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Ultrasonic Microwave-Assisted Extraction of Polyphenols, Flavonoids, Triterpenoids, and Vitamin C from *Clinacanthus nutans*

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Abstract

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Polyphenols, flavonoids, triterpenoids, and vitamin C from *Clinacanthus nutans* were extracted by an ultrasonic microwave-assisted method. The optimal extraction conditions were established in distilled water, solid-liquid ratio 1 : 55 g/ml, irradiation power 90 W, and extraction cycle with 75 seconds. Results revealed that the solid-liquid ratio exerted a significant effect on polyphenols, whereas the irradiation power played a key role in the extraction of flavonoids, triterpenoids, and vitamin C. Under these conditions the extraction yield of each component was: polyphenols 8.893, flavonoids 25 936, triterpenoids 16 789, and vitamin C 0.166 mg/g. These results showed that the ultrasonic microwave-assisted extraction was an efficient technology to extract bioactive substances from *Clinacanthus nutans* and *Clinacanthus nutans* could potentially be a source of natural antioxidants.

Keywords: herbaceous plant; bioactive substance; ultrasonic microwave-assisted extraction; yield

Clinacanthus nutans, a member of the family Acanthaceae, is a magnificent herbaceous plant which is widely distributed in China, Thailand, Vietnam, Malaysia, and Indonesia (Yi *et al*. 2012). Its leaves have important roles in clearing heat, diuresis, detumescence with wet, invigorating the circulation of hydrophobic, clearing moisture, and anti-tumour action (Wang *et al*. 2013). Malaysian local cancer patients found that *Clinacanthus nutans* had antitumor effects on the body, so that many medical researchers all over the world were attracted (Teshima *et al*. 1997). Studies showed that *Clinacanthus nutans* contained abundant bioactive substances, such as extensive sterols, polyphenols, flavonoids, triterpene glycosides, and sulphur compounds (Sakdaart *et al*. 2009). Zhang *et al*. 2014 extracted total flavonoids from *Clinacanthus nutans* by ultrasound-assisted extraction, but there are only a few literature sources about bioactive extracts from *Clinacanthus nutans* by ultrasonic-microwave method. Hence, the ultrasonic microwave-assisted method was chosen in this study to extract bioactive substances from *Clinacanthus nutans*. This technology combines the ultrasonic vibration cavitation with a high-energy effect of microwaves and completes the process under the conditions of low temperature and atmospheric pressure (Liu *et al*. 2014; Tang *et al*. 2017). Employing a sequential ultrasonic-microwave extraction could potentially minimise or prevent the degradation of extract (Shan *et al*. 2016). The combined techniques could get higher yields and are suitable for the extraction of thermally labile active compounds (Cristina & Timothy 2010; Chen *et al*. 2010).

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The aim of this study therefore is to optimise the process of extracting the bioactive substances from *Clinacanthus nutans* by ultrasonic microwave-assisted extraction. Various parameters including ultrasonic-microwave time, irradiation power, and solid-liquid ratio of the method were studied. The optimal extraction conditions have been determined to provide a reference for the further development and utilisation of *Clinacanthus nutans*.

MATERIAL AND METHODS

Materials and chemicals. *Clinacanthus nutans* was obtained from Wuzhishan Wanjiabao Science and Technology Co., Ltd (China). Gallic acid, rutin, oleanolic acid, and vanillic aldehyde were obtained from Yuanye Biological Science and Technology Co., Ltd (China). Forint phenol was purchased from Suolaibao Science and Technology Co., Ltd (China). All solvents and chemicals used in this study were of analytical grade.

Process flow. *Clinacanthus nutans* powder was mixed with distilled water. The mixture solution was placed in an ultrasonic-microwave synergistic extraction apparatus (CW-2000, China). The extracts were centrifuged at 4000 *g* for 10 min and the supernatant was obtained.

Design of extraction test. A single factor test: according to the results of preliminary experiments, microwave power (20, 40, 60, 80, 100, and 120 W, respectively), ultrasonic-microwave time (30, 60, 90, 120, 150, and 180 s, respectively), and solid-liquid ratio (1 : 30, 1 : 40, 1 : 50, 1 : 60, 1 : 70, and 1 : 80 g/ml, respectively) were selected as the factors of a single factor test. Under the condition that other factors are invariable, make a research on the influence of each factor on the parameters of yields. Polyphenols, flavonoids, triterpenoids, and vitamin C were selected to evaluate the effects of extraction of *Clinacanthus nutans*.

A three-factor and three-level orthogonal test was employed to investigate and optimise the effects of process variables on polyphenol, flavonoid, triterpenoid, and vitamin C yields from *Clinacanthus nutans*. The design of factors and levels is shown in Table 1. The irradiation power ranged from 70 W to 90 W with ultrasonic-microwave time between 75 and 105 s, while the solid-liquid ratio was varied between 1 : 45 and 1 : 55 g/ml. In all of the experiments, the extraction of samples was conducted in triplicates.

Table 1. Factors and their levels in an orthogonal array design

Level	Factor		
	A	B	C
1	70	75	1 : 45
2	80	90	1 : 50
3	90	105	1 : 55

A – irradiation power (W); B – ultrasonic-microwave time (s); C – solid-liquid ratio

Determination of polyphenol yield. The polyphenol yield (mg/g) was calculated using Equation (1):

$$\text{Polyphenol yield} = (C_1 \times V_1)/m \quad (1)$$

where C_1 – concentration of polyphenols (mg/ml); V_1 – volume of the fluid under test (ml); m – weight of *Clinacanthus nutans* (g)

The method to determine the yield of polyphenols was performed as in a previous report with some modifications (Singleton *et al*. 1965). The adsorption of different samples was measured with a UV spectrophotometer (Longnike Equipment Company, China) at 760 nm. The standard curve of polyphenols was obtained as follows: $A = 91.406\,C_1 + 0.0159$, $r^2 = 0.9958$

Determination of flavonoid yield. The flavonoid yield (mg/g) was calculated using the Equation (2):

$$\text{Flavonoids yield} = (C_2 \times V_2)/m \quad (2)$$

where C_2 – concentration of flavonoids (mg/ml); V_2 – volume of the fluid under test (ml); m – weight of *Clinacanthus nutans* (g)

The method to determine the yield of flavonoids was used according to a previous report with some modifications (Wang *et al*. 1995). The adsorption of different samples and extraction were measured with a UV spectrophotometer at 510 nm. The standard curve of flavonoids can be obtained as follows: $A = 5.8766\,C_2 + 0.0159$, $r^2 = 0.9955$.

Determination of triterpenoid yield. The triterpenoid yield (mg/g) was calculated using equation (3):

$$\text{Triterpenoids yield} = (C_3 \times V_3)/m \quad (3)$$

where C_3 – concentration of triterpenoids (mg/ml); V_3 – volume of the fluid under test (ml); m – weight of *Clinacanthus nutans* (g)

The method to determine the yield of triterpenoids was used according to a previous report with

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some modifications (Yao *et al*. 2009). The adsorption of different samples was measured with a UV spectrophotometer at 560 nm. The standard curve of triterpenoids can be obtained as follows: A = 42.899 C_3 –0.0162, r^2 = 0.9958.

Determination of vitamin C yield. The percentage of vitamin C yield (mg/g) was calculated using equation (4):

$$\text{Vitamin C yield} = [(V_2 - V_0) \times \text{T}]/m \quad (4)$$

where V_0 – volume of 2,6-dichloroindophenol that the blank solution consumed (ml); V_2 – volume of 2,6-dichloroindophenol that the titration consumed (ml); T – titre (mg/ml); m – quality of *Clinacanthus nutans* (g)

The method to determine the yield of vitamin C was used according to a previous report with some modifications (Stephane *et al*. 2005).

Statistical analysis. All results are presented as means ± SD (standard deviation). Statistical analysis was performed with one-way analysis of variance (ANOVA) by SPSS software (version 19.0 for Windows, SPSS Inc., USA). The figures were drawn by Origin (version 8.0 for Windows, Origin Lab Inc., USA).

RESULTS AND DISCUSSION

Effect of irradiation power on yield. The irradiation power affects extraction performance. The microwave power must be controlled to obtain a desired set of temperatures to avoid excessive temperature and overpressure in the extraction process. The effect of the microwave irradiation power on yields of polyphenols, flavonoids, triterpenoids, and vitamin C was evaluated. The rest of the variables were a solid-liquid ratio of 1 : 40 and each extraction cycle of 90 seconds. The yields of bioactive ingredients were gradually enhanced with an increase in the irradiation power (Figure 1). At a higher microwave power, the temperature of the solution increased to promote the diffusion of targeted compound. It was so because the higher irradiation power increased the motor speed of the material and sample cell molecular. Based on the encouraging literature, they absorbed more microwave energy which increased to damage the sample cell for leaching of the active ingredients (Liu 2010). However, the yields showed a declining trend when the microwave power was more than 80 W. In addition, the high microwave irradiation power did not result in high yields of bioactive ingredients,

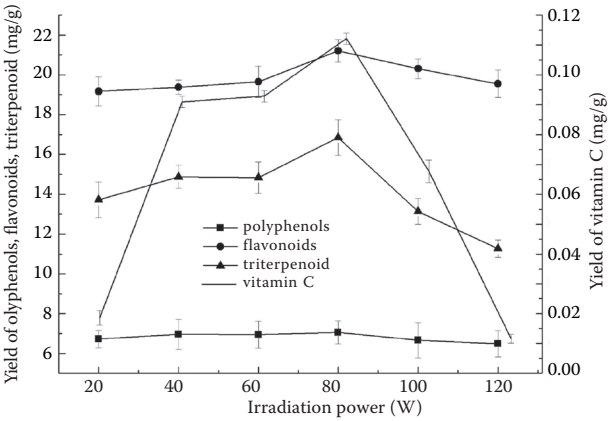

Figure 1. Effect of the microwave power on yield of polyphenols, flavonoids, and triterpenoid

and the yields of flavonoids, triterpenoids, and vitamin C decreased significantly. This fact is attributed to the increasing power of moisture content in the sample which destroyed the bioactive substances (Wang *et al*. 2010). Different bioactive substances presented different trends. This phenomenon was relevant with their molecular structure. The present work indicated that the microwave irradiation power of 80 W gave the highest yield.

Effect of ultrasonic-microwave time on yield. The main advantage of ultrasonic microwave-assisted extraction compared to a conventional method is time saving. Careful selection of irradiation time is crucial. The yields of polyphenols, flavonoids, triterpenoids, and vitamin C after various extractions are shown in Figure 2. The highest yield was obtained by the extraction at 80 W for 90 seconds. During the process, the solid-liquid ratio was 1 : 40 g/ml. Ultrasonic-microwave time played an important

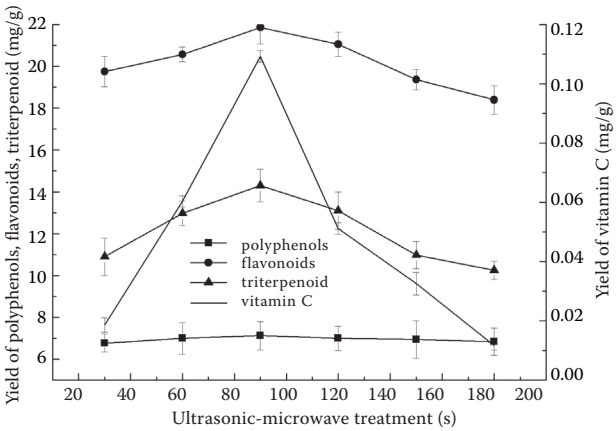

Figure 2. Effect of the ultrasonic-microwave treatment time on yield of polyphenols, flavonoids, triterpenoid, and vitamin C

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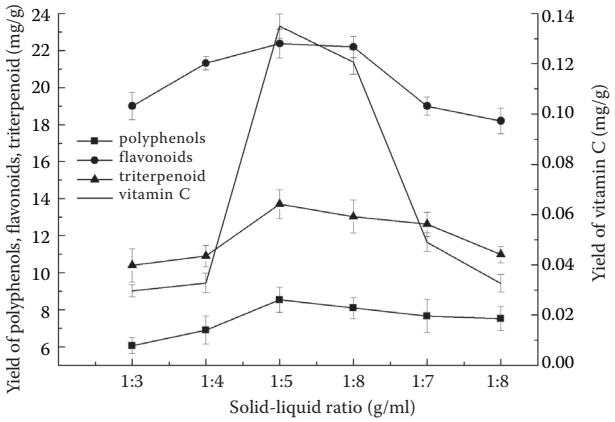

Figure 3. Effect of the solid-liquid ratio on yield of polyphenols, flavonoids, triterpenoid, and vitamin C

role in the yields. Before 90 s, yields increased as the extraction time increased. A short reaction time might be inadequate to bioactive substances. While after 90 s, extraction yields kept decreasing until 180 seconds. The irradiation time of 120 s gave decreasing yields (Figure 2). It is interesting to note that the longer irradiation time increased the microwave energy from the sample cell molecular. It indicated that an increase of the irradiation time promoted the destruction of the cell wall, so that the yields of the four active substances increased obviously. But the long time of microwave radiation damaged the structure of molecules with the decreases of the yields, which was unfavourable for the extraction of active substances. Thus, 90 s was deemed as the optimal ultrasonic-microwave irradiation time.

Effect of solid-liquid ratio on yield. In general, an appropriate solid-liquid ratio increases solubility in the solution. Thus, it is important to compare the effects of different solid-liquid ratios on yields of polyphenols, flavonoids, triterpenoids, and vitamin C.

Table 2. Orthogonal test

No.	A	B	C	Polyphenols	Flavonoids	Triterpenoid	Vitamin C
				(mg/g)			
1	1	1	1	7.549 ± 0.89	23.344 ± 0.89	14.315 ± 0.49	0.055 ± 0.009
2	1	2	2	8.125 ± 0.92	23.492 ± 0.89	15.006 ± 0.38	0.128 ± 0.007
3	1	3	3	8.270 ± 0.68	22.638 ± 0.93	14.605 ± 0.82	0.125 ± 0.006
4	2	1	2	8.641 ± 0.59	23.134 ± 0.74	16.665 ± 0.96	0.085 ± 0.009
5	2	2	3	8.335 ± 0.76	21.590 ± 0.97	16.468 ± 0.62	0.135 ± 0.009
6	2	3	1	7.326 ± 0.74	19.191 ± 0.88	15.096 ± 0.61	0.158 ± 0.008
7	3	1	3	8.904 ± 0.69	28.682 ± 0.86	12.938 ± 0.36	0.180 ± 0.009
8	3	2	1	7.900 ± 0.89	25.946 ± 0.69	13.250 ± 0.56	0.158 ± 0.007
9	3	3	2	8.378 ± 0.83	25.838 ± 0.97	13.046 ± 0.79	0.149 ± 0.008
Polyphenols (mg/g)	7.981	8.365	7.592	order		the optimal level	
	8.101	8.120	8.381	C > A > B		$A_3B_1C_3$	
	8.394	7.991	8.503				
	0.413	0.374	0.911				
Flavonoids (mg/g)	23.158	25.053	22.827	order		the optimal level	
	21.305	23.676	24.154	A > B > C		$A_3B_1C_3$	
	26.822	22.556	24.303				
	5.517	2.497	1.476				
Triterpenoid (mg/g)	14.642	14.639	14.220	order		the optimal level	
	16.076	14.908	14.906	A > C > B		$A_2B_2C_2$	
	13.078	14.249	14.670				
	2.998	0.659	0.686				
Vitamin C (mg/g)	0.103	0.107	0.124	order		the optimal level	
	0.126	0.140	0.121	A > B > C		$A_3B_3C_3$	
	0.162	0.144	0.147				
	0.059	0.037	0.026				

A – irradiation power (W); B – ultrasonic-microwave time (s); C – solid-liquid ratio

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The solid-liquid ratio should be controlled properly and the rest of the variables were a microwave irradiation power of 80 W and ultrasonic-microwave time of 90 seconds. The yields of bioactive substances increased dramatically with the increase of solid-liquid ratios from 1 : 30 to 1 : 50 (Figure 3). The volume of the solvent was insufficient during the range which led to the less ultrasound and microwave dielectric molecular (Yin *et al.* 2015), the higher solid-liquid ratio increased extraction efficiency. The yield would get the highest value when the solid-liquid ratio was 1 : 50. However, the yield would decrease when the ratio was over 1 : 50. There was dissolution equilibrium and adsorption equilibrium in the solution due to an increase of the solid-liquid ratio. The reason for the decrease was that the solvent molecules shared more energy (Wang *et al.* 2012). Thus, taking into account the consumption of the solvent, the solid-liquid ratio in the test was identified as 1 : 50.

Orthogonal test. An orthogonal experimental design was employed to optimise the ultrasonic microwave-assisted extraction process. As shown in Table 2, the optimal level of polyphenol yield was $A_3B_1C_3$; the optimal level of flavonoid yield was $A_3B_1C_3$; the optimal level of triterpenoid yield was $A_2B_2C_2$; the optimal level of vitamin C was $A_3B_3C_3$. Considering that the solid-liquid ratio had the most significant influence on flavonoids, so the best solid-liquid ratio 1 : 55 of flavonoids was selected as the optimal solid-liquid ratio. The microwave irradiation power had a significant influence on flavonoids, triterpenoids, and vitamin C. In order to extract the bioactive substances maximally, the microwave irradiation power of 90 W for flavonoids and vitamin C was selected as the optimal microwave irradiation power. Ultrasonic-microwave time had the most significant influence on flavonoids and vitamin C, so the best time of 75 s was selected as the optimal time from the perspective of energy saving. Thus, the optimum conditions for the ultrasonic microwave-assisted extraction of polyphenols, flavonoids, triterpenoids, and vitamin C from *Clinacanthus nutans* were the microwave irradiation power of 90 W, ultrasonic-microwave time of 75 s, and solid-liquid ratio of 1 : 55.

Confirmation experiments were carried out at these best conditions, and the yields of polyphenols, flavonoids, triterpenoids, and vitamin C were 8.893, 25.936, 16.789, and 0.166 mg/g, which was in good agreement with the predicted values.

CONCLUSION

In this study we characterised for the first time the ultrasonic microwave-assisted extraction process of *Clinacanthus nutans* in terms of bioactive substances (polyphenols, flavonoids, triterpenoids, vitamin C). The effects of solid-liquid ratio, irradiation power, and ultrasonic-microwave time were taken into account in the preliminary and completed optimisation of the proposed treatment. The results showed that the solid-liquid ratio has a key role in the extraction of polyphenol yield while the irradiation power was the key factor for the yields of flavonoids, triterpenoids, and vitamin C.

Thus, the optimum conditions for ultrasonic microwave-assisted extraction were determined to be: microwave irradiation power of 90 W, ultrasonic-microwave time of 75 s, and solid-liquid ratio of 1 : 55.

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