

Optimization of Extraction Process and Bioactivities of Polyphenols from *Cinnamomum Longepaniculatum* by Response Surface Method

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Abstract: Based on the results of single factor experiment, Box-Behnken response surface method was used to optimize the extraction process of polyphenols from *C. longepaniculatum* leaves. The influences of ethanol concentration, liquid to material ratio and extraction temperature on the extraction rate of polyphenols were investigated. The antioxidant and whitening activities of polyphenols from *C. longepaniculatum* leaves were evaluated by measuring the DPPH ·free radical scavenging rate and tyrosinase inhibition rate. The results showed that the optimal extraction conditions were as follows: ethanol concentration was 51%, liquid to material ratio was 30: 1 mL/g, extraction temperature was 71 °C, the extraction rate of polyphenols from *C. longepaniculatum* was (1.70 ±0.01) %. The DPPH free radical of 0.50 mg/mL polyphenols from *C. longepaniculatum* were 82.70%, and the tyrosinase inhibition rate of 10.00 mg/mL polyphenols from *C. longepaniculatum* were 91.78%. This shows that *C. longepaniculatum* leaves have good antioxidant and whitening effects.

Keywords: *Cinnamomum longepaniculatum*; Polyphenols; Anti-oxidation; Whitening

1. Introduction

Cinnamomum longepaniculatum (Gamble) N.Chao is an evergreen tree in the Lauraceae family thrives in Sichuan and western Shaanxi Province, China^[1]. The essential oil of *C. longepaniculatum* has been reported to have various pharmacological activities such as antibacterial^[2,3], antioxidant^[4], anti-inflammatory^[5] and other effects. *C. longepaniculatum* has attracted widespread attention because its leaves have large amounts of high-value-adding essential oils^[6]. However, a large amount of residue of *C. longepaniculatum* leaves will be produced after the removal of *C. longepaniculatum* essential oil, causing a waste of resources^[7]. Existing studies have shown that the leaf residue after essential oil extraction has antibacterial^[8], anti-inflammatory^[9] and anticancer^[10] effects. In addition to essential oil, *C. longepaniculatum* leaves also contain flavonoids, amino acids, peptides, proteins, polysaccharides and other chemical components^[11]. In this study, response surface was used to optimize the extraction process of polyphenols from *C. longepaniculatum* leaves, and the antioxidant and whitening functions of polyphenols from *C. longepaniculatum* leaves were studied by measuring the DPPH free radical scavenging rate and tyrosinase inhibition rate.

2. Materials and methods

2.1 Material of experiment

C. longepaniculatum leaves were collected from Zhanghai Town, Xuzhou District, Yibin City, Sichuan Province, China. The leaves are dried naturally after being picked. Extraction of essential oil from *C. longepaniculatum* leaves by steam distillation. After the essential oil was extracted, the residue of camphor leaves was collected and dried in the drying oven at 60 °C. The leaves were broken into powder with a wall breaking machine and sifted through 80 mesh.

2.2 Methods

2.2.1 The standard curve of gallic acid solution

The total polyphenol content (TPC) of the *C. longepaniculatum* leaf extract was evaluated by the Folin Ciocerteanu method according to the method of Singleton et al.^[12]. Weigh 25.0 mg of gallic acid standard accurately, add deionized water to dissolve and fix the volume to 25.0 mL to obtain 1.00 mg/mL gallic acid standard solution. Dilute with distilled water to obtain 0, 0.08, 0.16, 0.24, 0.32, 0.40 mg/mL polyphenol standard solution. Accurately aspirate 10 μ L of the standard solution and add it to the enzyme standard plate, then add 40 μ L of water and 10 μ L of Folin & Ciocalteu's phenol reagent. After 6 min reaction, add 100 μ L 150 g/L sodium carbonate solution and 80 μ L deionized water. The reaction was carried out for 2 h at room temperature, protected from light, and the absorbance was measured at 765 nm. The mass concentration of gallic acid (X) was used as the vertical coordinate, and the absorbance (Y) was used as the vertical coordinate. Standard curve equation: $Y=2.4171X+0.0706$ ($R^2=0.9953$). It shows that gallic acid has a good linear relationship with absorbance in the range of 0.00 ~ 0.40 mg/mL.

2.2.2 Determination of extraction rate of polyphenols from *C. longepaniculatum*

Weighed 0.100 g of *C. longepaniculatum* leaf powder was added into a test tube and extracted for 40 min at a certain concentration of ethanol, liquid to material ratio and temperature. After the extraction, the extract was filtered and fixed to 10.0 mL. The absorbance at 765 nm was measured according to the method of 2.1.1 and the blank was used as reference.

2.2.3 Optimization of extraction process of polyphenols from *C. longepaniculatum*

The basic conditions of the experiment were set as follows: ethanol concentration of 50%, liquid to material ratio of 20:1 mL/g, extraction temperature of 70 $^{\circ}$ C, and extraction time of 40 min. Single-factor experiments were used to investigate the effects of ethanol concentration (40, 50, 60, 70, 80%), liquid to material ratio (20: 1, 25: 1, 30: 1, 35: 1, 40: 1 mL/g), extraction temperature (30, 40, 50, 60, 70 $^{\circ}$ C) on the extraction rate of polyphenols from *C. longepaniculatum*, respectively.

2.2.4 Response surface optimization design experiment for the extraction of polyphenols from *C. longepaniculatum*

We designed a 3-factor, 3-level Box-Behnken's central combination experiment to optimize the extraction process of polyphenols from *C. longepaniculatum* based on the results of the single-factor test, and the experimental factor design levels are shown in Table 1.

Table 1: Test factors and levels of response surface optimization for the extraction of polyphenols from *C. longepaniculatum*

Level	Factor		
	Ethanol concentration/%	liquid to material ratio/(mL/g)	Extraction temperature/ $^{\circ}$ C
	A	B	C
-1	40	25	50
0	50	30	60
1	60	35	70

2.2.5 Determination of DPPH radical activity of polyphenols from *C. longepaniculatum*

The ethanol extract was concentrated to 1/4 of the original volume by rotary evaporator and then dissolved in water. Petroleum ether was added for extraction until the petroleum ether layer was colorless. Concentrate the water layer to 1/4 of its volume and add absolute ethanol to make the ethanol content 80%. Place the solution in a refrigerator at 4 $^{\circ}$ C for one night. The solution was filtered and the filtrate was concentrated into extract. Vacuum freeze-drying was performed to obtain crude polyphenols.

Antioxidant activity of extracts from *C. longepaniculatum* leaves was determined by DPPH (1, 1-diphenyl-2-trinitrohydrazine), referring to Brand-Williams et al.^[13]. Dissolve 1.0 mg DPPH in 25.0 mL ethanol to obtain 0.10 mmol/L DPPH ethanol solution. Dissolve 1.0 mg crude polyphenol in 2.0 mL of ethanol solution to obtain 0.50 mg/mL *C. longepaniculatum* polyphenol solution. Solutions of 0.40, 0.30, 0.20, and 0.10 mg/mL *C. longepaniculatum* polyphenol were obtained by dilution. Vitamin C was configured to the same concentration. The mixture was homogenized according to DPPH ethanol solution and the sample 1:1, and the absorbance was measured at 517 nm after 30 min reaction at room temperature and protected from light.

$$\text{Rate of clearance/\%} = \left[1 - \frac{A_i - A_j}{A_0} \right] \times 100 \quad (1)$$

A_i : absorbance value of sample solution + DPPH ethanol solution; A_j : absorbance value of sample solution + anhydrous ethanol solution; A_0 : absorbance value of anhydrous ethanol + DPPH ethanol solution.

2.2.6 Determination of tyrosinase inhibitory activity of polyphenols from *C. longepaniculatum*

The tyrosinase inhibition rate of polyphenol from *C. longepaniculatum* was determined according to the method of Lee et al.^[14]. Accurately weigh 0.0680 g L-tyrosine, add appropriate amount of water, add 4~5 drops of hydrochloric acid and heat in water bath. Adjust the pH to 7.0 with sodium hydroxide, and fix the volume to 25.0 mL. The concentration of L-tyrosinase solution prepared is 7.50 mmol/L. Weigh 0.0010 g tyrosinase dissolved in phosphate buffer, and fix the volume to 10.0 mL, and configure it into 100 U/mL tyrosinase solution. Weigh 0.0100 g of crude polyphenol and dissolve in 1.00 mL of dimethyl sulfoxide solution to obtain 10 mg/mL of *C. longepaniculatum* polyphenol solution. Dilute with ethanol to 8.00, 6.00, 4.00, 2.00 mg/mL *C. longepaniculatum* polyphenol solution. β -arbutin dissolved in dimethyl sulfoxide was configured to the same concentration as *C. longepaniculatum* polyphenol. Sodium phosphate buffer, different concentrations of the solution to be tested and tyrosinase solution were mixed well, and after standing for 10 min at room temperature, L-tyrosine solution was added to the reaction solution. After the reaction for 20 min, the absorbance was tested at 475 nm. Reaction system design of tyrosinase activity inhibition test are shown in Table 2.

Table 2: Reaction system design of tyrosinase activity inhibition test

Reagents	Standard control group	Negative control group 1	Test group	Negative control group 2	μL
Liquid to be tested	0	0	20	20	
Tyrosinase solution	40	40	40	40	
L-tyrosine solution	100	0	100	0	
Phosphate buffer	60	160	30	140	

$$\text{Rate of inhibition/\%} = \frac{(OD_A - OD_B) - (-OD_C - OD_D)}{(OD_A - OD_B)} \times 100 \quad (2)$$

OD_A : the absorbance of standard control group, OD_B : the absorbance of negative control group 1, OD_C : the absorbance of textl group, and OD_D : the absorbance of negative control group 2.

3. Results and discussion

3.1 Single factor experiment

3.1.1 Effect of ethanol concentration on the extraction rate of polyphenols from *C. longepaniculatum*

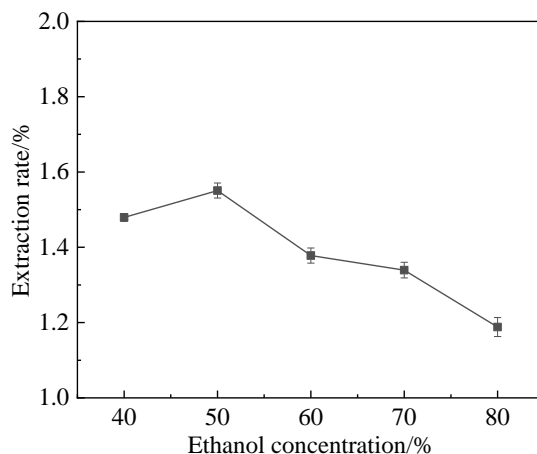


Figure 1: Effect of ethanol concentration on extraction rate of polyphenol

As can be seen from Fig. 1, with the increase of ethanol concentration, the rate of polyphenol extraction showed a trend of first increasing and then decreasing. When the ethanol concentration reached 50%, the highest rate of polyphenols extraction was $(1.56 \pm 0.02)\%$. The polarity of the extraction solution was similar to that of polyphenolic compounds, which made the polyphenols more soluble. When the concentration of ethanol was greater than 50%, the leaching rate of alcohol-soluble impurities, chlorophyll and other substances increased and competitively bound to ethanol, which affected the effect between polyphenolic compounds and the solvent^[15]. Water is a good solvent, but it is not easy to break the hydrogen bond. When ethanol is added to the aqueous solution it can break the hydrogen bond and improve the extraction rate of polyphenols^[16]. Therefore, 50 % ethanol solution is the best choice for the extraction of polyphenols from *C. longepaniculatum*.

3.1.2 Effect of liquid-material ratios on the extraction rate of polyphenols from *C. longepaniculatum*

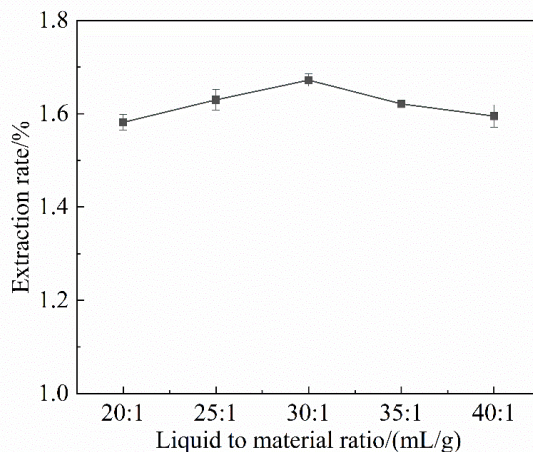


Figure 2: Effect of liquid to material ratio on extraction rate of polyphenol

We can see from Fig. 2, with the increase of liquid to material ratio, the extraction of polyphenols from *C. longepaniculatum* was firstly increased and then decreased, when the liquid to material ratio was 20~30 mL/g, the extraction rate of polyphenols increased, which might be due to the increase of contact area between *C. longepaniculatum* and solvent. And when the liquid to material ratio was greater than 30 mL/g, the dissolution of polyphenol from *C. longepaniculatum* had reached saturation state, which caused the decomposition of structurally unstable polyphenols and led to the decrease of polyphenol content^[17]. When the liquid to material ratio is higher, it leads to the separation of other components, which is not conducive to the subsequent concentration separation and purification^[18]. Therefore, we chose 30 mL/g as the condition for extracting polyphenols from *C. longepaniculatum*.

3.1.3 Effect of Extraction temperature on the extraction rate of polyphenols from *C. longepaniculatum*

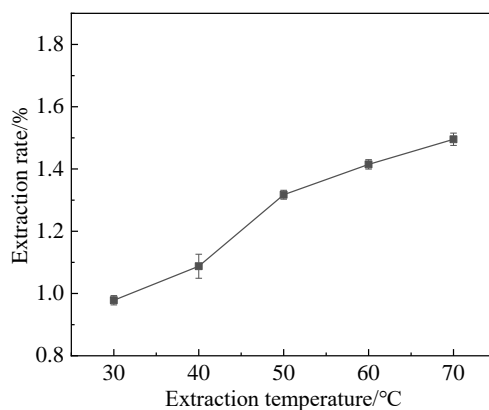


Figure 3: Effect of extraction temperature on extraction rate of polyphenol

As can be seen from Fig. 3, the polyphenol yield showed a gradual increase with the increase of the water bath temperature. This may be due to the fact that as the extraction temperature increases, the hydrogen bonds are easily broken, the rate of molecular movement is accelerated, and the rate of mass transfer and diffusion of materials is accelerated. Thus, the permeation, diffusion and dissolution of polyphenols are accelerated.

3.2 Response surface optimization design experiments

3.2.1 Response surface design and analysis of results

The ethanol concentration, liquid to material ratio and extraction temperature were used as independent variables, and the extraction rate of polyphenols from *C. longepaniculatum* was used as the response value. A total of 17 sets of experiments were conducted, and the response surface results are shown in Table 3.

Table 3: Results of Box-Behnken Response Surface test for Polyphenol Extraction from C. longepaniculatum

number	Ethanol concentration A	Liquid to material ratio B	Extraction temperature C	Extraction amount of polyphenols /%
1	0	0	0	1.66
2	0	0	0	1.64
3	-1	-1	0	1.49
4	-1	0	-1	1.45
5	1	-1	0	1.46
6	0	0	0	1.65
7	-1	1	0	1.48
8	1	1	0	1.46
9	0	-1	1	1.65
10	0	0	0	1.63
11	-1	0	1	1.61
12	1	0	-1	1.48
13	0	1	-1	1.49
14	0	0	0	1.65
15	0	-1	-1	1.49
16	1	0	1	1.60
17	0	1	1	1.68

Table 4: Analysis of variance for each term of developed quadratic regression model

Source	Sum of Squares	df	Mean Square	F-value	p-value	Level of significance
Model	0.1206	9	0.0134	49.59	< 0.0001	**
A	0.0001	1	0.0001	0.46	0.5183	
B	0.0000	1	0.0000	0.12	0.7422	
C	0.0474	1	0.0474	175.48	< 0.0001	**
AB	0.0000	1	0.0000	0.09	0.7670	
AC	0.0006	1	0.0006	2.13	0.1881	
BC	0.0003	1	0.0003	1.04	0.3424	
A ²	0.0491	1	0.0491	181.84	< 0.0001	**
B ²	0.0189	1	0.0189	69.95	< 0.0001	**
C ²	0.0000	1	0.0000	0.16	0.7025	
Residual	0.0019	7	0.0003			
Lack of Fit	0.0015	3	0.0005	5.44	0.0677	
Pure Error	0.0004	4	0.0001			
Cor Total	0.1225	16				

A. Ethanol concentration; B. Liquid to material ratio; C. Extraction temperature

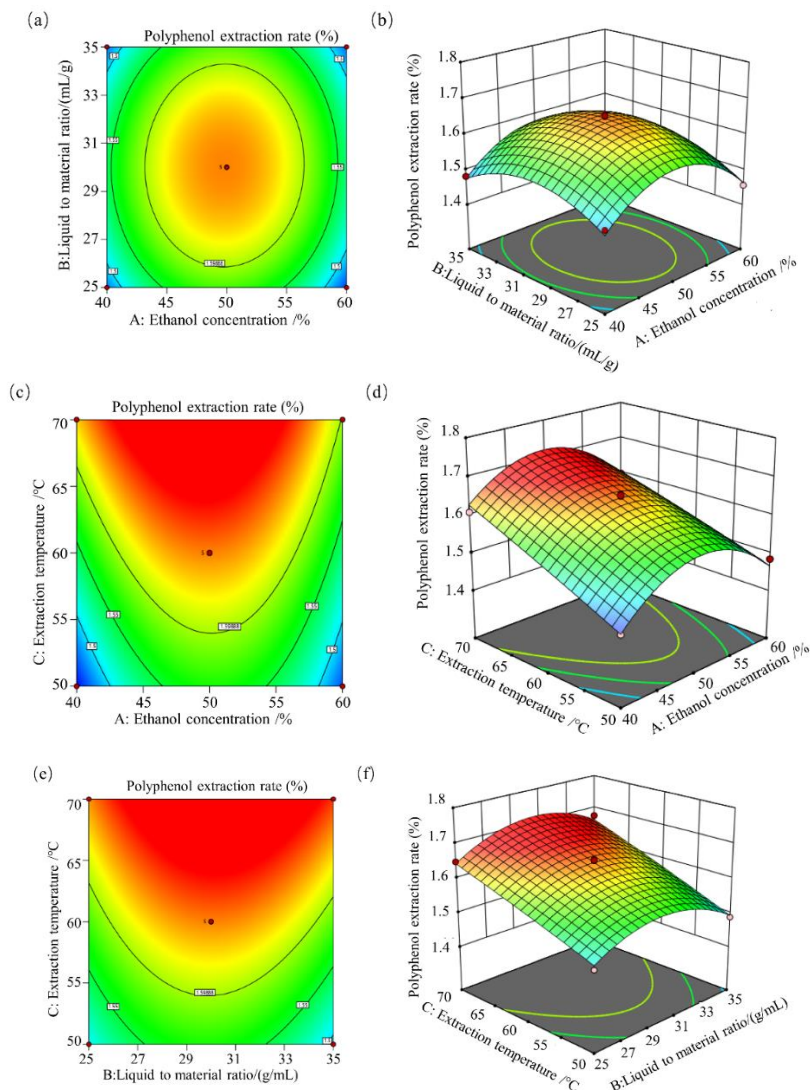
Note:* It indicates significant influence ($p < 0.05$), and ** indicates extremely significant influence ($p < 0.01$)

As can be seen from Table 3, the regression model has $p < 0.0001$, which indicates that the model is

extremely significant, while the correlation coefficient of the equation $R^2=0.9647$, which indicates that the predicted values of the model are highly correlated with the experimental values and the fit is good. The misfit of the model $F=5.44$, $p=0.0677>0.05$, indicating that the difference is not significant and has a small experimental error. The coefficient of variation (C.V. %=1.05) was $<5\%$, indicating that the stability of the model was good. The analysis of the above experimental results shows that the model can be used to predict and analyze the extraction process conditions of polyphenols from *C. longepaniculatum*.

According to the F value, the order of significance of the extraction effect of polyphenols from *C. longepaniculatum* was $C>A>B$, that is extraction temperature>ethanol concentration>liquid to material ratio. The effects of primary term C, secondary terms A^2 and B^2 were highly significant ($p<0.01$), and the effects of primary terms A and B, and interactive terms AB, AC, BC and C^2 were not significant ($p>0.05$); this indicated that the extraction rate of polyphenols from *C. longepaniculatum* was influenced by the interaction of various factors. The variance analysis and significance test of the model equation are shown in Table 4.

3.2.2 Response surface plot analysis



(a) (b): Effect of ethanol concentration and liquid to material ratio on extraction rate of polyphenols;
 (c) (d): Effect of ethanol concentration and extraction temperature on extraction rate of polyphenols;
 (e) (f): Effect of liquid to material ratio and temperature on extraction rate of polyphenols.

Figure 4: Response surface and contour plots of the effect of the interaction of different factors on the extraction rate of polyphenols from *C. Longepaniculatum*

The flatness and steepness of the response surface reflect the strength of the effect of a factor value on the response value. The steeper the slope, the stronger the effect of the factor value change on the

response value. From Fig. 4, it can be seen that among the three experimental factors, the extraction temperature was the main factor affecting the yield of polyphenols from *C. longepaniculatum*, and its surface had the largest slope and steepest slope; followed by the liquid to material ratio and ethanol concentration, which was consistent with the ANOVA results.

3.2.3 Determination and verification of optimal extraction conditions

Through software analysis, the optimal extraction process for extracting polyphenols from *C. longepaniculatum* is obtained: ethanol concentration 51.07%, Liquid to material ratio 29.91:1 mL/g, ultrasonic temperature 70.88 °C, under this condition, the extract rate of polyphenols from *C. longepaniculatum* is highest. Based on the actual situation, the ethanol concentration was optimized at 51%, the liquid-material ratio was 30:1 mL/g, and the ultrasonic temperature was 71 °C. The experiment was repeated three times under optimized conditions. The actual measured average yield of polyphenols from *C. longepaniculatum* was $(1.70 \pm 0.01)\%$, indicating that the experimental results are accurate and reliable.

3.2.4 Evaluation of antioxidant activity

Free radicals are prone to oxidation, and if the human body contains too many free radicals, it may lead to skin aging and many diseases, while antioxidants can scavenge free radicals, slow down aging and protect against many diseases^[19]. As shown in Fig. 5, the scavenging rate of DPPH free radicals increased gradually with the increase of extract concentration when the extract concentration was 0.1 ~ 0.2 mg/mL. When the extract concentration was 0.50 mg/mL, the scavenging rate reached 82.70%, which was similar to the positive control vitamin C scavenging rate. This indicates that the extract has a strong antioxidant capacity.

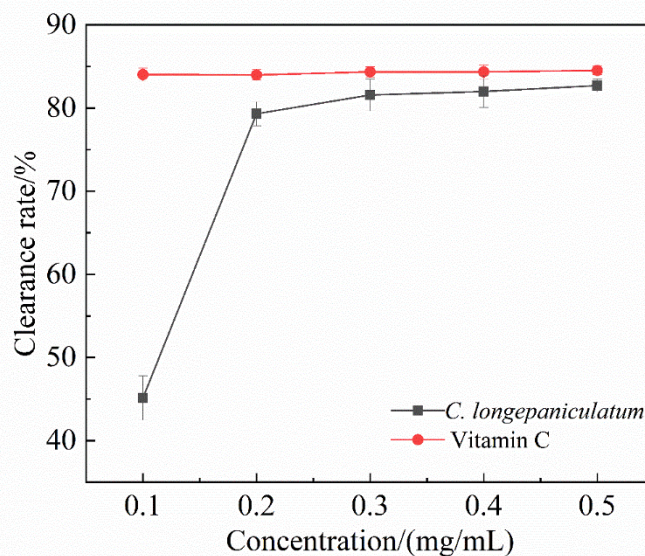


Figure 5: DPPH free radical scavenging rate of of polyphenol from *C. longepaniculatum*

3.2.5 Determination of tyrosinase inhibition

According to the experimental method in 2.1.6 the change of tyrosinase inhibition rate of *C. longepaniculatum* polyphenol with concentration is shown in Fig.6. With the increase of *C. longepaniculatum* polyphenol concentration, the inhibition rate of tyrosinase also increased gradually. When the concentration was 10.00 mg/mL, the inhibition rate could reach 91.78%, which could reach β -Arbutin has the same inhibitory effect. Tyrosinase is a key enzyme in melanin production. By inhibiting the activity of tyrosinase, melanin production can be inhibited, so as to achieve whitening effect. *C. longepaniculatum* polyphenol has a certain whitening effect^[20].

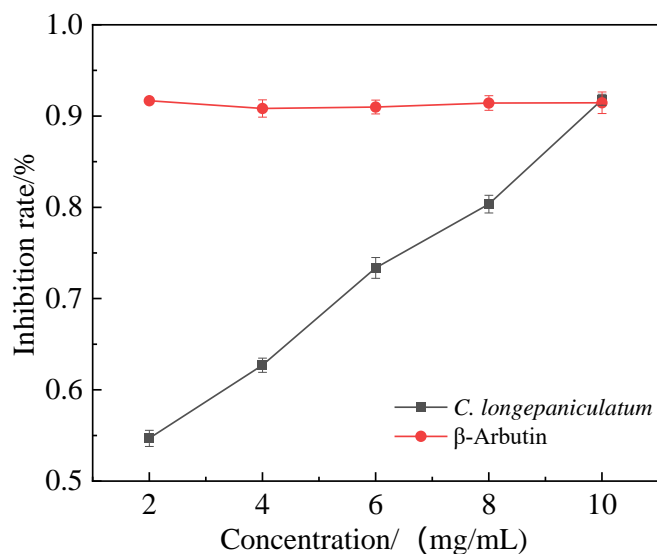


Figure 6: Tyrosinase inhibition rate of polyphenol from *C. longepaniculatum*

4. Summary

Optimize the test results of each single factor by response surface analysis. The concentration of ethanol was 51%, the ratio of liquid to material was 30:1 mL/g, and the extraction temperature was 71 °C, the extraction amount of polyphenols can reach $(1.70 \pm 0.01)\%$. The biological activity of polyphenols from *C. longepaniculatum* were analyzed, and the DPPH free radical scavenging rate of 0.5 mg/mL polyphenols from *C. longepaniculatum* were 82.70%, and the tyrosinase inhibition rate of 10 mg/mL polyphenols from *C. longepaniculatum* were 91.78%, indicating that the oil The polyphenolic extracts from *C. longepaniculatum* have good anti-oxidation and whitening effects. This provides a theoretical basis for the comprehensive development and utilization of polyphenols from *C. longepaniculatum*.

Acknowledgements

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