-biofilm activities [22]. Therefore, the antioxidant capacity of phenolic acids from *D. morbifera* has been extensively reported [10,23].

The response surface methodology (RSM) is a mathematical and statistical technique, and it is used to design experiments and optimize responses that are affected by multiple independent variables [24]. The optimization of extraction conditions by a traditional

single-factor has some problems such as time-consuming and labor-intensive and is easy to ignore the interaction of various factors, whereas that by RSM can be obtained information about the interaction between variable factors and multiple responses at the same time [25,26]. Therefore, RSM has been widely used in various fields such as physical science and engineering, food science, social science, and biological and chemical sciences [27]. Especially, it has been applied to determine the optimal extraction condition of polyphenols from plants [28–31]. The optimal extraction method for CA, which is predominant in plants, has been established for *Melissa officinalis* L. [30], *Cecropia glaziovii* [32], olive [33], and tobacco [34]. However, to the best of our knowledge, no information is available to date on the optimal extraction of CA content from *D. morbifera* leaves using RSM. Therefore, the purpose of this study is optimizing the operating conditions to extract CA from *D. morbifera* leaves using RSM to maximize its yield. In addition, the antioxidant capacity of *D. morbifera* leaves extracts obtained from the optimal extraction conditions were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.

2. Materials and Methods

2.1. Materials

The dried *D. morbifera* leaves were purchased from Nemo Food (Busan, South Korea). The leaves were ground using mortar and pestle, and passed through a 0.5 mm mesh sieve screen, and subsequently preserved at −60 °C until further use. The chemicals including caffeic acid, methanol, acetic acid, gallic acid, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma (St. Louis, MO, USA), and all other reagents and solvents were of analytical grade and used directly.

2.2. Extraction of D. morbifera Leaves

In single factor test, the solvent extraction process of *D. morbifera* leaves were optimized by comparing solvents such as methanol, ethanol, and water. Based on the results, methanol was selected as the extraction solvent in this study (Data not shown). *D. morbifera* leaves (1 g) were ground and extracted with 20 mL of different concentrations of aqueous methanol at varying temperatures and times. Furthermore, CA extraction from *D. morbifera* leaves was optimized by considering methanol concentration, extraction time, and temperature as the selected variables for RSM as described in the subsequent section.

After extraction, the extracts were centrifuged at 10,000 rpm for 10 min and filtered through 0.22 μm syringe filter (hydrophilic PTFE, Advantec, Dublin, CA, USA), and maintained at −20 °C prior to high-performance liquid chromatography (HPLC) analysis. The entire extraction procedure for each extraction condition was performed in triplicate.

2.3. HPLC Analysis

For CA analysis, 10 μL of the extract was injected into the HPLC coupled with an LC-20AD pump, an SPE-M20A diode array detector, a CTO-20A oven, a CBM-20A controller, and an SIL-20A autosampler (HPLC; Simazhu, Kyoto, Japan). A Symmetry C18 column (5 μm, 3.9 × 150 mm, Waters, Milford, MA, USA) was used to separate CA at 30 °C. The HPLC analysis was performed using isocratic elution method with 100% methanol and 0.5% acetic acid (*v/v* = 1:1) as mobile phase for 20 min with a flow rate of 1 mL/min, and CA was detected at 330 nm.

2.4. Response Surface Methodology

According to the principle of Box–Behnken center combination test, three factors were entered into Design-Expert 12 software to fit and optimize the method to extract CA from the leaves of *D. morbifera*. Methanol concentration (20–60%; 20, 40, and 60%), extraction time (1–3 h; 1, 2, and 3 h), and extraction temperature (80–100 °C; 80, 90, and 100 °C) were considered as independent variables, and were expressed in their coded forms as −1, 0, and 1, which represented low, center, and high levels of each independent variables, respectively. The software designed 15 experiments with CA yield as an index. The test

factors and levels are shown in Tables 1 and 2. Finally, multiple regression fitting was performed using Design-Expert 12.0 software [31].

Table 1. Factor levels and design matrix in the Box–Behnken central composite design model.

Independent Variable	Solvent Concentration (%)	Extraction Time (h)	Temperature (°C)
−1	20	1	80
0	40	2	90
1	60	3	100

Table 2. Box–Behnken design for the three independent variables (coded) for CA extraction from *D. morbifera* leaves.

Run Order	Solvent Concentration	Extraction Time	Extraction Temperature
1	0	−1	1
2	1	0	1
3	1	1	0
4	1	−1	0
5	0	0	0
6	−1	−1	0
7	−1	0	−1
8	0	−1	−1
9	0	0	0
10	0	0	0
11	0	1	−1
12	−1	0	1
13	0	1	1
14	1	0	−1
15	−1	1	0

2.5. Determination of Polyphenol Content and Antioxidant Capacity

2.5.1. Determination of the Content of Polyphenols

In this study, the contents of total polyphenols (TP), soluble tannins (TET), and total flavonoids (TFlav) in *D. morbifera* leaves were determined.

Folin–Ciocalteu method was used to determine TP and TET [35]. To estimate TP, ethanol extract (16 µL) and Folin–Ciocalteu reagent (60 µL) were mixed for 5 min, and subsequently treated with 60 µL sodium carbonate solution (60 g/L) and incubated at 25 °C for 90 min. After the reaction, the absorbance value of the mixture was measured at 725 nm, with methanol extract as blank using a microplate reader (1550, Thermo Fisher, Waltham, MA, USA). To determine TET [36], 110 mg of polyvinylpolypyrrolidone was added to 1 mL of the extract, mixed thoroughly, and centrifuged at 3000× *g* for 10 min at 4 °C to precipitate tannins. The tannin content was calculated using the following Equation (1):

$$\text{TET} = \text{TP} - \text{TP in supernatant}, \quad (1)$$

TP and TET content are expressed as milligrams of gallic acid equivalents per gram of the extract (mg GAE/g).

TFlav content was determined according to the method described by Roshanak [37]. Briefly, 20 µL of the extract was mixed with 6 µL $NaNO_2$ (5%) and 80 µL ddH_2O, and incubated for 6 min, thereafter treated with 6 µL $AlCl_3$ solution (10%), which was again incubated for 6 min, to which 80 µL NaOH solution (4%) was added subsequently. After incubating the mixture at 25 °C for 30 min in dark, the absorbance was measured at 510 nm using a microplate reader (1550, Thermo Fisher). Results are expressed as milligrams of quercetin equivalents per gram of the extract (mg QE/g).

2.5.2. Scavenging Effect on DPPH

DPPH radical scavenging activity was performed according to a method previously described with minor modifications [38]. Sample solutions (50 mg/mL) were diluted to different concentrations with 50% methanol. DPPH solution (100 µL) was mixed with sample solutions (100 µL) of different concentrations and incubated at 25 °C for 30 min in dark. The absorbance values of the mixture were measured at 518 nm and converted into percentage antioxidant activity (AA) using the following Equation (2):

$$AA\% = 100 - [(Ab\ sample - Ab\ blank \times 100)\ / Ab\ control], \quad (2)$$

where 100 µL of 50% methanol and 100 µL of sample extract solutions were used as blank; while, control is mixed with 100 µL of 50% methanol and 100 µL DPPH. The antioxidant IC50 value of *D. morbifera* leaves was analyzed using SPSS (IBM SPSS Statistics 25).

3. Results and Discussion

3.1. Box–Behnken Center Combination of RSM

The CA extraction conditions were optimized using Box–Behnken design of RSM. The central test design and the results are shown in Table 3.

Table 3. Central composition design and response values of the CA yield of the *D. morbifera* leaves extracts.

Run Order	Solvent Concentration (%, *v*/*v*)	Extraction Time (h)	Extraction Temperature (°C)	Actual Value (mg/g)	Predicted Value (mg/g)
1	40	1	100	17.64	18.02
2	60	2	100	16.44	16.32
3	60	3	90	16.23	16.73
4	60	1	90	18.48	18.23
5	40	2	90	20.13	20.35
6	20	1	90	18.16	17.66
7	20	2	80	17.04	17.17
8	40	1	80	17.23	17.61
9	40	2	90	20.35	20.35
10	40	2	90	20.56	20.35
11	40	3	80	18.32	17.94
12	20	2	100	17.62	17.75
13	40	3	100	16.08	15.70
14	60	2	80	18.85	18.73
15	20	3	90	16.92	17.17

CA yield ranged from 16.08 to 20.56 mg CA/g depending on the extraction conditions. The Equation (3) of ternary quadratic polynomial regression model was obtained by analyzing the CA (Y) yield of the *D. morbifera* leaves extract versus the considered independent variables: methanol concentration (A), extraction time (B), and extraction temperature (C).

$$Y = 20.35 + 0.032A - 0.50B - 0.46C - 0.25AB - 0.75AC - 0.66BC - 1.36A^2 - 1.53B^2 - 1.49C^2, \quad (3)$$

The positive and negative coefficients of the quadratic term in the equation reflect their influence [39]; a positive coefficient indicates a positive correlation with the response value, while a negative coefficient indicated negative correlation. Since the quadratic coefficient of independent variables was all negative, it was inferred that the paraboloid represented by the equation was open downwards, and therefore had a maximum value point, which was consistent with the practical significance, and thus could be optimized and analyzed. The model was examined by a significant variance analysis.

The regression equation lack-of-fit test was not significant, indicating that the unknown factors interfered slightly with the test results (Table 4).

Table 4. Analysis of variance of RSM for CA yield.

Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	*p*-Value Prob > F	Significant
Model	28.54	9	3.17	11.68	0.0073	**
A-Solvent	0.00845	1	0.00845	0.031	0.8669	
B-Time	1.96	1	1.96	7.22	0.0435	*
C-Temperature	1.67	1	1.67	6.17	0.0556	
AB	0.26	1	0.26	0.94	0.3770	
AC	2.24	1	2.24	8.23	0.0350	*
BC	1.76	1	1.76	6.47	0.0517	
A^2	6.88	1	6.88	25.32	0.0040	**
B^2	8.7	1	8.7	32.03	0.0024	**
C^2	8.25	1	8.25	30.38	0.0027	**
Residual	1.36	5	0.27			
Lack of Fit	1.27	3	0.42	9.12	0.1004	
Pure Error	0.092	2	0.046			
Core Total	29.9	14				
R-Squared					value: 0.9546	

* Indicates significant at the $p < 0.05$ level, ** indicates extremely significant at the $p < 0.01$ level.

The fitting test was extremely significant, with the R-squared (R^2) value reaching 0.95, indicating that the empirical model fits well with the actual data, and better reflected the relationship between the CA yield and the extraction time, extraction temperature, and methanol concentration. Therefore, the obtained regression equation satisfactorily predicted the variations of CA extraction rate with different parameters.

3.2. Analysis of Response Surface Test Results

The corresponding response surface graph and contour graph generated according to the regression model explored various factors influencing CA yield. The optimal level for each factor was determined in the area near the vertex of the response surface. In the response surface graph, the steepness of the slope reflects the influence of the degree of change of processing factors on the response value. The steeper the slope, the greater the influence; in contrast, smoother slope indicates less influence. The level of influence of the interaction between the two factors on the response value could also be determined by the shape of the contour line. An ellipse indicates significant interaction between the two factors, and a circle indicates that the interaction was not significant [40,41]. The magnitude of the influence was reflected by the vertical distance to the same point in the contour.

As shown in Figures 1A, 2A and 3A, the response surface graphs of this experiment are all open downwards; the three constraints and the extraction rate exhibit a parabolic relationship, and the maximum value appears within the experimental range. In the case of controlling the temperature (90 °C), we analyzed the combined influence of different solvents and time of CA yield. As shown in Figure 1A, a gradual increase in methanol content increased the extraction time, which gradually increased the extraction rate of CA. At solvent concentration of 40.53% and time 1.84 h, the CA yield reached the maximum value at 20.39 mg CA/g. A continued increase in methanol concentration and time decreased the extraction rate of CA. These results imply that the polarity of the aqueous methanol solution should be optimized to ensure effective CA extraction. In previous research, CA was observed to decompose after prolonged heat treatment [23]. As shown in Figure 1B, the practically circular contour line indicates that the interaction between methanol concentration and time is not significant.

As shown in Figure 2A, when the extraction time is set to 2 h, a maximum value of 20.39 mg CA/g was obtained at 41.15% methanol concentration and 88.32 °C. These findings indicate that optimal methanol concentration and temperature are essential for efficient extraction of CA due to the polarity of the solvent and degradation at high tem-

perature. Figure 2B shows elliptical contours indicating a significant interaction between methanol concentration and temperature.

From Figure 3A, methanol concentration fixed at 40% yielded 20.41 mg CA/g at 1.87 h extraction time and 88.76 °C. An increase in temperature can lead to a decrease in solvent viscosity and an increase in solvent permeability, which has a significant impact on the mass transfer process. At the same time, the higher temperature makes the plant cell structure more degraded, thus making the cells more permeable [42]. However, a mechanistic route of caffeic acid decomposition over 90 °C conditions has been reported by Andueza et al. [43]. This is consistent with our results. These results indicate that high temperature and prolonged extraction cause the degradation and oxidation of CA. Therefore, the interaction between time and temperature is not significant (Figure 3B and Table 4).

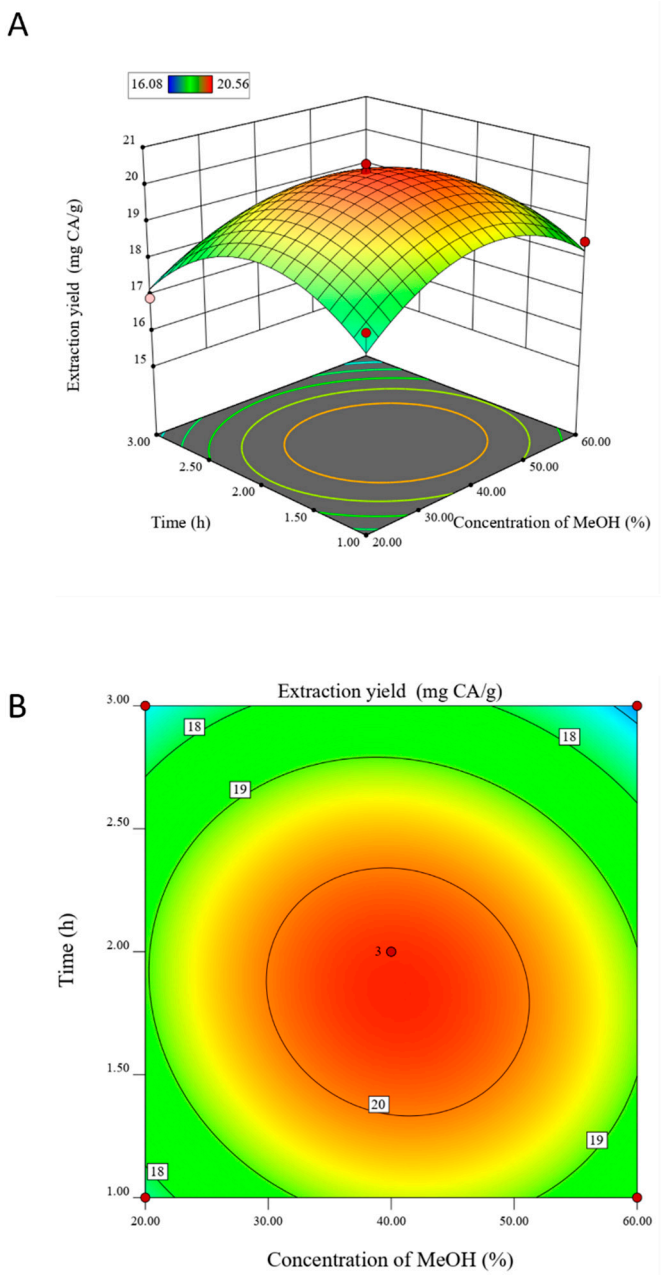

Figure 1. Response surface analysis based on solvent and time. (**A**) is the response surface graph and (**B**) is the corresponding contour graph.

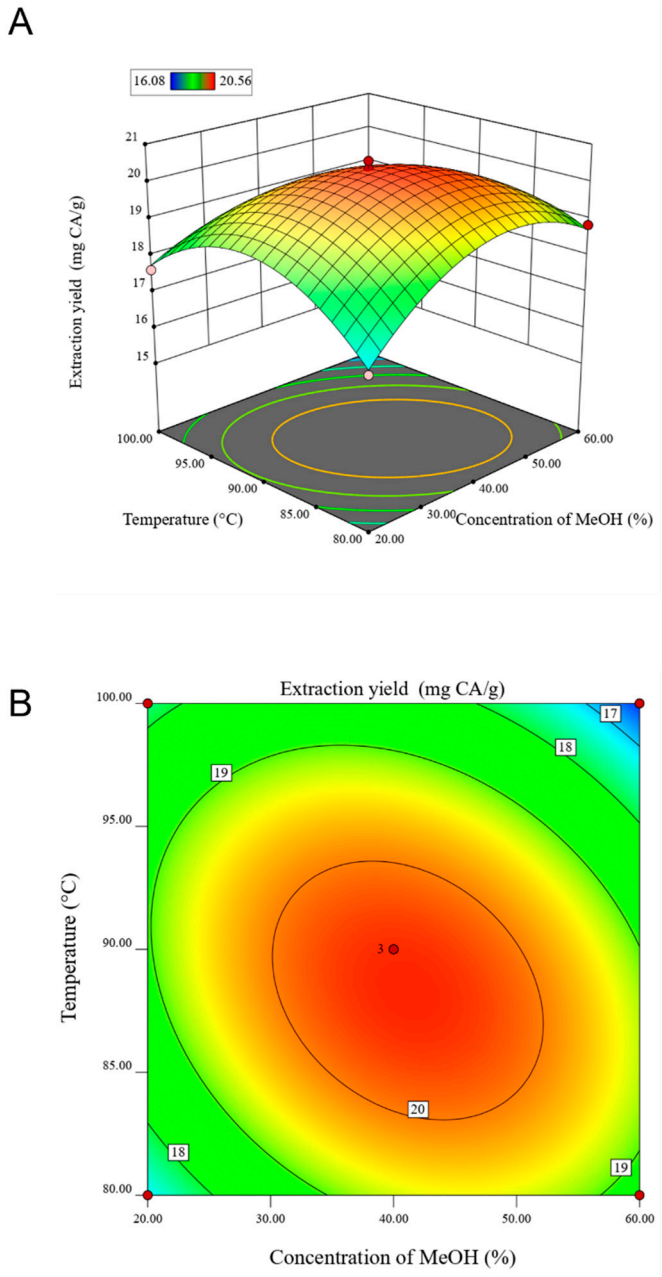

Figure 2. Response surface analysis based on solvent and temperature. (**A**) is the response surface graph and (**B**) is the corresponding contour graph.

The Box–Behnken central combination test was used to optimize the process parameters. The optimal extraction conditions to produce CA from the leaves of *D. morbifera* were: methanol concentration at 41.25 %, extraction time of 1.86 h, and extraction temperature of 88.61 °C, which yielded 20.35 mg/g CA, which was close to the theoretical value (20.41 mg/g), with a difference of only 0.29%, thereby providing an optimized design for actual production.

Certain previous studies extracted CA from aromatic herbs such as coriander, tarragon, and fennel, where the content of CA was approximately 0–0.4 mg/g [44], and according to Wang et al., the CA concentration in peanut was 2.10 mg/g [45]. In particular, there was no CA in the *D. morbifera* leaves, although CA was detected in debarked stems (0.92 ± 0.01 mg/g), branches (0.94 ± 0.01 mg/g), and bark (1.07 ± 0.01 mg/g) [5]. The other studies also showed that contents of CA in aqueous extract of *D. morbifera* leaves and in *D. morbifera* branch were 0.1 mg/g [46] and 31.96 μg/g [47], respectively. Compared

with the previous research results, these results indicate that the CA content in *D. morbifera* leaves was relatively high with an excellent utilization value and our extraction method is more effective.

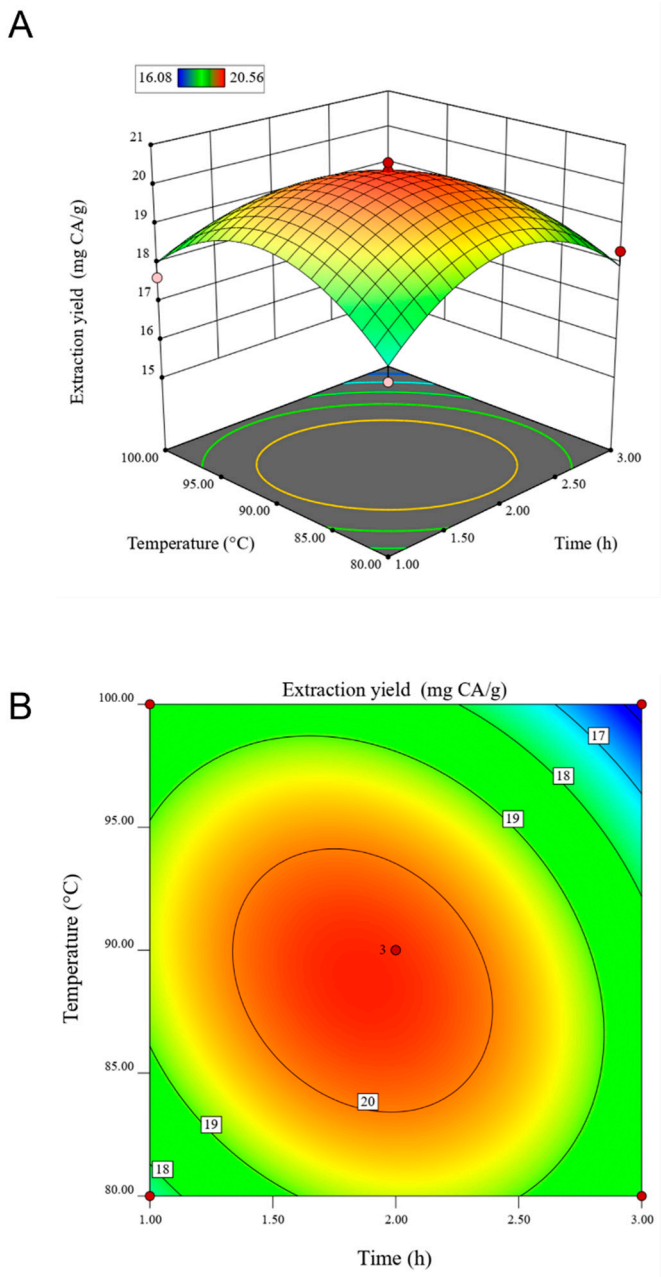

Figure 3. Response surface analysis based on time and temperature. (**A**) is the response surface graph and (**B**) is the corresponding contour graph.

3.3. Antioxidant Capacities of D. morbifera

Several previous studies have shown that the antioxidant capacity is closely related to the polyphenols content [48,49], and CA is a major subgroup of polyphenols that exhibits antioxidant properties [50–52]. Therefore, in this study, we determined the total polyphenols content and in vitro antioxidant properties of the extract that was produced using the optimized extraction conditions of CA.

3.4. Content of Polyphenols

The content of TP, TET, and TFlav in the *D. morbifera* leaves was 32.48 ± 2.05 mg GAE/g, 17.61 ± 2.61 mg GAE/g, and 9.14 ± 0.17 mg QE/g, respectively (Table 5).

Table 5. The content of polyphenols compounds in the *D. morbifera* leaves.

TP (mg GAE/g)	TET (mg GAE/g)	TFlav (mg QE/g)
32.48 ± 2.05	17.61 ± 2.61	9.14 ± 0.17

TP content in other plant extracts such as *Limnophila aromatica* extract, ginger, senna, nettle, and parsley was 20.20 ± 0.38, 11.84 ± 0.004, 24.14 ± 1.00, 14.61 ± 0.50, and 11.06 ± 1.50mg GAE/g, respectively, indicating that the polyphenol content in this extract was relatively higher than that in the other plant extracts, wherein a similar extraction method was used [53–55]. Furthermore, TET content in *Psidium Guajava* L. leaves determined using different extraction methods ranged from 1.728 to 2.315 mg/g [56]. Compared to other study, the TFlav content in methanol extract of *Catharanthus roseus* and *Ageratum conyzoides* was 5.24 ± 0.46 and 6.58 ± 0.46 mg QE/g, respectively, indicating that the TFlav content in *D. morbifera* leaves was relatively high compared to the other plants [57].

3.5. DPPH Test

The determination of free radical scavenging ability of the extract is an effective method to detect its antioxidant capacity. DPPH have been widely used to determine the free radical scavenging ability [58,59]. DPPH forms a stable violet solution in methanol at room temperature. When the antioxidant is mixed with DPPH solution, the color of the reaction liquid changes from light yellow to colorless due to the breakage of DPPH chain [60].

The free radical scavenging ability of *D. morbifera* leaves extract was expressed as IC50 (concentration of the sample required to scavenge 50% DPPH free radicals). As shown in Figure 4, the IC50 of *D. morbifera* leaves extract and ascorbic acid is 6.92 mg/mL and 0.099 mg/mL, respectively; therefore, the ascorbic acid equivalent (AAE) antioxidant capacity of 14.3 mg AAE/g is considerably greater than the antioxidant capacity of 11 fruits (IC50 values ranged from 0.135 to 2.18 mg AAE/g [61]) and 50 most popular food in the United States (IC50 values ranged from 0.068 to 5.207 mg AAE/g [62]) previously reported, which indicates a good free radical scavenging ability of *D. morbifera* leaves extracts.

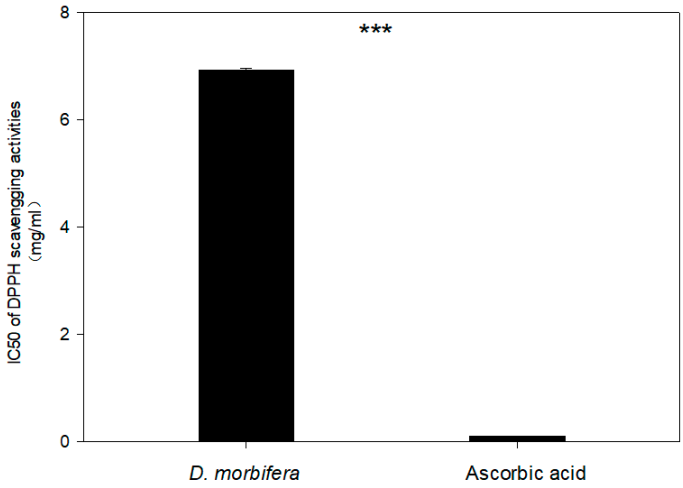

Figure 4. IC50 of DPPH scavenging activities of *D. morbifera* extract and ascorbic acid. The data are shown as the mean ± standard deviation and represent three replicate measurements. Significant difference set as *** $p < 0.001$.

4. Conclusions

D. morbifera has been used as a medicinal plant in Korea, and widely applied in folk and modern medicines. In this experiment, the main purpose of this experiment was to optimize a method to extract an effective biological component from the leaves of *D. morbifera*–CA. Here, methanol concentration at 41.23 %, extraction time of 1.86 h, and extraction temperature of 88.61 °C were observed to be the most suitable extraction conditions, which produced a maximum CA content of 20.35 mg/g. Under similar conditions, content of polyphenols compounds and antioxidant capacity of the extract were higher than those of other plants, presenting excellent antioxidant activity of *D. morbifera*. Therefore, the leaves of *D. morbifera* can be used as a promising candidate to extract CA and can also be used as a source of natural antioxidants. In further studies, the separation and purification of CA from *D. morbifera* should be investigated and various physiological activities of CA should be evaluated. This study can be used for further isolation of CA from *D. morbifera* leaves. In further studies, the purification of CA from *D. morbifera* leaves and preclinical evaluation of CA should be investigated for application in production of functional food and pharmaceuticals.

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