RESPONSE SURFACE METHODOLOGY OPTIMIZATION OF MICROWAVE IRRADIATION PRETREATMENT EXTRACTION OF FLAVONOIDS FROM *CLINOPODIUM GRACILE* (BENTH.) MATSUM., AND A STUDY OF ANTIMICROBIAL ACTIVITIES

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Abstract. *Clinopodium gracile* is one of the commonly used medicinal herbs of the She ethnic minority in China. The whole plant of *C. gracile* is rich in flavonoids. This study pioneers the investigation to improve flavonoid yield by the use of response surface methodology (RSM) and microwave irradiation pretreatment method. The antimicrobial activities of its extracts was also tested. The significant parameters of extraction conditions of the whole plant of *C. gracile* flavonoids (WPCF) were as follows: wetted time of 25.514 min, microwave radiation pretreatment time of 38.024 s, microwave power of 411.557 w, and mass of vaporization solvent of 1.841 mL. For the convenience of the experiment, the optimum process parameters were modified as follows: wetted time of 25 min, microwave radiation pretreatment time of 38 s, microwave power of 420 w, and mass of vaporization solvent of 1.8 mL. Under these conditions, the yield of WPCF was 145.677 mg/g, which was increased by 25.450% compared to the traditional water bath extraction, a significant difference was observed. Antimicrobial experimentations showed that the extracts of ethyl acetate exhibited good activities against *Pseudomonas aeruginosa* (ATCC9027), *Staphylococcus aureus* (ATCC29213), *Staphylococcus aureus* (SA1) with minimal inhibitory concentration (MIC) value of 16 mg/mL, 8 mg/mL, 8 mg/mL, respectively. Therefore, the processing technology in this study was successful and microwave irradiation pretreatment provided some practical significance for the extraction of WPCF. The results also suggest that the extracts from *C. gracile* could be used as a potential antimicrobial source.
Keywords: *Clinopodium gracile, RSM design, microwave irradiation pretreatment, extracts of ethyl acetate, antibacterial activity*

Introduction

Clinopodium gracile (Benth.) Matsum., one of the commonly used medicinal herbs of the She ethnic minority in China belonging to the Labiatae family, is mainly used to treat snake bites (Lei and Li, 2007). Recent studies have indicated that *C. gracile* contains some important compounds including flavonoids (Guo et al., 2000), terpenoids, steroids and saponins (Yamamoto et al., 1993; Chi and Lu, 2006). Besides, a total of 34 components of the essential oil from *C. gracile* aerial parts were characterized by our group in previous studies, in which the sesquiterpenoids were the principal compounds (70.49%) (Chen et al., 2013).

Previous investigation have demonstrated that the extracts of a plant from *Clinopodium* possessed various biological functions, such as antibacterial effects in the cases of *C. vulgare*, *C. ascendens*, *C. chinense* (Opalchenova and Obreshkova, 1999;

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Castilho et al., 2007; Stefanovic et al., 2011; Yang et al., 2009) and anticancer effects in human cancer cells (A2058, HEp-2 and L5178Y) in case of *C. ascendens* (Dzhambazov et al., 2002). The total saponins and flavonoids are the most effective active ingredients in the genera of *Clinopodium* (Chi and Lu, 2006), however, there is a lack of research on flavonoids from *C. gracile.* Few flavonoids in case of apigenin-7-*O*-β-*D* glucopyranoside, luteolin-7-*O*-β-*D* glucopyranoide and Vanillin were reviewed. (Zhong et al., 2012).

Attention has been focused on the optimization of flavonoids from plants due to their important activities. The response surface methodology (RSM) has been widely applied in the optimization process and proved to be an effective method to evaluate the key experimental parameters (Shangguan et al., 2023; Sun and Chen, 2023). Microwave irradiation pretreatment extraction has attracted extensive attention recently (Wang et al., 2016). This method is different from microwave-assisted extraction, the dry material is wetted to a certain extent first, and then place the plant in microwave irradiation to destroy the cell structure, then extracted by traditional methods. It is a time- and cost-effective means (Edgar et al., 2008). However, little effort has been made to optimize flavonoids from the whole plant of *C. gracile*.

Therefore, in this study, microwave irradiation pretreatment was used to research the extraction yield of WPCF and the experimental parameters were decided by RSM. The experimental parameters studied were wetted time, microwave radiation pretreatment time, microwave power and mass of vaporization solvent. Furthermore, the antimicrobial activity of WPCF obtained under microwave irradiation pretreatment conditions and extracted by ethyl acetate was evaluated.

Materials and methods

Plant materials

The whole plant of *C. gracile* was collected in Lishui, Zhejiang province, China, from May to June (excluding single factor experiments on mass of vaporization solvent, which is from March to April) and then air-dried for three weeks. The sample was powdered by a grinding mill passed through a 40-mesh sieve, and stored in a cool, dry place.

Microwave irradiation pretreatment (MIP)

Dried powder (5 g) of *C. gracile* was put in a glass conical flask (volume 250mL). the MIP parameters were MIP time (0, 30, 60, 90, and 120 s), power (140, 280, 420, 560, and 700 w), mass of vaporization solvent (purified water, 0, 2.5, 5, 10, and 20 mL) and wetted time (mixed thoroughly with purified water, 10, 20, 40, 60, and 80 min), of which single factor experiments were performed, respectively. The above experiments were done in a domestic microwave oven (P70F20CN3P-SR(WO) Galanz Microwave Life Electrical Co., Ltd., Guangdong, China). After MIP the sample was placed into a glass bottle with ethanol, then extracted by traditional methods. The traditional extraction time of 90 min, the temperature of 70℃, liquid to materials ratio of 40 mL/g, and the ethanol percentage of 65% (v/v) were chosen as fixed factors for experiments performed in a water bath (Grant SUB Aqua 12 Plus, Grant Instruments (Cambridge) Ltd., United Kingdom). Triplicate experiments were carried out.

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The extraction yield of WPCF

The extraction yield of total flavonoids was determined by following with some modifications (Lei and Chen, 2021), and rutin was selected as a reference. Briefly, 100 mg of dried rutin was weighed accurately, dissolved with 75% ethanol and transferred into a 50 mL volumetric flask by adding ethanol to the scale. 10 mL were precisely transferred to 100 mL volumetric flasks by adding water to scale. A series of soluted rutin (0.0, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 mL) were precisely transferred to 25 mL volumetric flasks by adding water to 6.0 mL. Then, 1 mL of 5% sodium nitrite was added for 6 min, followed by mixing with 1 mL of 10% aluminum nitrate very evenly. 10 mL of 4% sodium hydroxide was added to the volumetric flasks after 6 min, then, water was added to 25 mL and kept for 15 min. The absorbance was measured at 510 nm using a UV752N spectrophotometer (Jingke Experimental Instrument Co., Ltd., Shanghai, China). The linear response was as follows ($A = 10.64B - 0.001$, $R^2 = 0.9998$). C was the concentration of total flavonoids (mg/mL) at the wavelength of 510 nm. The total flavonoid yield was calculated by the following equation:

$$Y = \frac{C \times V \times M}{W} \times 100\% \quad \text{(Eq.1)}$$

where Y is total flavonoids yield (mg/g), V is the volume of extraction (mL), M is the dilution multiple of extraction and W is the weight of the sample (g).

RSM design

RSM (Design-Expert, trial version 8.0.6) was applied to optimize the conditions of extraction of WPCF based on the single-factor experiments. Four independent factors (X_1, wetted time; X_2, MIP time; X_3, MIP power; X_4, mass of vaporization solvent) and three levels (−1, 0, 1) were performed using Box-Behnken design. The yield of WPCF (Y) was chosen as the response of the experiments. A quadratic model was taken as follows:

$$Y = k_0 + \sum_{i=1}^{4} k_i x_i + \sum_{i=1}^{4} k_{ii} x_i^2 + \sum_{i<j=2}^{4} k_{ij} x_i x_j \quad \text{(Eq.2)}$$

where Y is the predicted yield of WPCF; k_0 is a constant; k_i is the coefficients of the linear; k_{ii} represents quadratic; k_{ij} represents interaction terms; and X_i and X_j represent the response variables.

Antimicrobial experiments

Antimicrobial experiments were performed using the agar dilution method formulated by the Clinical and Laboratory Standards Institute (CLSI) to obtain the minimum inhibitory concentrations (MICs) (Clinical and Laboratory Standards Institute, 2017; Lu et al., 2018). Three replications were tested. The ethanol extract was preprocessed by MIP from *C. gracile* and then extracted with ethyl acetate, the solvent was recycled by a rotary evaporator (RV8, IKA, Germany). The strains including *Staphylococcus aureu*s (ATCC29213), *Staphylococcus aureu*s (SA1), *Pseudomonas*

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aeruginosa (ATCC9027), *Pseudomonas aeruginosa* (PA1) and *Escherichia coli* (ATCC25922) were obtained from school of medicine and health, Lishui University, Lishui, China. PA1 and SA1 were isolated from clinical practice.

Statistical analysis

Design–Expert, version 8.0.6 was used for the experiment design. The data obtained from experiments were expressed as mean ± standard deviation and triplicate. Statistical analysis was achieved using an independent samples t-test in *Table 4* by SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). p-value < 0.01 was determined as a highly significant difference.

Results

Effect of mass of vaporization solvent

As shown in *Figure 1A*, the effect of different masses of vaporization solvent on the yield of WPCF was studied when the following factors were chosen as uniform conditions, MIP time 60 s, power 420 w, and wetted time 5 min, respectively. The yield increased slightly from 221.961 mg/g to 222.222 mg/g at the mass of vaporization solvent between 0 mL and 2.5 mL but decreased rapidly to the minimum level (195.698 mg/g) at 5 mL. The yield of WPCF increased inconspicuously from 201.284 mg/g to 202.068 mg/g between 10 mL and 20 mL. Thus, the range of mass of the vaporization solvent used in RSM analysis was 0-5 mL.

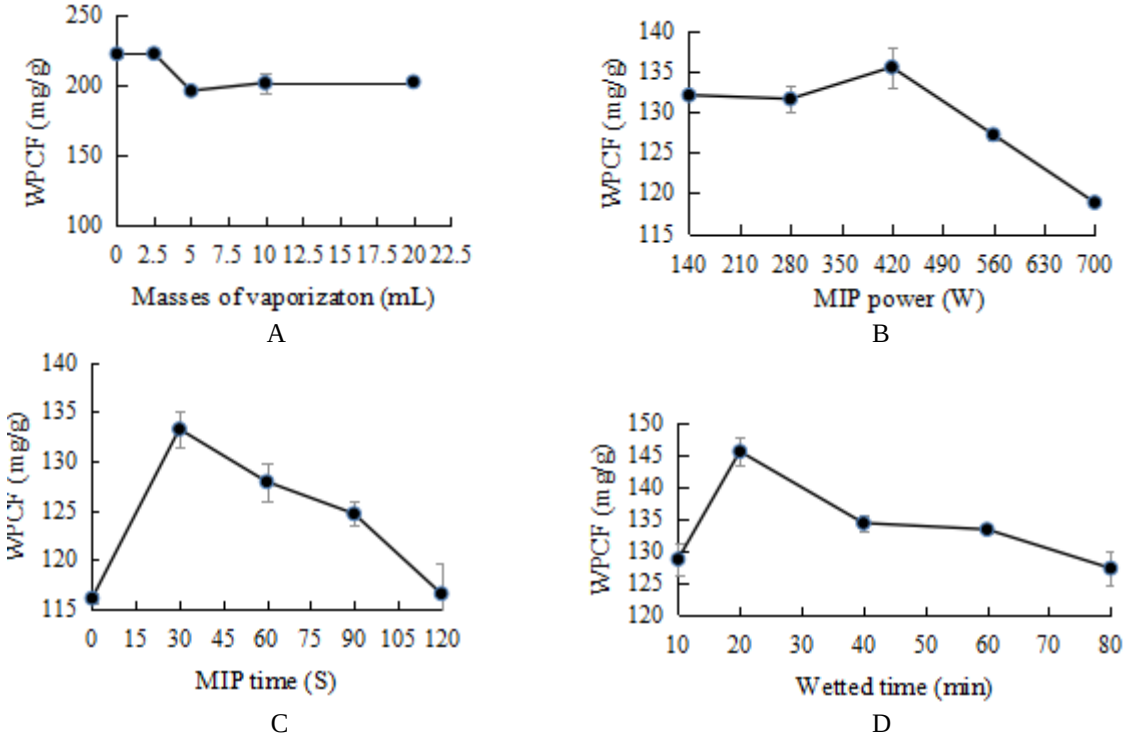

Figure 1. *The effects of masses of vaporization, MIP power, MIP time and Wetted time on the whole plant of C. gracile flavonoids (WPCF)*

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Effect of MIP power

As shown in *Figure 1B*, the effect of different MIP power on the yield of WPCF was studied when the following factors were chosen as uniform conditions, MIP time 60 s, mass of vaporization solvent 2.5 mL, and wetted time 5 min, respectively. The change of the yield was not obvious from 140 to 280 w but increased up to a maximum (135.495 mg/g) at 420 w. The yield finally dropped to the minimum at 700 w (118.839 mg/g). Thus, the range of MIP power used in RSM analysis was 280-560 w.

Effect of MIP time

As shown in *Figure 1C*, the effect of different MIP times on the yield of WPCF was studied when the following factors were chosen as uniform conditions, MIP power 420 w, mass of vaporization solvent 2.5 mL, and wetted time 5 min, respectively. The yield of WPCF increased rapidly when MIP time increased from 0 s to 30 s, and then decreased obviously when exceeding 30 s. The yield finally dropped to a low level at 120 s (116.594 mg/g). Thus, the range of MIP time used in RSM analysis was 0-60 s.

Effect of wetted time

As shown in *Figure 1D*, the effect of different wetted times on the yield of WPCF was studied when the following factors were chosen as uniform conditions, MIP power 420 w, mass of vaporization solvent 2.5 mL, and MIP time 30 s, respectively. The yield of WPCF increased up to a maximum (145.468 mg/g) at 20 min and then decreased when wetted time ascended from 20 min to 80 min. Thus, the range of wetted time used in RSM analysis was 10-40 min.

Analysis of RSM model

The significant parameters including wetted time (X_1), MIP time (X_2), MIP power (X_3), and mass of vaporization solvent (X_4) were analyzed using BBD based on single-factor results. A total of 29 runs of different combinations were shown in *Table 1*. The predicted regression model could be expressed as follows:

$$Y = 28.22702 + 1.74379X_1 + 1.29928X_2 + 0.33931X_3 + 2.56884X_4 + 0.00315960X_1X_2 + 0.000313030X_1X_3 + 0.000872414X_1X_4 - 0.000217560X_2X_3 - 0.020190X_2X_4 + 0.00205143X_3X_4 - 0.039083X_1^2 - 0.016479X_2^2 - 0.000416384X_3^2 - 0.72448X_4^2$$

The ANOVA results are shown in *Table 2*. According to the analysis of ANOVA, the model was highly significant (*F*-value 55.54, *p-value* < 0.0001) and the lack of fit was not significant (*F*-value 3.84, *p-value* < 0.1034). It indicated that the model equation was appropriate for predicting the yield of WPCF. The values of R^2 and R^2_{adj} were 0.9823 and 0.9646, respectively, indicating that the model had a good fit which was also supported by the low value of coefficient of variation (*C.V.* = 1.35%).

As a result, the coefficients of X_2, X_4, X_1^2, X_2^2, X_3^2, and X_4^2 were highly significantly different (*p-value* < 0.01), which showed that they had effects on the yield of WPCF.

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Analysis of 3D response surface

Three-dimensional (3D) graphs were generated to depict the interactions between two factors when the other factors were set to zero level. In this study, the results of the yield of WPCF affected by the four factors of MIP are shown in *Figure 2*.

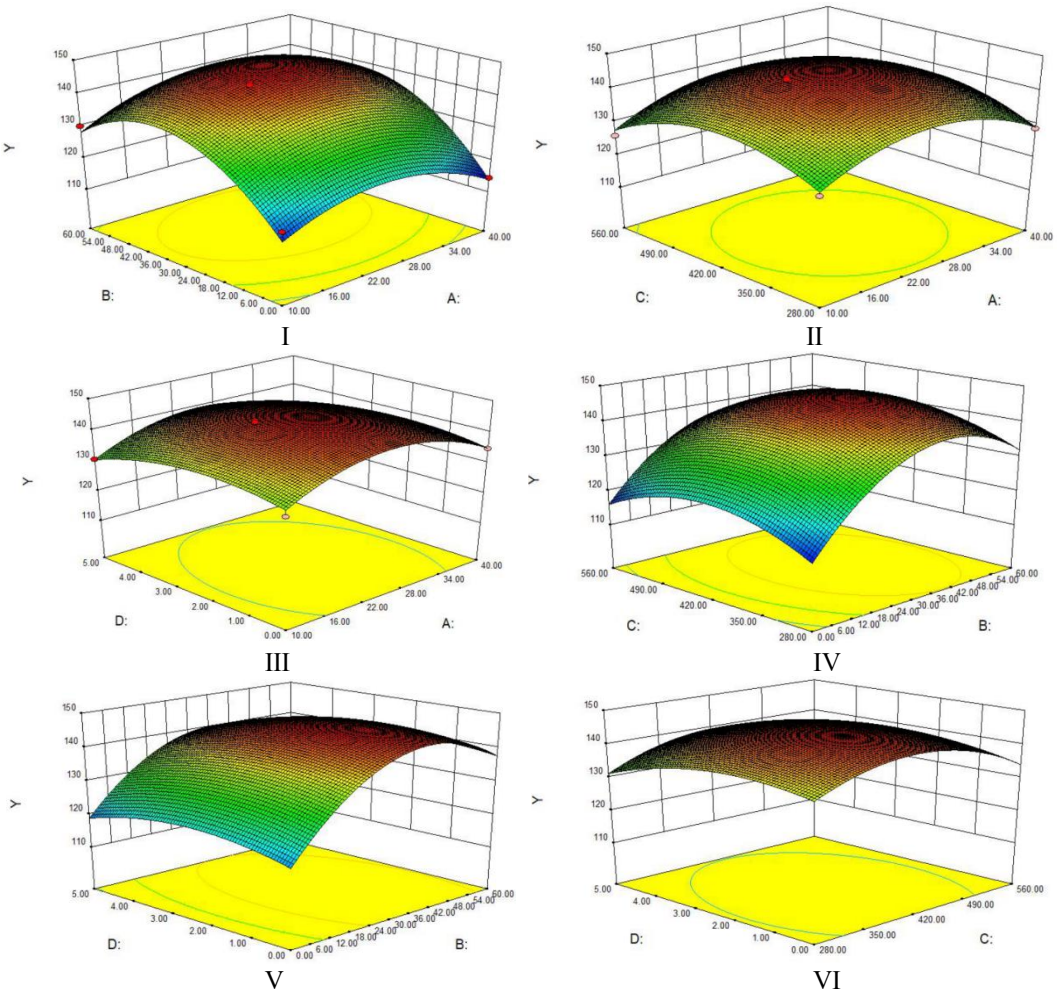

Figure 2. *3D response surface map of total flavonoids extraction. A: wetted time, B: MIP time, C: MIP power, D: mass of vaporization solvent*

Figure 2 Ⅰ shows the effects of wetted time and MIP time on the yield of WPCF at a MIP power of 420 w and mass of vaporization solvent at 2.5 mL. MIP time had an influence on the yield, but wetted time had a slight influence on the yield, and the interactions between them were not significant (p-value = 0.1170). The yield of WPCF increased obviously with the increase of MIP time from 0 to 30 s, indicating that a further increase of MIP time would not increase the yield.

Figure 2 Ⅱ showed the effects of wetted time and MIP power on the yield of WPCF at MIP time 30 s and mass of vaporization solvent 2.5 mL, showed that both of the two factors had slight influences on the yield, and the interactions between them were not significant (p-value = 0.4527).

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Figure 2Ⅲ shows the effects of wetted time and mass of vaporization solvent on the yield of WPCF at MIP time 30 s and MIP power 420 w. The mass of the vaporization solvent had an influence on the yield, but wetted time had a slight influence on the yield, and the interactions between them were not significant (p-value = 0.9699). The yield of WPCF decreased with the mass of the vaporization solvent exceeding 5.0 mL, indicating that a further increase in the mass of the vaporization solvent would not increase the yield.

Figure 2Ⅳ shows the effects of MIP time and MIP power on the yield of WPCF at a wetted time of 25 min and mass of vaporization solvent 2.5 mL. MIP time had an influence on the yield, but MIP power had a slight influence on the yield, and the interactions between them were not significant (p-value = 0.3178).

Figure 2 Ⅴ shows the effects of MIP time and mass of vaporization solvent on the yield of WPCF at a wetted time of 25 min and MIP power 420 w. Both MIP time and mass of vaporization solvent had influences on the yield, but the interactions between them were not significant (p-value = 0.1081).

Figure 2Ⅵ shows the effects of MIP power and mass of vaporization solvent on the yield of WPCF at a wetted time of 25 min and MIP time at 30 min. The mass of the vaporization solvent had an influence on the yield, but MIP power had a slight influence on the yield, and the interactions between them were not significant (p-value = 0.4293).

Table 1. *BBD design and results for RSM*

Run	Variables				Yield of WPCF (mg/g)
	X_1	X_2	X_3	X_4	
1	0	0	1	-1	134.973 ± 2.903
2	0	-1	1	0	115.497 ± 1.010
3	0	0	-1	-1	138.210 ± 2.934
4	-1	-1	0	0	119.100 ± 0.548
5	-1	1	0	0	130.065 ± 1.229
6	-1	0	1	0	125.940 ± 1.867
7	-1	0	0	1	130.644 ± 1.297
8	1	-1	0	0	114.401 ± 0.832
9	0	0	0	0	145.520 ± 1.039
10	0	0	0	0	146.094 ± 1.542
11	1	0	-1	0	128.864 ± 0.656
12	0	-1	0	-1	117.325 ± 0.627
13	0	0	1	1	130.535 ± 1.979
14	0	0	0	0	143.484 ± 2.396
15	0	1	1	0	127.141 ± 1.584
16	-1	0	0	-1	132.495 ± 1.347
17	1	1	0	0	131.579 ± 1.469
18	0	-1	-1	0	114.557 ± 1.249
19	0	1	0	-1	135.495 ± 1.347
20	0	-1	0	1	116.855 ± 1.039

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21	1	0	1	0	128.759 ± 1.786
22	0	0	0	0	144.632 ± 2.809
23	0	1	0	1	128.968 ± 1.018
24	0	1	-1	0	129.856 ± 1.273
25	1	0	0	1	132.049 ± 1.062
26	0	0	0	0	145.468 ± 1.689
27	-1	0	-1	0	128.968 ± 1.175
28	0	0	-1	1	130.900 ± 1.350
29	1	0	0	-1	134.816 ± 1.542

Table 2. *ANOVA for regression model analysis of WPCF yield*

Source	**Sum of squares**	**df**	**Mean square**	***F*-value**	***p*-value**	**Significance**
Model	2419.50	14	172.82	55.54	< 0.0001	**
X_1	0.88	1	0.88	0.28	0.6025	Not significant
X_2	582.17	1	582.17	187.08	< 0.0001	**
X_3	3.34	1	3.34	1.07	0.3175	Not significant
X_4	39.67	1	39.67	12.75	0.0031	**
X_1X_2	8.69	1	8.69	2.79	0.1170	Not significant
X_1X_3	1.86	1	1.86	0.60	0.4527	Not significant
X_1X_4	0.004598	1	0.004598	0.001478	0.9699	Not significant
X_2X_3	3.34	1	3.34	1.07	0.3178	Not significant
X_2X_4	9.17	1	9.17	2.95	0.1081	Not significant
X_3X_4	2.06	1	2.06	0.66	0.4293	Not significant
X_1^2	373.86	1	373.86	120.14	< 0.0001	**
X_2^2	1426.81	1	1426.81	458.50	< 0.0001	**
X_3^2	432.03	1	432.03	138.83	< 0.0001	**
X_4^2	132.99	1	132.99	42.74	< 0.0001	**
Residual	43.57	14	3.11			
Lack of Fit	39.45	10	3.95	3.84	0.1034	Not significant
Pure Error	4.11	4	1.03			
Cor Total	2463.06	28				
R^2	0.9823					
R^2_{adj}	0.9646					
C.V.%	1.35					

**Highly significant ($p < 0.01$)

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Antimicrobial activity

The antimicrobial activities of ethanol extract preprocessed by MIP after extracted with ethyl acetate were evaluated by agar dilution method. Concentrations of 16, 8, 4, 2, 1 and 0.5 mg/mL of ethyl acetate extract were tested. As shown in *Table 3*, The MIC to ATCC25922 and PA1 exceeded 16 mg/mL, indicating that they had a high resistance level. ATCC9027 had a high MIC level (16 mg/mL), and the value was twice ATCC29213 and SA1, which indicated that the extract had good activities against ATCC29213 and SA1.

Table 3. *Antimicrobial activity of ethyl acetate extract from WPCF (MIC, mg/mL)*

	ATCC9027	**PA1**	**ATCC29213**	**SA1**	**ATCC25922**
MIC of ethyl acetate extract	16	/	8	8	/

Discussion and conclusions

As shown in this article, microwave irradiation pretreatment was an efficient extraction method that has been used in the extraction of WPCF. It can better dissolve total flavonoids and improve extraction efficiency by damaging the plant cell wall without damaging the extract (Yang, 2008; Wang et al., 2016). But, the processing range of each factor was crucial for extraction. In single-factor results, the scholar found the yield of flavonoids from *Toona sinensis* (A. Juss.) Roem increased up to a maximum at 2.5 mL of vaporization solvent but decreased obviously when exceeding 2.5 mL (Wang et al., 2016). The results were consistent with ours. The excessive amount of vaporizer made some vaporizers free from the raw material and absorbed part of the microwave. As a result, the microwave which reached the inside of the raw material decreased and the yield of flavonoids decreased. The MIP power results were consistent with a previously published study (Wang et al., 2016), High microwave power destroyed total flavonoids. MIP time had a positive effect on the yield of total flavonoids, but with the increment of the time, a negative effect on the yield occurred (Qin et al., 2008).

The extract of *C. gracile* showed good inhibitory effect on *Staphylococcus aureu*s. Its MIC was significantly lower than the inhibitory effect of total flavonoids from *Actinidia arguta* (Siebold & Zucc.) Planch. ex Miq., the MIC of the latter was greater than 25 mg/mL (Zhang et al., 2012). The inhibitory effect of purified total flavonoids from *Catalpa ovata* G. Don root bark on *Staphylococcus aureus* was similar to our result (Shao et al., 2017). The antibacterial effect of *C. gracile* extract was also better than that of *Eupatorium odoratum* Linn. total flavonoid extract, and *Cinnamomum longepaniculatum* (Gamble) N. Chao leaf total flavonoid extract (Zheng et al., 2015; Du et al., 2015).

RSM was performed to analyze and optimize the significant experiment parameters for MIP. This method had been proven to be feasible. The extraction conditions of WPCF were as follows: wetted time of 25.514 min, microwave radiation pretreatment time of 38.024 s, microwave power of 411.557 w, and mass of vaporization solvent of 1.841 mL. For the convenience of the experiment, the optimum process parameters were modified as follows: wetted time of 25 min, microwave radiation pretreatment time of 38 s, microwave power of 420 w, and mass of vaporization solvent of 1.8 mL. In *Table 4*, under the modified extraction conditions, the yield of WPCF was 145.677 mg/g, which was increased by 25.450% compared to the traditional water bath extraction, and a highly significant difference was observed (p-*value* < 0.01, t-test).

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Table 4. *The extraction yields of WPCF by MIP compared with traditional water bath*

Method	MIP intervention	Traditional water bath
Wetted time (min)	25	0
Microwave radiation pretreatment time (s)	38	0
Microwave power (w)	420	0
Mass of vaporization solvent (ml)	1.8	0
Ethanol percentage (%)	65	65
Water bath temperature (℃)	70	70
Liquid to materials ratio (ml/g)	40	40
Extraction yields of WPCF (mg/g)	145.677 ± 2.113^{a}	116.124 ± 0.562^{b}

Different lowercase letters in extraction yields indicate a highly significant difference between the data ($p < 0.01$ t-test)

The extracts obtained from ethanol extract which extracted with ethyl acetate from *C. gracile* exhibited good antimicrobial activity against ATCC29213 and SA1. So, the analysis of bioactive compounds of the extract from *C. gracile* deserves attention in further study.

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