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Optimization of the Extraction of Antioxidant Components from *Curculigo orchioides* Gaertn Rhizomes by Ultrasound-Assisted Enzymatic Extraction

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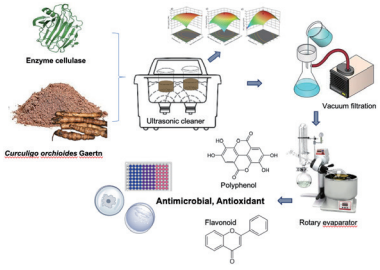
Abstract: This study investigated the application of ultrasound-assisted enzymatic extraction (UAEE) to obtain bioactive compounds from *C. orchioides*. The major natural compounds, including phenolics, alkaloids, saponins, flavonoids, and terpenoids, were identified in the extracts. Cellulase concentration, temperature, and material/solvent ratio were determined to be the main factors affecting the extraction efficiency. Using Response surface methodology (RSM), the optimal conditions were established to maximize the bioactive compounds. The major natural compounds, including phenolics, alkaloids, saponins, flavonoids, and terpenoids, were identified in the extracts. The optimal parameters included a cellulase concentration of 2.63%, an extraction time of 10 minutes, a solvent ratio of 75.36, and a temperature of 38.86°C. The total polyphenol content was 48.571 mg GAE/g DW ($R^2 = 0.9903$) and the total flavonoid content was 16.664 mg QE/g DW ($R^2 = 0.9842$). The antioxidant activity assessed by DPPH, ABTS, FRAP, and RP assays showed lower values than the positive controls ascorbic acid and trolox. These findings indicate that although the antioxidant potential of *C. orchioides* extracts is modest, they are still promising as a source of natural bioactive compounds for potential applications in functional foods and pharmaceuticals.



Key words: *Curculigo orchioides* Gaertn, ultrasound-assisted enzymatic, bioactive, extraction

1 Introduction

Curculigo orchioides Gaertn. belongs to the Amaryllidaceae family. *C. orchioides* is a traditional medicinal plant widely used in Asian countries for its well-known health benefits. *C. orchioides* is a plant rich in bioactive compounds, including phenolics, alkaloids, saponins, flavonoids, and terpenoids, were identified in the extracts. The phenolic and flavonoid compounds present in *C. orchioides* play an important role in neutralizing free radicals, helping to reduce oxidative stress and protect cells from oxidative damage. Yan Nie et al.(2013)studied the plants of the genus Curculigo and found that they mainly contained phenolic compounds and phenolic glycosides, lignans and lignan glycosides, along with triterpenes and triterpenoid glycosides[1)]. The plant fractions of *C. orchioides* exhibited significant antioxidant activity by scavenging free radicals with a DPPH radical scavenging assay of 52.93 ± 0.66 μg/mL[2)]. Studies have also documented the antibacterial activity of *C. orchioides* against a variety of pathogenic microorganisms, including Gram-positive and Gram-negative bacteria. The antibacterial activity of *C. orchioides* rhizomes is attributed to the presence of bioactive phenolic compounds[3)]. Notably, the ethanol extract from the rhizome of this species has been shown to inhibit the growth of methicillin-resistant *Pseudomonas aeruginosa*, with a minimum inhibitory concentration(MIC)of 49 μg/mL, while other Gram-negative bacteria were not affected(Marasini et al., 2015). In addition, the ethanol

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extract of *C. orchioides* showed the strongest antibacterial effect against *Streptococcus pyogenes*, with a similar MIC of 49 μg/mL[4]. However, the efficient extraction and optimization of these bioactive components are still important to fully exploit its medicinal value. Traditional extraction methods, such as maceration and reflux extraction, are widely used but often require longer extraction times and higher solvent volumes, which can lead to thermal degradation of the bioactive compounds[5, 6]. Microwave-assisted extraction (MAE) is another innovative method that uses microwave energy to rapidly heat the plant material and solvent, improving extraction efficiency[7]. However, MAE is not always suitable for thermally unstable compounds due to local overheating. Supercritical fluid extraction (SFE), which uses CO_2 as a solvent, is highly efficient and environmentally friendly but requires specialized equipment and incurs higher operating costs[5].

The use of advanced extraction techniques, such as ultrasound-assisted extraction (UAE), combined with enzyme-assisted extraction (EAE), offers a promising approach to improve the yield and bioactivity of plant extracts. UAE enhances mass transfer and cell disruption through ultrasound, while EAE uses specific enzymes such as cellulases to hydrolyze plant cell walls, facilitating the release of intracellular bioactive compounds[8]. These complementary techniques not only increase extraction efficiency but also reduce processing time and solvent usage, making them suitable for sustainable operations.

In addition, to achieve optimal extraction conditions for bioactive compounds from *C. orchioides*, Response surface methodology (RSM) is a valuable statistical tool. RSM allows systematic evaluation and optimization of multiple parameters, such as enzyme concentration, ultrasound power, extraction time, and temperature, to maximize the extraction yield and bioactivity. While previous studies have reported on the antioxidant and antibacterial properties of *Curculigo orchioides*, the results have been inconsistent due to variations in raw materials, extraction methods, solvents, and experimental conditions. The antioxidant and antibacterial activities are crucial factors in determining the potential practical uses of *Curculigo orchioides* extracts in pharmaceuticals, functional foods, and cosmetics. Focusing on these two activities helps to confirm the medicinal benefits of *Curculigo orchioides* and lays the foundation for refining the extraction process to produce extracts with high biological activity for product development purposes. This study aimed to extract bioactive components from *C. orchioides* by synergistically combining ultrasound-assisted extraction and enzyme-assisted extraction with cellulase. The extraction process was optimized through response surface methodology, focusing on the key factors influencing the yield and bioactivity. Furthermore, the biological activities of the extracts, including antioxidant and antibacterial properties, were evaluated to highlight their potential applications in functional foods, pharmaceuticals, and cosmetics.

2 Experimental Procedures

2.1 Materials

C. orchioides is harvested in Lai Chau province by local people who select good quality plants and ensure there are no pests or diseases. The harvested samples are accurately identified at the *C. orchioides* and Medicinal Materials Center in Ho Chi Minh City. After harvesting, the raw materials are carefully packaged and transported to the laboratory for processing. They are washed under running water to remove sand and impurities on the surface, and any damaged, rotten, or substandard parts are removed. The raw materials are dried at a temperature of 50℃ to ensure the retention of active ingredients and prevent mold. The dried materials are finely ground and then packed in sealed zip bags for storage.

2.2 Chemicals

Folin & Ciocalteu's phenol reagent (2N), 1,1-diphenyl-2-picrylhydrazyl (DPPH＞97%), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS＞98%), Trolox＞97%, and 2,4,6-Tris (2-pyridyl) -s-triazine (TPTZ＞99%) were purchased from Sigma Aldrich Co. LLC. (St. Louis, MO, USA). Ethanol, gallic acid, ascorbic acid, kali ferricyanide, ferric chloride, sodium acetate, aluminum chloride hexahydrate,... and some analytical chemicals were provided by Xilong Scientific Co., Ltd. (Guangzhou, China). Other test chemicals used in this study were of analytical purity.

2.3 Experimental

2.3.1 Single-factor experiments

Enzymatic extraction was performed according to the method of Feng Li et al. (2017) with some modifications[9]. The extraction was carried out with the assistance of a temperature- and time-controlled ultrasonic machine with a fixed frequency of 40 kHz. In this study, 30% ethanol with a pH value of 5.0 adjusted with HCl was chosen as the extraction solvent. The powder of *C. orchioides* (1.0 g) was placed in a 100 mL Erlenmeyer flask. Next, the flask was fixed in the same position in water (the water depth was fixed at 100 mm) in an ultrasonic machine at a frequency of 40 kHz, 250 W, and extracted according to the experimental design. The designed extraction temperature was adjusted by a temperature controller, and the actual temperature of the solution was monitored by a thermometer. Finally, the clear liquid was filtered using Whatman Φ90 mm filter paper and kept in a brown bottle at refrigerator temperature until analysis. To evaluate the effect of each factor on the extraction yield from *C. orchioides*, different cellulase concentrations (from 0% to 3%), sonication time

(10 to 40 min), sonication temperature (30 to 70℃), and solvent ratio (10 to 50 mL/g) were studied as single factors to be used as a basis for further optimization.

2.3.2 Optimization of the extraction process of *C. orchioides* using Response surface methodology (RSM)

The optimization process of parameters affecting the extraction process of *C. orchioides* uses the RSM (Response surface methodology) model with Box–Behnken design. The factors in the model are divided into levels, with three input levels being the central variable (0), low level (−1) and high level (+1). With 3 input factors as in this case (Cellulase enzyme concentration (A), solvent/material ratio (B), extraction temperature (C), the Box–Behnken design will create an experimental matrix consisting of 17 experiments (Table 1).

2.4 Analysis method

Total polyphenol content was determined using the Folin-Ciocalteu colorimetric method at a wavelength of 765 nm with a UV–Vis spectrophotometer, based on the standard curve of gallic acid[10]. Accurately weigh 0.1 g of *C. orchioides* extract sample, dissolve it in ethanol solvent, and make up to 10 mL in a volumetric flask. Filter the mixture through filter paper to collect the extract. Micropipette 0.1 mL of the extract into a test tube, then add 0.5 mL of 10% Folin-Ciocalteu reagent. After 5 minutes, add 0.4 mL of 7.5% Na_2CO_3 solution to the mixture. Shake the mixture well with a vortex and incubate for 1 hour. Calculate the total polyphenol content as mg gallic acid equivalents (GAE)/g DW.

The total flavonoid content was determined using the colorimetric method with aluminum chloride, measured at 415 nm, following the study by Mahboubi et al. (2013). Quercetin was used as a standard[11]. Accurately weigh 0.1 g of extract, dilute it with ethanol solvent, and make up to 10 mL in a volumetric flask. Micropipette 0.5 mL of the extract into a test tube, add 4.3 mL of ethanol, then add 0.1 mL of 10% $AlCl_3$ solution and 0.1 mL of 1 M CH_3COOK solution. Shake the mixture well with a vortex machine and incubate for 30 minutes at room temperature. After incubation, measure the absorbance of the mixture at 510 nm with a spectrophotometer. Repeat the experiment 3 times. Express the total flavonoid content as milligrams of quercetin equivalents per gram of dry extract (mg QE/g DW).

The antioxidant capacity of the extracts of the *C. orchioides* samples was determined by the DPPH free radical scavenging method according to the modified procedure of[12]. The reaction mixture was prepared by adding 1.5 mL of DPPH solution (6×10^{-4} M, diluted in ethanol, with $OD_{517\ nm} = 1.1 \pm 0.02$) to each test tube containing 0.5 mL of extracts at different concentrations. The extracts were diluted with ethanol to reach concentrations ranging from 310.5 to 5000 μg/mL. After mixing, the test tubes were kept in the dark for 30 min, and then the absorbance was measured at 517 nm. Ascorbic acid solution was used as a

Table 1 Box–Behnken empirical matrix for the three survey factors.

No	Encoded Variables			Actual Variables		
	A	B	C	A (%)	B (g/mL)	C (℃)
1	+1	+1	0	3	80	40
2	+1	0	−1	3	75	30
3	−1	+1	0	2	80	40
4	0	0	0	2.5	75	40
5	0	0	0	2.5	75	40
6	−1	0	−1	2	75	30
7	0	−1	−1	2.5	70	30
8	0	+1	+1	2.5	80	50
9	−1	0	+1	2	75	50
10	−1	−1	0	2	70	40
11	3	0	+1	3	75	50
12	0	0	0	2.5	75	40
13	0	0	0	2.5	75	40
14	3	0	0	3	70	40
15	0	−1	+1	2.5	70	50
16	0	+1	−1	2.5	80	30
17	0	0	0	2.5	75	40

positive control. The antioxidant capacity of the samples was expressed as IC_{50} value, which is the concentration of antioxidant required to inhibit 50% of DPPH free radicals.

The ABTS free radical scavenging ability of the extracts was evaluated by a modified method based on the procedure of[13]. $ABTS^+$ free radical was generated by mixing ABTS solution (7.4 mM) with potassium persulfate solution (2.6 mM) and letting the reaction take place in the dark at room temperature for 12-16 hours. The mixture was diluted with water to achieve an absorbance value at 734 nm in the range of 1.1 ± 0.02. In each experiment, 1.5 mL of the prepared $ABTS^+$ solution was mixed with 0.5 mL of extracts (0.1 g/10 mL) of different concentrations. After incubation for 30 min at room temperature, the absorbance at 734 nm was measured. The results were compared with the positive control ascorbic acid. The ABTS radical scavenging ability was expressed as IC_{50} value, which is the sample concentration required to reduce the signal intensity of $ABTS^+$ by 50%. The iron reducing capacity (RP) of the ethanol extract of *C. orchioides* was evaluated based on the method of[14]. The reaction mixture consisted of 0.5 mL of *C. orchioides* extract at the tested concentrations, 0.5 mL of phosphate buffer solution (0.2 M, pH 6.6), and 0.5 mL of 1% $K_3Fe(CN)_6$ solution. The mixture was incubated at 50℃ for 20 min, then 0.5 mL of 10% CCl_3COOH solution was added, and centrifuged at 3000 rpm for 10 min. After centrifugation, 0.5 mL of the supernatant was taken, 0.5 mL of water and 0.1 mL of 0.1% $FeCl_3$ solution were added, and the mixture was shaken well. The absorbance of the reaction mixture was measured at 700 nm. Trolox solution was used as a positive control. The antioxidant activity of the extracts was evaluated by the ability to reduce iron using the modified FRAP method[15]. This method is based on the principle of reducing the ferric-tripyridyltriazine complex to the ferrous form. Extracts at different concentrations (10 μL) were reacted with the FRAP solution (990 μL) in the dark for 30 min. Then, the optical density was measured at 593 nm. Trolox was used as a positive control. The antioxidant efficacy of the extracts at different concentrations was compared with that of the standard, based on the concentration (μg/mL) at which the OD value reached 0.5 ($OD_{0.5}$).

2.5 Data processing

Data were processed and analyzed using Microsoft Excel and Origin 8.5.1 software. Design-Expert software (version 11, State Ease, Minneapolis, USA) was used to optimize the parameters in the study. The combination of Design Expert and Origin software provides a powerful tool to evaluate the effectiveness of optimization processes and ensure that statistical results are highly accurate and reliable.

Table 2 Preliminary results of phytochemical composition of *C. orchioides* extract.

Parameters	Phenomenon	Results
Phenolic	Blue-black precipitate	+
Alkaloid	Red-brown precipitate	+
Saponin	Stable foam (HCl)	+
Flavonoids	Yellow precipitate	+
Tannins	Blue-black precipitate	+ +

Note: (-) negative, (+) positive.

3 Results

3.1 Preliminary assessment of medicinal quality

To make a preliminary assessment of the raw *C. orchioides* material before proceeding with the extraction process, the analytical parameters of moisture, ash content, and some points related to the qualitative determination of the chemical composition of *C. orchioides* were determined. The analytical results show that the moisture content of *C. orchioides* powder is $7.51 \pm 0.095\%$. This moisture level is within the appropriate range for preserving raw powder because moisture content lower than 10% helps limit the growth of microorganisms and molds, ensuring quality during storage. The very low moisture content of *C. orchioides* extract is $0.47 \pm 0.04\%$, indicating that the extract has been well concentrated, helping to minimize the risk of damage caused by microorganisms or oxidation during storage. The total ash content is $5.62 \pm 0.06\%$, showing that *C. orchioides* contains a certain amount of minerals, possibly including trace and macronutrients with nutritional value. The insoluble ash in HCl was $0.11 \pm 0.027\%$, reflecting the amount of insoluble inorganic impurities, usually silica or impurities from sandy soil. The low level indicates that *C. orchioides* has been well processed, with little contamination from the environment. Phytochemical screening of the root extract of *C. orchioides* confirmed the presence of a variety of phytochemical compounds, which are secondary metabolites that play an important role in the biological activities of the plant. Phytochemical screening was performed according to a modified method Hossain et al. (2013)[16] and Tiwari et al. (2011)[17]. The detected phytochemicals included phenolics, tannins, terpenoids, flavonoids, alkaloids, and saponins, as shown in Table 2. Similar to the publication of Agrahari et al. (2010) that found the presence of phenols and flavonoids, carbohydrates, alkaloids, steroids, and saponins in the methanol extract of *C. orchioides* roots[18].

3.2 Effect of extraction conditions on the extraction of polyphenols and flavonoids from *C. orchioides*

Enzyme-assisted extraction is considered an environmentally friendly solution, using biological enzymes to break down the plant cell structure, helping to release bio-

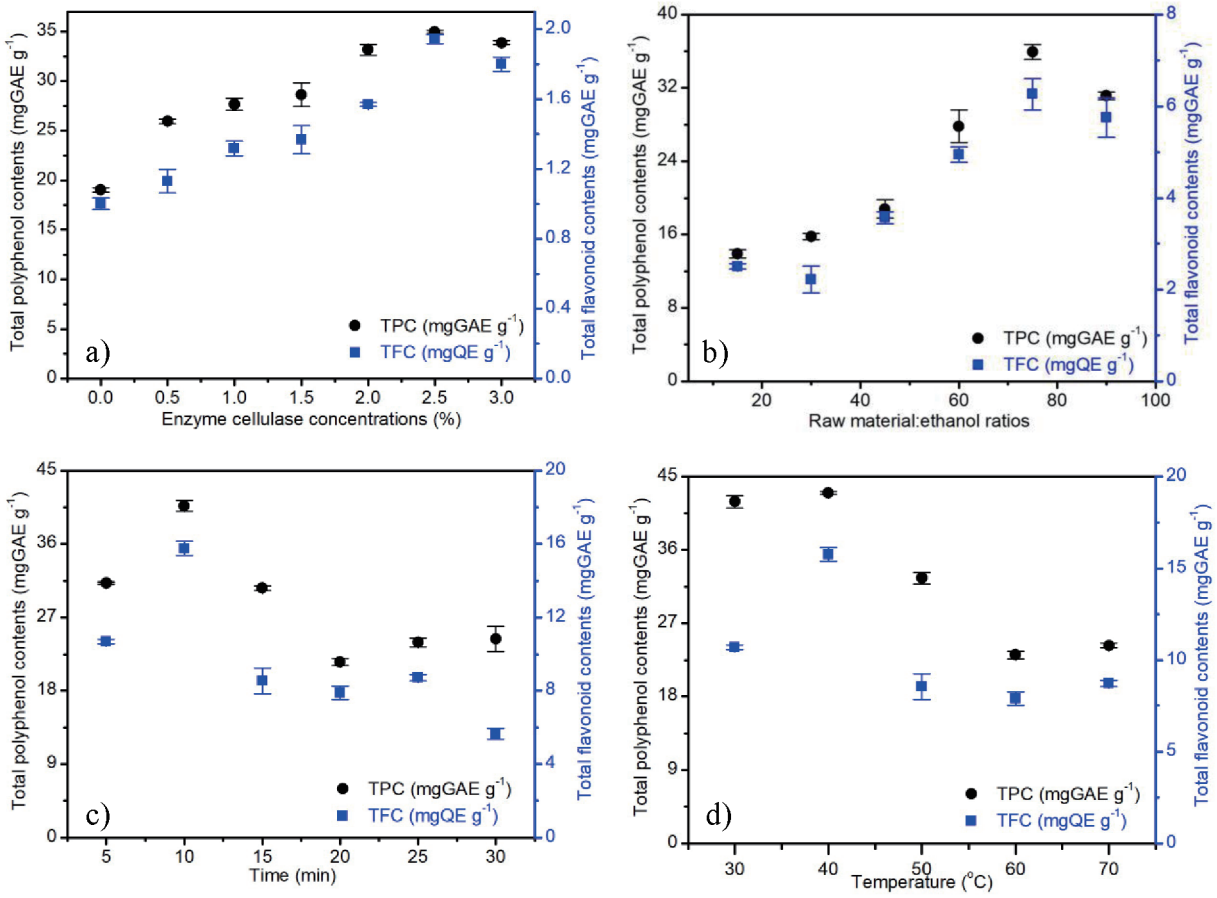

Fig. 1 Polyphenol and flavonoid content in *C. orchioides* extract under the following conditions: Cellulase enzyme concentration (a), Raw material: solvent ratio (b), Time (c) and Temperature (d).

active compounds such as flavonoids, polyphenols, and antioxidants. Different concentrations of cellulase enzyme (0 – 3%) were used to study the effect of cellulase content on TPC and TFC of *C. orchioides*, and the extraction conditions were fixed as raw material: ethanol ratio 50 mL/g, ultrasonic power 40 W, pH 5, temperature 50℃, and ultrasonic time 30 min. Figure 1a shows that TPC increases with increasing cellulase content from 0% to 2.5% with TPC content increasing from 19.016 mg GAE/g DW to 34.976 mg GAE/g DW and TFC increasing from 1.001 mg QE/g DW to 1.944 mg QE/g DW, but TPC and TFC begin to decrease when cellulase enzyme is added to 3%. When reaching a certain concentration of cellulase, the reaction rate may be saturated if all the active sites on the enzyme are occupied by cellulase. In this case, further increase in enzyme concentration will not bring higher efficiency. Therefore, the best cellulase enzyme concentration is 2.5%.

The ratio of raw materials and solvent is an important factor that directly affects the extraction efficiency in extraction methods. As can be seen in Fig. 1b, TPC and TFC were increased from 13.904 ± 0.443 to 35.925 ± 0.823 mg GAE/g DW and 2.502 ± 0.056 to 6.266 ± 0.034 mg QE/g DW, with the increase of liquid-solid ratio from 15 to 90 mL/g. However, TPC and TFC remained almost unchanged when the liquid-solid ratio was greater than 75 mL/g. As the liquid-solid ratio (solvent/raw material) increased, the amount of solvent in contact with the surface of the raw material increased, increasing the solubility and diffusion of the substances to be extracted. However, this was only effective to a certain extent. At low liquid-solid ratios, the solvent may not have permeated the entire surface of the material, so increasing the solvent will improve the extraction efficiency. A larger amount of solvent facilitates the diffusion of soluble substances from the inside of the material to the outside of the solvent, which speeds up the reaction. In addition, excess solvent can lead to increased costs without improving efficiency.

The extraction time in the presence of cellulase enzyme is an important factor determining the efficiency of cellulose degradation and extraction of desired compounds (Fig. 1c). In the initial stage (5–10 min), when the time is increased, the extraction efficiency increases, the TPC and TFC content is easily extracted into the solvent due to

strong diffusion from the material surface into the solvent. Easily soluble polyphenols and flavonoid compounds are quickly released when the cellulose structure is broken down by cellulase enzyme. The 10-minute time may be the best time when the TPC and TFC contents reached 23.958 ± 0.522 mg GAE/g DW and 8.725 ± 0.166 mg QE/g DW. However, prolonged exposure to enzymes or solvents may promote polyphenol degradation or transformation. When the time is prolonged, undesirable compounds such as cellulose, lignin, or other substances may dissolve into the solvent, diluting or masking the polyphenol compounds. Temperature is an important factor affecting the extraction efficiency when using cellulase enzymes. Temperature not only affects the enzyme activity but also affects the solubility of the compounds and their stability. Based on the results in Fig. 1d, at low temperatures (30–40℃), the TPC and TFC contents increased slightly from 41.927 ± 0.757 mg GAE/g DW to 43.019 ± 0.198 mg GAE/g DW and 10.693 ± 0.14 mg QE/g DW to 15.762 ± 0.381 mg QE/g DW. At 40℃, the cellulase enzyme reached its optimum activity level, which effectively broke down the cellulose structure and released polyphenols and flavonoids from the raw material. At high temperatures, undesirable compounds such as lignin or hemicellulose could dissolve into the solvent, affecting the measurement results. In addition, the viscosity of the extraction medium decreased with increasing temperature, and thus the bioactive compounds were more soluble. However, excessively elevated temperatures can determine the change of the three-dimensional enzyme configuration, inhibiting hydrolysis processes and breaking down bioactive compounds (Muniglia et al., 2014). Ni et al. (2015) analyzed and confirmed the active nature of the enzyme at low temperatures, with catechin from tea (*Camellia sinensis* L.) successfully hydrolyzed by tannase at temperatures ranging from 40 to 60℃ [19].

3.3 Optimization of extraction and recovery of polyphenols and flavonoids from *C. orchioides* using Response surface methodology (RSM)

The total polyphenol and total flavonoid contents in *C. orchioides* were optimized based on the Box-Behnken design, and the optimization process (RSM) was obtained by applying the quadratic polynomial equation. Based on the survey of factors affecting the extraction conditions of TPC and TFC by enzyme-assisted ultrasonic extraction, the factors of cellulase enzyme concentration, temperature and raw material: solvent ratio were the factors that significantly affected the extraction process of TPC and TFC in *C. orchioides* medicinal powder (Table 3).

The results of optimizing the extraction process of *C. orchioides* using the RSM method with R^2 values for total polyphenols (TPC) and total flavonoids (TFC) of 0.9903 and 0.9842, respectively, showed that the R^2 value was close to 1, reflecting that the regression model was able to explain the variation in experimental data very well. The high R^2 value proves that the model is reliable in optimizing the ex-

Table 3 Values of experimental and predicted variables.

No	Independent variables			TPC (mgGAE/g DW)		TFC (mgGAE/g DW)	
	A (%)	B (g/mL)	C (℃)	Reality	Prediction	Reality	Prediction
1	3	80	40	40.03	39.96	12.02	12.02
2	3	75	30	39.01	39.96	10.14	10.71
3	2	80	40	34.94	35.10	8.078	8.16
4	2.5	75	40	48.57	47.83	16.66	16.14
5	2.5	75	40	47.66	47.83	15.65	16.14
6	2	75	30	44.88	45.59	12.10	12.58
7	2.5	70	30	39.94	39.15	10.80	10.32
8	2.5	80	50	33.79	34.58	9.24	9.73
9	2	75	50	27.87	26.93	5.58	5.02
10	2	70	40	31.42	31.50	6.90	6.91
11	3	75	50	45.43	44.72	15.81	15.34
12	2.5	75	40	47.30	47.83	16.56	16.14
13	2.5	75	40	48.54	47.83	16.17	16.14
14	3	70	40	38.96	38.81	11.58	11.50
15	2.5	70	50	28.64	29.51	11.04	11.61
16	2.5	80	30	39.70	38.84	14.52	13.97
17	2.5	75	40	47.0	47.83	15.61	16.14

traction process. This is especially important when using RSM to predict and select optimal conditions. Both R^2 values exceeded the threshold of 0.9, indicating a strong correlation between independent variables (such as temperature, solvent concentration, raw material/solvent ratio) and dependent variables (TPC and TFC). The method applied was to analyze the regression of experimental data, obtaining a quadratic polynomial model predicting TPC and TFC as follows:

$$Y_{Polyphenol} = -1707.99556 + 54.71215A + 45.57279B - 1.55533C - 0.245190AB + 1.17094AC + 0.026921BC - 15.41640A^2 - 0.305327B^2 - 0.046731C^2$$

$$Y_{Flavonoid} = -822.65051 + 55.20731A + 19.34541B + 1.86026C - 0.073686AB + 0.609492AC - 0.027623BC - 13.96739A^2 - 0.119787B^2 - 0.017323C^2$$

In which: A: Cellulase enzyme concentration, B: Raw material: solvent ratio, C: Temperature.

The results of ANOVA analysis are shown in Table 4. From the results table, it can be seen that for polyphenol content, the F-value of the model is 79.07, the *p*-value of the model is <0.0001, proving that the model is significant. The regression coefficient $R^2 = 0.9903$, the closer the regression coefficient R^2 is to 1, the more significant the model is. This result shows that 99.03% of the experimental data is compatible with the predicted data of the model. Furthermore, the value of the coefficient of variation (CV) of the TPC objective function is 2.58%, giving the proposed model less than 10% with high accuracy and reliability of the testing process[20].

Analysis of variance (ANOVA) for the second-order response surface model was determined by the F value and *p*-value in Table 5. The model analysis results showed a high F value of 48.56 and *p*-value <0.0001, and the value of the coefficient of determination $R^2 = 0.9842$, indicating a high correlation between the experimental and predicted values. The variation of total flavonoid content was due to the influence of independent variables such as temperature function, ratio of raw materials to extraction solvent, and concentration of cellulase enzyme. This result showed that 98.42% of the experimental data were compatible with the predicted data of the model. The relative value of C.V. = 5.5 (coefficient of variation) indicated better reliability of the experimental values.

The 3D response surface plots of the relationships between extraction parameters and TPC and TFC values are presented in Figs. 2 and 3, illustrating the effects of cellulase concentration and solvent-raw material ratio on TPC and TFC values when the sonication temperature is fixed at 40℃ (zero level). The plot shows a downward parabolic curve, in which the polyphenol values gradually increase as the parameters reach an optimum region, and then gradually decrease as they move beyond this optimum region. In Fig. 2b, the curve continues to show a nonlinear relationship between the parameters (cellulase enzyme concentration and temperature) and the polyphenol values. The peak area of the plot, representing the highest polyphenol value, is located in the middle of the curve, not at the edge. When the sonication time is fixed at 10 min, and the extraction temperature was kept at a lower level, the TPC and TFC values initially increased and then decreased slowly as the cellulase concentration increased. However, the TPC and TFC values decreased significantly as the extraction temperature increased. The graph in Fig. 2c shows a parabolic curve with the peak near the center, the red

Table 4 ANOVA data of the optimal model for total polyphenol content.

Source	Sum of Squares	Degrees of Freedom	F-Value	*p*-Value	Statistic Value
Model	768.86	85.43	79.07	<0.0001	
A-Concentration	73.89	73.89	68.39	<0.0001	
B-Rate	11.29	11.29	10.45	0.0144	
C-Temperature	96.54	96.54	89.35	<0.0001	
AB	1.50	1.50	1.39	0.2767	
AC	137.11	137.11	126.91	<0.0001	Std.Dev. = 1.04
BC	7.25	7.25	6.71	0.0359	Mean = 40.22
A^2	62.54	62.54	57.89	0.0001	C.V.% = 2.58
B^2	245.33	245.33	227.08	<0.0001	$R^2 = 0.9903$
C^2	91.95	91.95	85.11	<0.0001	AP = 26.2105
Residual	7.56	1.08			
Lack of Fit	5.60	1.87	3.80	0.1149	
Pure Error	1.96	0.4911			
Cor Total	776.42				

Table 5 ANOVA data of the optimal model for total flavonoid content.

Source	Sum of Squares	Degrees of Freedom	F-Value	*p*-Value	Statistic Value
Model	199.01	22.11	48.56	＜0.0001	
A-Concentration	35.68	35.68	78.34	＜0.0001	
B-Rate	1.56	1.56	3.42	0.1068	
C-Temperature	4.32	4.32	9.50	0.0178	
AB	0.1357	0.1357	0.2981	0.6021	
AC	37.15	37.15	81.57	＜0.0001	Std.Dev. = 0.6748
BC	7.63	7.63	16.75	0.0046	Mean = 12.47
A^2	51.34	51.34	112.73	＜0.0001	C.V.% = 5.5
B^2	37.76	37.76	82.92	＜0.0001	R^2 = 0.9842
C^2	12.63	12.63	27.74	0.0012	AP = 21.4822
Residual	3.19	0.4554			
Lack of Fit	2.22	0.7413	3.08	0.1531	
Pure Error	0.9640	0.2410			
Cor Total	202.20				

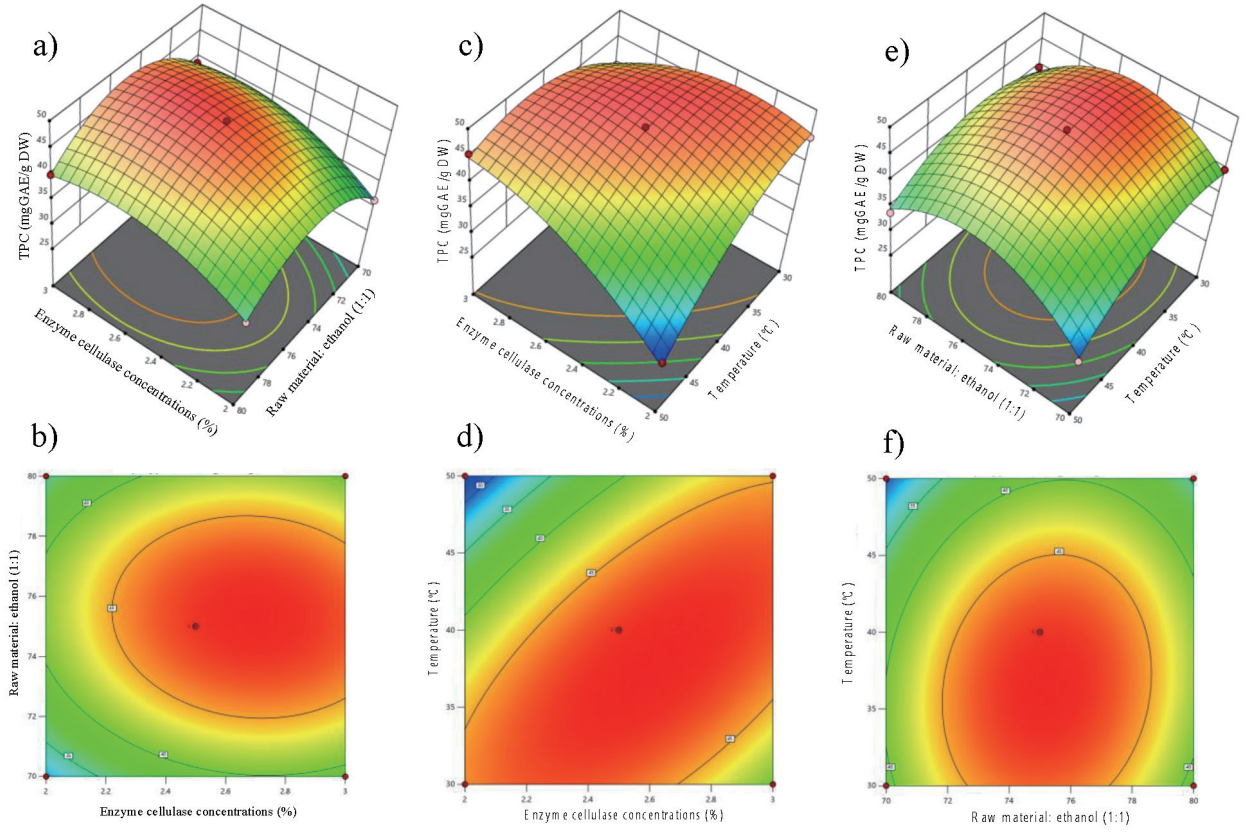

Fig. 2 The influence of 2 factors on TPC including experimental - predicted values: (a, b) cellulase enzyme concentration and raw material: solvent ratio, (c, d) cellulase enzyme concentration and temperature, (e, f) raw material: solvent ratio and temperature (d).

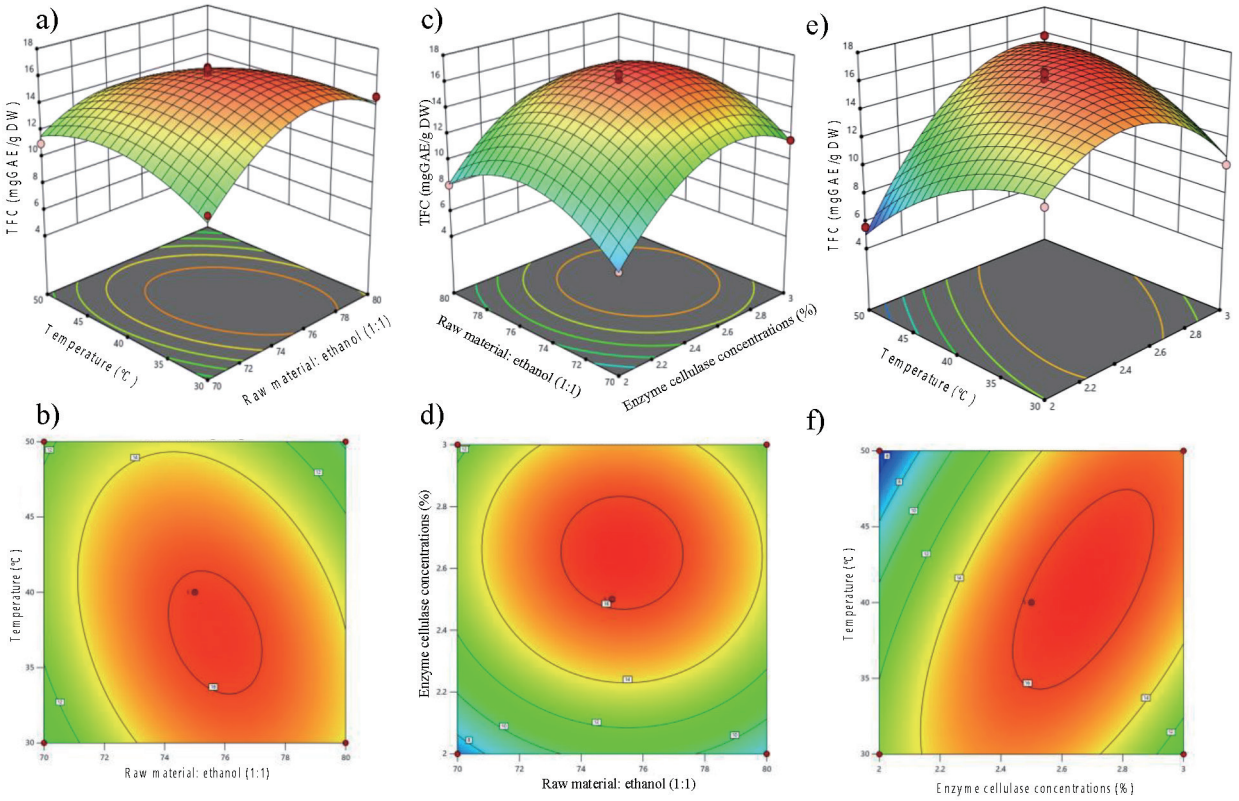

Fig. 3 The influence of 2 factors on TFC including experimental - predicted values: (a, b) cellulase enzyme concentration and raw material: solvent ratio, (c, d) cellulase enzyme concentration and temperature, (e, f) raw material: solvent ratio and temperature (d).

area (near the peak) represents the highest polyphenol values, while the green and blue areas (at the edges) represent lower values. As both the ratio and temperature parameters increased from low to medium levels, the polyphenol and flavonoid values increased, reaching an optimum value at the peak. Conversely, when either parameter increased too much, the polyphenol and flavonoid values began to decrease (indicated by the slope of the surface). The contour lines below the graph are elliptical, indicating a specific optimum region. The central region of the ellipse, corresponding to the lighter colored area (red/yellow) on the response surface, is where the highest polyphenol and flavonoid values are achieved. The green and blue regions at the edges of the graph indicate lower polyphenol and flavonoid values.

3.4 Antioxidant activity of *C. orchioides* extract

The antioxidant activity of the ethanol extract was compared with that of ascorbic acid (Table 6). As shown in Fig. 3, the higher the concentration of the extract of *C. orchioides*, the better the DPPH inhibitory ability. At a concentration of 5000 μg/mL, the DPPH radical scavenging activity of the ethanol extract of *C. orchioides* (65.057%) was higher than that of *C. orchioides* (IC_{50} = 2281.45 μg/mL) but the activity was lower than that of ascorbic acid (IC_{50} = 9.392 μg/mL). The IC_{50} value of *C. orchioides* was significantly higher than that of strong antioxidants such as ascorbic acid, which indicates that the antioxidant activity of *C. orchioides* was moderate or low. A high IC_{50} value may reflect that the polyphenol or flavonoid content in the extract was not high, or that these compounds did not have strong antioxidant activity[21, 22].

Table 6 IC_{50} value of *C. orchioides* extract.

Sample	IC_{50} DPPH (μg/mL)	IC_{50} ABTS (μg/mL)
C. orchioides	2281.45	1633.01
Ascorbic acid	9.392	8.875

The ABTS free radical scavenging activity of *C. orchioides* is an important criterion for evaluating the antioxidant capacity of this plant. The IC_{50} value of 1633.01 μg/mL for the ABTS free radical scavenging activity of *C. orchioides* shows that the antioxidant capacity of *C. orchioides* extract is through the ABTS free radical scavenging mechanism. Compared with the IC_{50} value of DPPH (2281.45 μg/mL), this activity is better but still at an average level, indicating that *C. orchioides* extract has a better ABTS free radical scavenging ability. This may be due to the different reaction mechanisms between ABTS and DPPH, or the chemical composition of *C. orchioides* has a stronger ac-

tivity against $ABTS^{+\cdot}$ radicals. Strong antioxidants such as ascorbic acid have an IC_{50} value of 8.875 μg/mL. Therefore, *C. orchioides* extract has antioxidant potential but is not in the strong group. DPPH is active in hydrophobic environments (mainly ethanol, methanol) and reacts with nonpolar antioxidant compounds. ABTS can be active in both hydrophobic and aqueous environments, thus it can scavenge free radicals with a wider range of compounds, including polar compounds. Ratnam et al. (2013) employed the soaking and percolation technique to extract the roots of *C. orchioides*. The ethanolic root extract of *C. orchioides* exhibited significant free radical scavenging, reducing power, and antioxidant properties in a dose-dependent manner[23]. Tuz Mia Nur Akhi et al. (2025) utilized a Soxhlet apparatus with ethanol as the solvent, followed by fractionation with ethanol (ECO), *n*-hexane (HCO), and chloroform (CCO). The findings indicated that the HCO fractionated extract displayed superior antioxidant, α-amylase inhibitory, and antidiarrheal activities[24].

The ferric reducing ability (FRAP) of *C. orchioides* reflects its antioxidant potential based on the electron exchange mechanism. The FRAP iron-reducing activity of *C. orchioides* provides further evidence of the antioxidant potential of this herb. Based on Figs. 4a and 4b, the increase in the concentration of *C. orchioides* extract was directly proportional to the iron-reducing ability of the sample, with the FRA value increasing from 2.18 to 40.91 (mg/g DW). The increase in absorbance shows that the ability of *C. orchioides* to reduce iron ions Fe^{3+} to Fe^{2+} increases with increasing sample concentration. This is consistent with the mechanism of action of antioxidants: as the concentration of antioxidant compounds in the sample increases, the number of electrons provided to reduce iron ions also increases, resulting in higher absorbance. The $OD_{0.5}$ value of *C. orchioides* was 2861 μg/mL, when compared with the OD value of the standard Trolox ($OD_{0.5}$ at 190.313 μg/mL), which suggests that the antioxidant activity of *C. orchioides* through the iron reduction mechanism is moderate and may depend on the specific bioactive compounds (such as polyphenols or flavonoids).

RP iron reduction activity of *C. orchioides* is an important index in assessing its antioxidant potential. However, to have a comprehensive view of the antioxidant capacity, it should be combined with other methods such as DPPH, ABTS, and FRAP. This method helps determine the ability of the sample to resist oxidation by donating electrons to

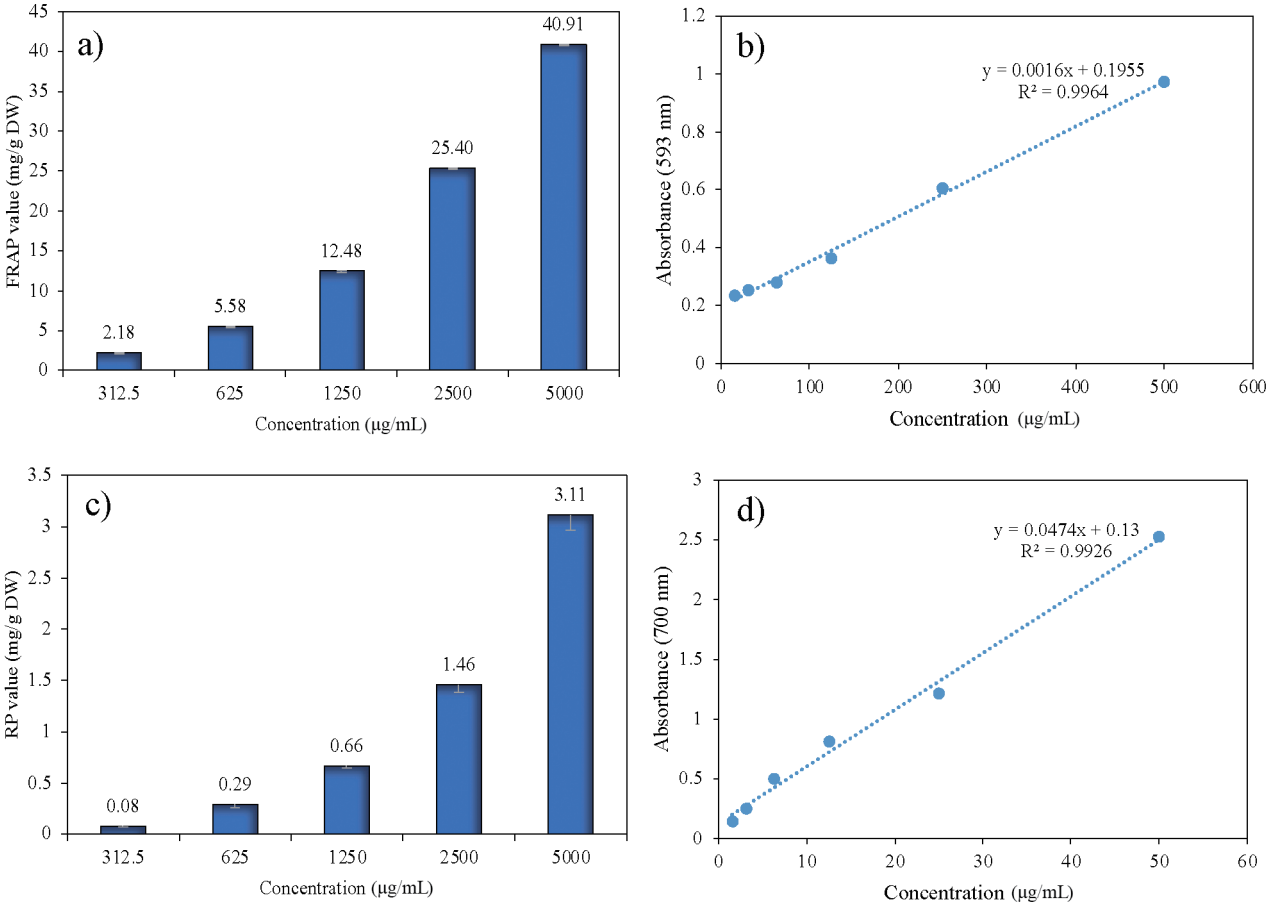

Fig. 4 Standard curve of trolox (b), ascorbic acid (d) with experimental values of FRAP (a) and RP (c).

metal ions. The increase in iron reduction with RP value increased from 0.08 to 3.11 (mg/g DW) as the *C. orchioides* sample concentration increased, with $OD_{0.5}$ = 1431.66 μg/mL compared to $OD_{0.5}$ = 7.806 μg/mL of ascorbic acid, indicates that the ability of the sample to reduce Fe^{3+} to Fe^{2+} is improved with increasing concentration (Fig. 4c, 4d). As the concentration of the antioxidant compound increases, the electron-donating ability of the sample also increases, resulting in higher absorbance (OD) values. *C. orchioides* requires a concentration approximately 183 times greater than ascorbic acid to achieve the same level of uptake. This indicates that the iron-reducing activity of *C. orchioides* is much lower than that of ascorbic acid, a very strong standard antioxidant. However, *C. orchioides* still exhibits significant iron-reducing ability within the range of natural plant compounds.

4 Conclusion

The study demonstrated the effectiveness of the enzyme-assisted ultrasound extraction method in recovering the extract from the plant, with a yield of 7.88%. Important natural compounds including phenolics, alkaloids, saponins, flavonoids, and terpenoids were identified in the extract. The optimal extraction conditions determined by RSM included: cellulase enzyme concentration of 2.63%, extraction time of 10 min, solvent ratio of 75.36 mL at a temperature of 38.86℃. The obtained extract contained total polyphenols of 48.571 mg GAE/g DW and total flavonoids of 16.664 mg QE/g DW with high precision (R^2 of 0.9903 and 0.9842, respectively). The antioxidant capacity of the extract was also confirmed through the IC_{50} indexes of DPPH (2281.45 μg/mL) and ABTS (1633.01 μg/mL). The study showed that the antioxidant activity of the extract of *C. orchioides* was evaluated through four methods: DPPH, ABTS, FRAP, and RP, all of which were relatively lower than the positive control. However, the results still showed a certain potential of *C. orchioides* as a natural source, with potential applications in the fields of functional foods and pharmaceuticals.

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