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Optimization of microwave-assisted extraction for polyphenols and antioxidants from *Cordyceps militaris* base

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ABSTRACT

Cordyceps militaris is a valuable fungus. However, most research considers fruiting bodies while the solid growth medium is ignored. This study investigated microwave-assisted extraction (MAE) parameters for improving the recovery of phenolic compounds, as chemical markers, and associated antioxidant activity, as functional response, from the medium. To support MAE optimization, preliminary factors affecting efficiency were first evaluated, including particle size determination and establishing magnetic stirring conditions. Powders <0.25 mm produced higher total phenolic content (3.51 ± 0.36 mg GAE/g dry weight), total flavonoid content (6.03 ± 1.23 mg QE/100 g), and antioxidant capacity (9.9 ± 1.38 µmol TE/g). Combinations of temperature (50-70 °C) and time (5-20 min) with stirring were examined using principal component analysis, showing optimal conditions at 60 °C for 15 min. Finally, MAE factors were optimized using a factorial design. ANOVA and response surface methods showed that 560 W for 8 min produced the highest extraction values.

Keywords: *Cordyceps militaris base, microwave-assisted extraction, polyphenols, flavonoids, antioxidant, response surface methodology*

INTRODUCTION

Cordyceps militaris (C. militaris) is a fungus that was classified in the Clavicipitaceae family. However, recent studies have moved it to the Cordycipitaceae family [1]. It is highly valued for its rich bioactive compounds [2]. While *Cordyceps sinensis* is rare and expensive at high altitudes, *C. militaris* is cultivable and is currently produced in many countries, including China, Korea, Vietnam, Japan, and Thailand. *C. militaris* has become a great alternative for *Cordyceps sinensis* [3].

The fungus exhibits many pharmacological benefits, such as antioxidant, anti-inflammatory, and protective effects [4]. These effects are primarily driven by the bioactive constituents, including polyphenols, flavonoids, polysaccharides, and cordycepin [5]. Many ethnopharmacological uses have been scientifically supported. A study by Xiong *et al*. [6] shows that *C. militaris* extracts protect intestinal structure, reduce inflammation, and balance the gut microbiota. The anti-cancer potential of *C. militaris* ethanolic extract has also been studied by Thepmalee *et al.* [7].

The fungus cultivation yields two parts: fruiting bodies and solid medium (base). The fruiting body is extensively studied for its bioactive substances and therapeutic effects [8-10]. In contrast, the solid medium is treated as byproducts. Although the solid growth medium is normally thrown away after cultivation, previous studies have reported that it has residual constituents [10]. These constituents may be phenolic compounds, flavonoids, polysaccharides, and other low-molecular-weight metabolites associated with antioxidant activity [10]. Total phenolic content (TPC) and total flavonoid content (TFC) are commonly used to indicate such compounds and have been linked with antioxidant, anti-inflammatory, and protective effects against oxidative stress-related disorders [10-12]. In relation with TPC and TFC, antioxidant capacity assays such as Trolox equivalent antioxidant capacity (TEAC) show the functional ability of extracts to neutralize free radicals. It is relevant to the prevention of cellular

damage and chronic diseases [13]. The concentrations can be lower than those in the fruiting bodies. However, their presence suggests that solid medium is still a potential source of functional ingredients. Thus, suitable extraction strategies are needed to recover these compounds from this byproduct.

Many extraction methods have been applied to recover bioactive compounds from Cordyceps species, including hot water extraction, solvent maceration, ultrasonic-assisted extraction, and subcritical water extraction [14-16]. Conventional methods like boiling water or maceration are convenient yet inefficient [17]. Advanced methods such as subcritical water extraction, ultrasonic-assisted extraction, and supercritical fluid extraction have increased efficiency and yield [2, 5]. However, these methods need specific equipment and expertise. They are not economical or accessible to all laboratories or companies. In comparison, MAE is a good alternative for both labs and industries. It offers rapid heating, enhanced mass transfer, reduced solvent consumption, and operational simplicity. Its yield is normally higher than traditional methods [17]. MAE is a practical and efficient approach for extracting phenolic compounds and antioxidant activity from solid matrices. Nevertheless, limited research has systematically optimized MAE parameters specifically targeting the solid medium base of *C. militaris*.

Given the needed information, the study aimed to optimize MAE conditions for improving the recovery of phenolic compounds and their associated antioxidant activity from the solid medium of *C. militaris*. To support MAE optimization, the effects of powder particle size and magnetic stirring temperature and time on extraction efficiency were first evaluated. Later, MAE parameters were optimized to enhance phenolic and flavonoid yields using response surface methodology.

EXPERIMENTAL

Materials: The solid medium of *C. militaris* was purchased from growing agents in Binh Thuy district, Can Tho city, Vietnam (Supplementary Fig. **S1**). Ethanol (analytical grade, ≥ 99.7%) was used as the solvent for extraction. The reagents used for quantification of TPC, TFC, and TEAC included Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), gallic acid (GAE standard), aluminum chloride ($AlCl_3$), potassium acetate (CH_3COOK), quercetin (QE standard), DPPH, and Trolox (TE standard). All chemicals were of analytical grade and were purchased from Merck, Germany.

Sample preparation and preliminary extraction conditions: The samples were first sliced and dried at 70 °C (Shellab CE3F-2, Sheldon Manufacturing Inc., USA) until the moisture content was below 10%. They were then ground into fine particles of various sizes. After that, a baseline extraction was conducted to characterize the initial bioactive content of the solid medium before optimization experiments. Briefly, five grams of sample were extracted with 70% ethanol (1:20 w/v) at 60 °C for 90 minutes [18]. The extract was then filtered. The filtrate was collected for the analysis.

Preliminary experiments were conducted to evaluate particle size and magnetic stirring conditions because these factors influence mass transfer and heat distribution before MAE. Regarding particle size, the ground powder was classified using standard sieves of 0.5, 0.25, and 0.125 mm to obtain five size fractions: (A1) < 0.5 mm, (A2) 0.25-0.5 mm, (A3) < 0.25 mm, (A4) 0.125-0.25 mm, and (A5) < 0.125 mm. Each fraction was extracted with the baseline conditions above.

In terms of magnetic stirring time and temperature, the experiment used three temperatures (50, 60, and 70 °C) and four stirring periods (5, 10, 15, and 20 minutes), with three replications per treatment, for a total of 36 samples. All experiments were conducted under the baseline conditions described above.

Effect of microwave time and power on the extraction efficiency of bioactive content using MAE: This experiment aimed to determine how microwave power and extraction time affect the extraction of bioactive content (polyphenols, flavonoids, and antioxidant activity) from the *C. militaris* solid medium. A randomization design with two factors and three replications was performed. The first factor was microwave power, which was set at five levels: 80, 240, 400, 560, and 800 W. The second one was extraction time, which was set at six intervals: 2, 4, 6, 8, 10, and 12 minutes. Each treatment was done in triplicate, which resulted in 90 treatment combinations. For each trial, 5 grams of *C. militaris* medium powder (based on the optimal particle size determined in the preliminary extraction experiments) were used with 70% ethanol. The stirring condition was set as the optimal condition determined in the preliminary extraction experiments. After extraction, samples were filtered. The extracts were analyzed for TPC, TFC and TEAC.

Determination of total phenolic content (TPC): The TPC of *C. militaris* base was determined by a modified method based on Luo ***et al***. [3] with the Folin-Ciocalteu colorimetric assay. 0.1 mL of extract was mixed with 1.5 mL of 10% Folin-Ciocalteu reagent and left for 5 minutes. Then, 4.0 mL of 20% Na_2CO_3 was added. The resulting mixture was adjusted to a final volume of 10 mL with distilled water and incubated under dark conditions at room temperature for 30 minutes. Absorbance was recorded at 738 nm using a spectrophotometer. Gallic acid served as the calibration standard, and a standard curve was generated (y=0.1237x+0.0512, R^2=0.9957). TPC was quantified as milligrams of gallic acid equivalents (mg GAE) per gram of dry weight (DW).

Determination of total flavonoid content (TFC): The TFC of *C. militaris* was measured with a modified method from Shawon ***et al*** [19]. 1 mL of the extract was combined with 3 mL methanol, 0.2 mL of 10% aluminum chloride, 0.2 mL of 1 M sodium acetate and 5.8 mL of distilled water. After 30 minutes of incubation at room temperature, the mixture's absorbance was measured

at 415 nm. A standard curve was established using quercetin (y=0.0054x+0.0026, R^2=0.9995). The results were presented as milligrams of quercetin equivalents (mg QE) per 100 grams of dry weight (DW). This was done to be comparable scales with TPC (mg GAE per gram dry weight) in plotting.

Determination of antioxidant activity (TEAC Assay)

The TEAC was determined using a modified DPPH test based on Jakubczyk ***et al*** [20]. 0.1 mL of extract was combined with 4.0 mL of 0.1 mM DPPH solution. Ethanol was added to reach a volume of 5.0 mL. The mixture was incubated in the dark at room temperature for 30 minutes. Absorbance was measured at 517 nm. A standard curve was created with Trolox (y = -0.087x + 0.7467, R^2= 0.9849). Trolox equivalents (µmol TE/g dry weight) were used to express results.

Statistical analysis

Results were analyzed using IBM SPSS Statistics 29.0.2.0 (SPSS Inc., Chicago, IL, USA). They are presented as mean ± standard deviation. Statistical comparisons were conducted using the LSD test, with significance set at p<0.05. Multivariate analysis of variance (MANOVA) was performed with five powder sizes as independent variables and TPC, TFC, and TEAC as dependent variables. Principal component analysis (PCA) was conducted on 13 time-temperature combinations from the stirring process using TPC, TFC, and TEAC data in RStudio (2024.12.0-467) with R version 4.4.2. The ggplot2 and factoextra packages were used for visualization and analysis [21, 22]. Hierarchical cluster analysis was also carried out in SPSS to cluster 30 treatments of times and temperatures during microwave assistance on TPC, TFC, and TEAC. Response surface methodology (RSM) was applied to model and predict optimal time and power levels for TPC using the rsm package in R [23].

RESULTS AND DISCUSSION

Preliminary evaluation of extraction conditions: The extraction of bioactive compounds from *C. militaris* solid medium powder depends on different factors. Among them, the quality and content of the raw material play an important role in the extraction amount and efficacy. Table 1 presents the results of preliminary content of *C. militaris* base powder. The low moisture content (<10%) in this study was consistent with earlier results under similar drying circumstances [11]. This level could help maintain its stability and shelf life. The extract exhibited 6.88 ± 0.74 µmol TE/g DW of TEAC, 3.05 ± 0.43 mg QE/100 g DW of TFC, and 1.76 ± 0.36 mg GAE/g DW of TPC. It suggests that beneficial compounds, particularly polyphenols, are still present in this byproduct. This shows the potential application of *C. militaris* medium in health products and food.

Additionally, Table 2 illustrates that particles smaller than 0.5 mm had the highest yield. The fraction <0.25 mm had a high yield of 67.41 ± 0.08%. Meanwhile, the smallest fraction (<0.125 mm) was only 23.87 ± 0.21%. Particle size may influence extraction efficiency. Smaller particles could increase surface area for solvent contact. Also, breaking down cell structures by grinding the powder increases the release of bioactive compounds. Given the information, particle sizes were optimized before MAE.

Table 1. Preliminary content of *C. militaris* base powder.

Parameters	Content
Moisture (%)	9.22±0.41
TPC (mg GAE/g dw)	1.76±0.36
TFC (mg QE/100 g dw)	3.05±0.43
TEAC (µmol TE/g dw)	6.88±0.74

Note: The data in the table are the mean values of three replicates.

Table 2. The yield of powder by particle size.

Parameters	Content
A1: powder passing through a 0.5 mm sieve (< 0.5 mm)	100±0.00a
A2: particles retained on the 0.25 mm sieve (0.25-0.5 mm)	32.48±0.27d
A3: powder passing through a 0.25 mm sieve (< 0.25 mm)	67.41±0.08b
A4: particles retained on the 0.125 mm sieve (0.125-0.25 mm)	43.40±0.39c
A5: powder passing through a 0.125 mm sieve (< 0.125 mm)	23.87±0.21e

Note: Values in the same column followed by the same letter are not significantly different according to the LSD test at a 95% confidence level. A1 (<0.5 mm) = A2 (0.25–0.5 mm) + A3 (<0.25 mm) and A3 = A4 (0.125–0.25 mm) + A5 (<0.125 mm).

MANOVA was conducted to examine whether there was an overall significant difference among five *C. militaris* sizes. The analysis showed that there was a statistically significant multivariate effect of particle size on all dependent variables. The Pillai's Trace determined was 1.684, $F(12, 30) = 3.199$, p = 0.005, while the Wilks' Lambda was 0.051, $F(12, 21.458) = 3.732$, p = 0.004. The analysis came with a large effect size (Partial η^2 = 0.561-0.841) and high observed power (≥0.926). These results indicated that the model was reliable and different particle sizes made significantly different extraction outcomes via TPC, TFC, and TEAC. Post hoc comparisons with LSD test were conducted to determine the specific differences among variables (see Fig. 1). The post hoc analysis with the LSD test suggests that there were statistically significant differences in the bioactive contents across different powder sizes. Smaller particle sizes can shorten extraction time, increase diffusion rates, and achieve maximum yield of bioactive compounds [17]. The smaller the particle size, the bigger the surface area of contact between the material and the solvent. Besides, it is also possible that the uniformity of the samples is influencing the extraction efficiency. After grinding, the

dried *C. militaris* base presents different particle sizes. Finer particles may settle on the surface of unbroken coarse particles. They may potentially prevent solvent diffusion. Additionally, excessively fine particles may stick together, reducing solvent accessibility.

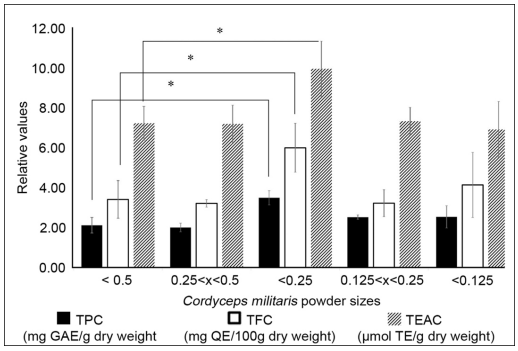

Fig. 1. TPC, TFC, and TEAC by sizes (mm). * $p < 0.05$ shows significant differences among the *C. militaris* powder sizes (post hoc LSD test).

The results indicate that powders with particle sizes smaller than 0.25 mm yielded significantly higher bioactive contents compared to other groups, with TPC and TFC values of 3.51 ± 0.36 mg GAE/g DW and 6.03 ± 1.23 mg QE/100 g DW, respectively. In contrast, the 0.25 < x < 0.5 mm group had the lowest means, with TPC at 2.02 ± 0.22 mg GAE/g DW and TFC at 3.23 ± 0.19 mg QE/100 g DW. Sample group A3 (<0.25 mm) was chosen for further experiments.

Before MAE, stirring time and temperature were evaluated because of their impact on heat and mass transfer. The stirring speed was set at 1500 rpm. Temperatures of 50, 60, and 70 °C and extraction times of 5, 10, 15, and 20 minutes were used. The experimental results are presented in Table 3. Magnetic stirring-assisted treatment significantly gave a higher yield and antioxidant activity of bioactive compounds (TPC, TFC, TEAC) than traditional soaking methods. This improvement is attributed to better mass transfer during stirring, which reduces the boundary layer around plant particles, improving solvent penetration and compound release [24]. Similar conclusions were drawn by da Silva ***et al***., [25] who found that stirring increased extraction efficiency in medicinal plant studies. In an extraction process, both temperature and time influence mass transfer and solubility.

When comparing static extraction (Experiment 1), results showed that increasing the temperature from 50 °C to 60 °C improved TPC and TFC yields, especially during the first 15 minutes (Table 3). For example, at 50 °C for 15 min, TPC and TFC reached 3.79 ± 0.68 mg GAE/g DW and 9.31 ± 1.75 mg QE/100 g DW. At 60 °C for 15 min, they peaked at 5.92 ± 0.56 and 11.70 ± 0.32, respectively. At 70 °C, the increase in compound levels stopped or dropped after 10 minutes, likely due to heat-related degradation or instability. This matches findings by Antony and Farid [26], who noted that higher temperatures help release polyphenols by breaking down cell walls and aiding solvent diffusion. Moderate heating can also free polyphenols from proteins or polysaccharides. However, longer extraction times (like 20 minutes) may cause degradation due to oxidation and exposure to light or air [26].

The trends can be seen clearly in the PCA biplot (Fig. 2). The two components account for 99% of total variance. Thus, the plot presents most of the information from the dataset. PC1 alone takes up to 86.3%, so it is the primary axis differentiating the treatments. All TPC, TFC, and TEAC arrows' directions point to the right side. This shows that they are all correlated. Treatments on the right side, the same directions with these arrows, have higher bioactive contents. In the 50 °C group, the 5-, 10-, and 15-minute treatments show a gradual shift to the right, indicating increasing TPC, TFC, and TEAC values. However, 50 °C_20 min treatment is located in the opposite direction, indicating lower TPC, TFC, and TEAC. As previously mentioned, this is possibly due to the degradation of bioactive compounds. A similar trend can be observed with the 60 °C group.

Table 3. Bioactive contents under different magnetic stirring temperatures and times.

Temp. (°C)	Time, (mins)	TPC, (mg GAE/g DW)	TFC, (mg QE/100 g DW)	TEAC, (µmol TE/g DW)
50	5	3.53 ± 0.41^{cA}	7.40 ± 1.12^{bA}	17.32 ± 0.37^{abB}
	10	3.59 ± 0.59^{cA}	7.71 ± 1.87^{bA}	19.81 ± 0.42^{abA}
	15	3.79 ± 0.68^{cA}	9.31 ± 1.75^{bA}	21.11 ± 0.39^{abA}
	20	3.06 ± 0.56^{cB}	6.16 ± 1.36^{bB}	15.71 ± 1.04^{abB}
60	5	5.18 ± 0.68^{aA}	8.55 ± 0.15^{aA}	18.33 ± 0.33^{aB}
	10	5.41 ± 0.12^{aA}	9.29 ± 0.11^{aA}	19.41 ± 0.96^{aA}
	15	5.92 ± 0.56^{aA}	11.70 ± 0.32^{aA}	25.41 ± 0.19^{aA}
	20	4.18 ± 0.54^{aB}	7.34 ± 1.11^{aB}	16.72 ± 0.33^{aB}
70	5	5.20 ± 0.30^{bA}	8.63 ± 0.11^{bA}	15.94 ± 2.04^{bB}
	10	5.50 ± 0.35^{bA}	10.44 ± 1.16^{bA}	21.01 ± 2.02^{bA}
	15	3.68 ± 0.37^{bA}	5.91 ± 1.12^{bA}	15.47 ± 1.23^{bA}
	20	3.12 ± 0.08^{bB}	5.47 ± 0.45^{bB}	14.21 ± 0.80^{bB}

Note: Values in the same column followed by different lowercase letters indicate statistically significant differences between temperatures ($p < 0.05$), while values followed by different uppercase letters indicate statistically significant differences between stirring times.

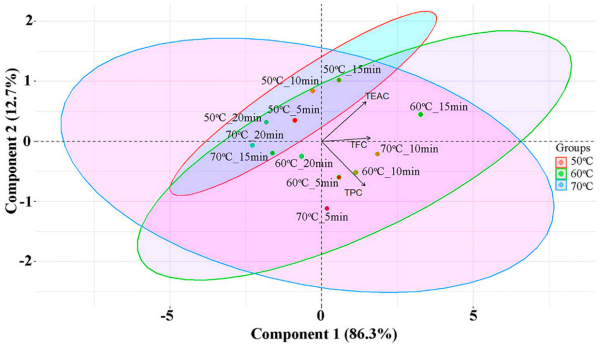

Fig. 2. PCA analysis on TPC, TFC, and TEAC across different stirring treatments.

On the other hand, treatments in the 70 °C group, especially 70 °C_15 min and 70 °C_20 min, appear on the left side of the biplot, indicating lower bioactive contents. This supports the possibility that degradation may occur after 10 minutes at 70 °C. The treatment of 60 °C_15 min is located furthest to the right, suggesting this combination gave higher TPC, TFC, and TEAC than others. Thus, it was selected for further experiments.

Effect of microwave time and power on the extraction efficiency of bioactive content using MAE: Particle size and stirring conditions were maintained at levels determined during preliminary evaluation. The extraction was carried out using a microwave, with different power levels and times. There were 5 power levels used: 80, 240, 400, 560, and 800 W. The extraction times were 2, 4, 6, 8, 10, and 12 minutes. The results of the experiment were recorded in Table 4. The results show that increasing the microwave power led to higher yields of TPC and TFC. For example, at the same 2-minute extraction, an increase in the power from 80 W to 560 W significantly offered higher bioactive yields. Specifically, at 80 W, TPC and TFC were 3.11 ± 0.15 mg GAE/g and 3.68 ± 0.04 mg QE/100 g, respectively. These values increased to 3.50 ± 0.05 mg GAE/g and 5.05 ± 0.06 mg QE/100 g at 560 W. This improvement is attributed to the rise in temperature caused by higher microwave energy, which accelerates the release of bioactive compounds into the solvent [26]. However, the efficiency peaked at 560 W.

Table 4. The TPC, TFC, and TEAC contents based on power (W) and time with extracts.

Time, (minutes)		TPC, (mg GAE/g DW)	TFC, (mg QE/100 g DW)	TEAC, (μmol TE/g DW)
80	2	3.11±0.15^{dD}	3.68±0.04^{cBC}	9.79±0.52^{dC}
	4	3.14±0.33^{dCD}	3.92±0.13^{cA}	10.87±0.49^{dB}
	6	3.14±0.17^{dB}	4.10±0.23^{cA}	11.79±0.05^{dA}
	8	3.42±0.19^{dA}	4.15±0.12^{cA}	12.08±0.05^{dA}
	10	3.32±0.05^{dAB}	4.29±0.29^{cAB}	11.12±0.32^{dB}
	12	3.17±0.15^{dBC}	4.25±0.68^{cC}	10.99±0.44^{dB}
240	2	3.13±0.02^{cdD}	4.59±0.33^{bBC}	10.95±0.68^{cC}
	4	3.24±0.05^{cdCD}	5.04±0.39^{bA}	11.36±1.07^{cB}
	6	3.39±0.40^{cdB}	5.10±0.06^{bA}	12.07±0.04^{cA}
	8	3.56±0.12^{cdA}	5.12±0.51^{bA}	12.23±0.13^{cA}
	10	3.62±0.11^{cdAB}	4.75±0.23^{bAB}	11.75±0.81^{cB}
	12	3.42±0.08^{cdBC}	4.35±0.36^{bC}	11.42±0.25^{cB}
400	2	3.46±0.14^{bD}	4.95±0.13^{bBC}	11.51±0.81^{bC}
	4	3.54±0.05^{bCD}	5.08±0.49^{bA}	11.83±0.30^{bB}
	6	3.82±0.60^{bB}	5.16±0.51^{bA}	12.23±0.08^{bA}
	8	3.95±0.26^{bA}	5.21±0.40^{bA}	12.66±0.17^{bA}
	10	3.81±0.08^{bAB}	4.90±0.53^{bAB}	12.10±0.16^{bB}
	12	3.71±0.45^{bBC}	4.55±0.13^{bC}	12.04±0.34^{bB}
560	2	3.50±0.05^{aD}	5.05±0.06^{aBC}	11.75±0.81^{aC}
	4	3.61±0.05^{aCD}	5.46±0.25^{aA}	11.90±0.23^{aB}
	6	4.02±0.07^{aB}	5.74±0.68^{aA}	12.75±0.30^{aA}
	8	4.63±0.60^{aA}	6.19±0.21^{aA}	13.68±0.19^{aA}
	10	4.16±0.19^{aAB}	5.61±0.07^{aAB}	12.83±0.31^{aB}
	12	3.88±0.08^{aBC}	4.86±0.22^{aC}	12.49±0.35^{aB}
800	2	3.17±0.07^{bcD}	4.60±0.17^{bBC}	10.95±0.36^{dB}
	4	3.37±0.04^{bcCD}	4.94±0.13^{bA}	11.52±0.15^{dB}
	6	3.75±0.30^{bcB}	4.89±0.18^{bA}	12.62±0.27^{dA}
	8	4.02±0.12^{bcA}	4.80±0.12^{bA}	11.94±0.46^{dA}
	10	3.62±0.80^{bcAB}	4.72±0.71^{bAB}	10.24±0.39^{dB}
	12	3.51±0.54^{bcBC}	4.38±0.25^{bC}	9.91±0.35^{dB}

Note: Within the same column, means followed by different lowercase letters (a, b, c, d) differ significantly by power, while uppercase letters (A, B, C, D) indicate significant differences by time ($p<0.05$).

At a higher power of 800 W, both TPC and TFC decreased. The values at 2 minutes in 800 W treatment were 3.17 ± 0.07 mg GAE/g and 4.60 ± 0.17 mg QE/100 g, respectively. This suggests that excessive energy may degrade sensitive compounds.

At the same microwave power, extending the extraction time from 2 to 8 minutes also improved the yield of phenolic and flavonoid compounds. Nonetheless, further increasing the time to 10 or 12 minutes led to a notable decrease in polyphenol content. This decline is likely due to heat degradation, especially in high power and long duration conditions. While extending extraction time might enhance yield in some cases, it does not always lead to better results and may even produce negative effects [27].

The study showed that extracting bioactive compounds from *C. militaris* powder using MAE at a power level of 560 W for 8 minutes gave the best results. Under these conditions, TFC and TPC obtained were 6.19 ± 0.21 mg QE/100 g dry weight and 4.63 ± 0.60 mg GAE/g dry weight, respectively.

Hierarchical cluster analysis was conducted to examine similar treatments with 560 W_8 min across three variables of TPC, TFC, and TEAC. Fig. 3 illustrates the grouping of different treatments. As seen in the plot, there are four main clusters including extremely low-performing, low-performing, moderate-performing, and high-performing treatments. The extremely low-performing group has 80 W_2 min treatment. The treatments that share similarities with the chosen 560 W_8 min are 560 W_6 min and 560 W_10 min. The two treatments indeed had high TPC, TFC, and TEAC of above 4 mg GAE/g dry weight, 5.5 mg QE/100 g dry weight, and 12.5 µmol TE/g dry weight.

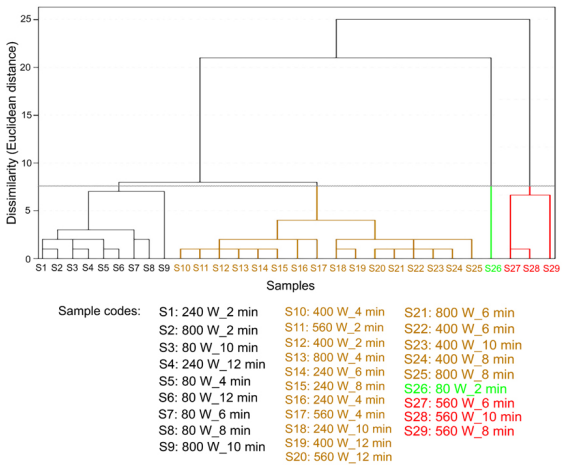

Fig. 3. Hierarchical cluster analysis dendrogram. Samples are labeled as S1–S29 for clarity. Colors indicate clusters at the selected cut-off distance.

Response surface methodology (RSM) was attempted to determine the optimized treatment. The Pearson correlation between TPC and TFC was R = 0.77 while that for TPC and TEAC was R = 0.75. Since TPC, TFC, and TEAC were moderately correlated, only TPC was used as a dependent variable in this analysis. Figure 4 presents the RSM model. The Regression equation determined was:

$$TPC = 1.377 + 0.081X_1 + 0.030X_2 - 0.038X_1X_2 - 0.111X_1^2 - 0.012X_2^2$$

where: X_1 is the coded value for Power (W) and X_2 is the coded value for Time (minute).

Model fit statistics included R^2=0.59, Adjusted R^2=0.51, F=6.99 , and p = 0.0004 (significant). The model had a moderate R^2 of 0.51, supporting its use for trend exploration. Power had a significant positive linear effect and a significant negative quadratic effect. Power was the main factor.

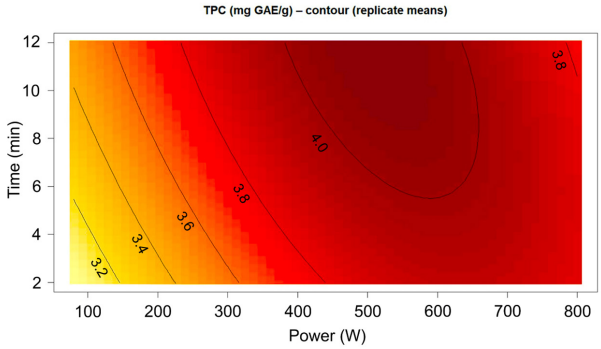

Fig. 4. Response surface methodology of time and power on TPC.

The plot indicates that higher TPC yields were generally observed in the range of 500-600 W and 7-11 minutes, suggesting this zone as a practical target for optimized extraction conditions. The optimal predicted point was 570 W and 7.34 minutes, which is consistent with the high-performing group from hierarchical cluster analysis dendrogram. However, this is only predictive and experimental studies are recommended.

CONCLUSIONS

The study shows that although *C. militaris* base is a byproduct, it still contains significant bioactive contents. Preliminary evaluations identified a powder particle size below 0.25 mm and magnetic stirring at 60 °C for 15 minutes as suitable conditions to support efficient MAE. Under these conditions, MAE was optimized through ANOVA and response surface analysis. TPC, TFC, and TEAC levels were predicted to be highest with extraction at 570 W and 7.34 minutes. This research provides useful extraction parameters for higher bioactive contents from *C. militaris* base. Future research should evaluate the biological activity of these extracts *ex-vivo* or *in vivo* models for their functional potential confirmation.

AUTHOR CONTRIBUTIONS

Conceptualization: TBL, TTNB, HLN; Methodology: TBL, TTNB, HLN; Investigation: TBL, TTNB; Formal analysis: TBL, TTNB; validation, TBL, TTNB; Writing-original draft preparation: TBL, HLN; Writing - review and editing: TBL, TTNB, NTBD, LNB, VTKH, NTTT; Supervision: TBL.

DATA AVAILABILITY STATEMENT

Data contained within the article.

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CONFLICT OF INTEREST

No conflict of interest to declare.

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