Microwave-assisted Extraction of Bioactive Substance from Clinacanthus nutans

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Abstract. Polyphenols, flavonoids, triterpenoid and VitaminC from Clinacanthus nutans were extracted by microwave-assisted extraction (MAE) technology. The optimal extracting conditions were established as distilled water as solvent, solid-liquid ratio 1:45 (g/ml), irradiation power 160W, and every extraction cycle of 50s. Results revealed that microwave time exert a significant effect on triterpenoid and VitaminC, whereas irradiation power plays a key role in the extraction of flavonoids. Furthermore, the effect of solid-liquid ratio on polyphenols is obvious. Under these conditions the extraction yield of each component were: polyphenols 8.382 mg/g, flavonoids 24.928 mg/g, triterpenoid 14.058 mg/g, vitaminC 0.129 mg/g. This study shows that MAE is an efficient technology to extract bioactive substances from leaves and Clinacanthus nutans could potentially be a resource for natural antioxidants.

Introduction

Clinacanthus nutans, a member of Acanthaceae family, commonly called Torsional order flowers or The Green Arrow, is a magnificant herbaceous plant which originates from Malaysia and Java[1]. In the southern provinces of China, it has been cultivated as a medicinal plant. According to the survey, more than 75.33 hectares of Clinacanthus nutans has been planted in Hainan province. Although there are several reports describing the kinds of Clinacanthus nutans, primarily including Clinacanthus nutans tea, Clinacanthus nutans fresh leaves and Clinacanthus nutans powder. Related research of deep processing for Clinacanthus nutans is less. Zhang Weihua extracts total flavonoids from Clinacanthus nutans by ultrasonic-assisted technology[2]. Besides, Liu Xu finds that n-butanol extract of Clinacanthus nutans has inhibitive effect on Heps liver cancer in mice[3]. Clinacanthus nutans also contains some other high value bioactive components, including polyphenols, flavonoids, triterpene and vitamins, and so on. Its extracts are described as presenting anti-aging, antioxidant and immune modulation effects, and these effects are associated with the presence of polyphenols, flavonoids, triterpene and vitamins[4-8]. Microwave-assisted extraction (MAE) is a process that it improves solvent extraction temperature and isolates the required compounds from sample matrix through microwave energy[9]. The application of microwave energy for sample preparation was first applied in the gossypol from cottonseed in 1986. Microwave energy is a non-ionizing radiation, which can penetrate into certain materials and interact with the polar components to generate heat. Heating of microwave energy acts directly on molecules by ionic conduction and rotation of dipoles. In contrast, extracts obtained with the conventional Soxhlet method were very poor in minority bioactive compounds. One of the main advantages of using MAE is the reduction of extraction time, which can mainly be attributed to the difference in the heating performance of microwave and conventional heating. Since then MAE has attracted growing interest, and it is called Green extraction technology because of its high efficiency, low consumption and less pollution[10,11]. Thus, the aims of this study is to optimize the process of extracting the bioactive substances from Clinacanthus nutans by microwave-assisted. Various parameters of MAE were studied including microwave time, irradiation power and solid-liquid ratio. The optimal extracting conditions have been determined to provide a reference for the further development and utilization of Clinacanthus nutans.

MATERIALS and METHODS

Materials and Chemicals

Clinacanthus nutans powder: Wuzhishan Wanjiabao science and technology company limited in Hainan; Gallic acid standard, Rutin standard, Oleanolic acid, Vanillic aldehyde: Yuanye biological science and technology company limited in Shanghai; the Forint phenol standard: Suolaibao science and technology company limited in Beijing; Others' pure is analytical; The water is distilled water.

Instruments preparationMethods

NJL07-3 experiment for microwave oven: Jiequan microwave equipment company limited in Nanjing; 7200 visible spectrophotometer: Longnike equipment company limited in Shanghai; BS124S electronic scales: Sartorius company; HH-S26S electric-heated thermostatic water bath: Dadi automation instrument company in Jintan; TDZ5-WS pipe rack automatic balance centrifuge: Xiangyi laboratory instrument development company limited in Hunan; SW-CJ-IF single double-sided clean bench: Suzhou purification equipment limited company; BCD-180 refrigerators: Zhujiang refrigerator factory in Guangdong.

Methods

Determination method of the polyphenols yield

The method to determine the yield of polyphenols was according to Xu's[12] with some modifications. 20mg gallic acid was dissolved into a 200 mL volumetric flask with distilled water to be stock solution. 0.0, 0.1, 0.2, 0.3, 0.4 and 0.5mL stock solution were moved to another vitro and diluted with water into 0.5mL. Add 10% FC reagent 2.5mL and shake completely. After 5min 7.5% $NaCO_3$ is added in it. Fetch it into Water bath pot for 5min under 50□. Keep it in dark place for 1h at room temperature . The adsorption of different samples were measured by UV spectrophotometer at 760nm. Standard curve of polyphenols can be obtained, as follows:

$$A=91.406C_1+0.0159,\ r^2=0.9958. \quad (1)$$

In accordance with the above standard curve measuring method, 0.5mL extracting solution was moved into the colorimetric wareand the absorbance was measured by UV spectrophotometer. The polyphenols concentration was obtained by Eq.1.

Polyphenols yield(mg/g)=$100C_1\times V_1/m$ (C_1: the quality concentration of the polyphenols, V_1: the volume of fluid under test, m: the qualityof Clinacanthus nutans).

Determination method of the flavonoids yield

The method to determine the yield of flavonoids was according to Li's[13] with some modifications. 0.0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0mL Rutin were moved into the volumetric flasks respectively. 0.3mL 5% sodium nitrite solution was added in it, shaked completely and standed for 6min. Then add 10% aluminum nitrate solution 0.3mL, shake completely and stand for 6min. 4mL 4% sodium hydroxide solution was moved in it and diluted with 60% ethanol into 10mL. Stand for 12min. The adsorption of different samples were measured by UV spectrophotometer at 510nm. Standard curve of flavonoids can be obtained, as follows:

$$A=5.8766C_2+0.0159,\ r^2=0.9955. \quad (2)$$

In accordance with the above standard curve measuring method, 1mL extracting solution was moved into the colorimetric wareand the absorbance was measured by UV spectrophotometer. The flavonoids concentration was obtained by Eq.2.

Flavonoids yield (mg/g) =$100C_2\times V_2/m$ (C_2: the quality concentration of the flavonoids, V_2: the volume of fluid under test, m: the qualityof Clinacanthus nutans).

Determination method of the triterpenoid yield

The method to determine the yield of triterpenoid was according to Huang's[14] with some modifications. 17mg Oleanolic acid was moved into volumetric flask accurately. 0.1, 0.2, 0.3, 0.4, 0.5, 0.6mL standard solution were dissolved into vitros respectively with anhydrous ethanol to 1mL. 1mL anhydrous ethanol was added into the blank sample. All the vitros were put in a boiling water bath until the solvent volatile completely. 5% vanillin-ice acetic acid 0.4mL, perchlorate 1.6mL were moved in it respectively and blended quickly. Afterwards 70□ water bath for 15 min,

cool it to the room temperature with ice water. After 8mL ethyl acetate was added, the adsorption of different samples were measured by UV spectrophotometer at 560nm. Standard curve of triterpenoid can be obtained, as follows:

$A=42.899C_3-0.0162$, $r^2=0.9958$. (3)

In accordance with the above standard curve measuring method, 0.5mL extracting solution was moved into the colorimetric wareand the absorbance was measured by UV spectrophotometer. The triterpenoid concentration was obtained by Eq.3.

Triterpenoid yield (mg/g) $=100C_3\times V_3/m$ (C_3: the quality concentration of the triterpenoid, V_3: the volume of fluid under test, m: the qualityof Clinacanthus nutans).

Determination method of the Vitamin C yield

The method to determine the yield of VitaminC was according to Zhao's[15] with some modifications. 0.2 mg/mL 2,6-dichloro indophenol solution was stored at 4□. It was calibrated by 0.05mg/mL ascorbic acid solution (it was prepared by 2% oxalic acid) before each using it. The titer was calculated by Eq.4:

$T=CV/(V_1-V_0)$ (4)

C: concentration of standard ascorbic acid, V: the volume of standard ascorbic acid solution that have taken, V_0: the volume of 2,6-dichloro indophenol that the blank solution consumed, V_1: the volume of 2,6-dichloro indophenol that the titration consumed.

10mL extracting solution was moved into conical flask and titrated by 2,6-dichloro indophenol. Write down the consumed volume. Vitamin C (mg/100mg) yield was calculated according to Eq.5:

$VC(mg/100mg)=5(V_2-V_0)T/M$ (5)

V_0: volume of 2,6-dichloro indophenol that the blank solution consumed, mL; V_2: the volume of 2,6-dichloro indophenol that the titration consumed, mL; T: the titer, mg/mL; M: the quality of Clinacanthus nutans,g.

Design of extraction test

The single factor test: according to the results of preliminary experiments, microwave power(80W, 160W, 240W, 320W, 400W), microwave time(20s, 40s, 60s, 80s, 100s) and solid-liquid ratio(1:20, 1:30, 1:40, 1:50, 1:60) were elected to the factors of the single factor test. Under the condition that other factors are invariable, make a research on every factor's influence on the yield of polyphenols, flavonoids, triterpenoid and VC from Clinacanthus nutans.

Orthogonal test: based on the result of the single factor test, process of microwave-assisted extraction is optimized by the orthogonal experiment. The design of factors and levels is shown in Table1.

Table 1 Factors and their levels in orthogonal array design

level	factor		
	A irradiation power(W)	B Microwave time(s)	C solid-liquid ratio
1	120	50	1 : 35
2	160	60	1 : 40
3	200	70	1 : 45

RESULTS and DISCUSSION

Results and discussion of the single factor test

Effect of irradiation power on yield

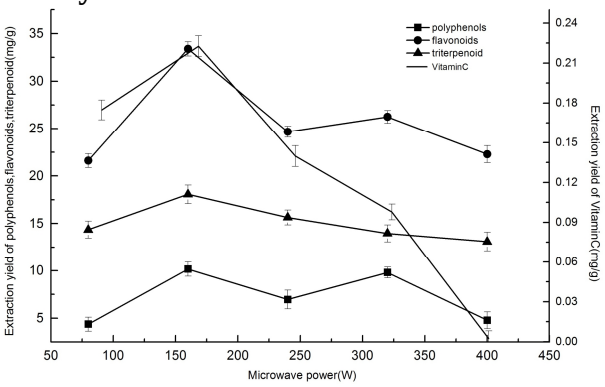

Fig.1 Effect of microwave power on yield

The effect of microwave irradiation power on yields of polyphenols, flavonoids, triterpenoid and VitaminC was evaluated. The rest of the variables were a solid-liquid ratio of 1:40 g/mL and every extraction cycle of 60s each. Figure 1 shows that as the power increased, the yields of bioactive ingredients were gradually enhanced. This is perhaps because that microwave power become higher, the material can absorb more microwave energy, which increases damage of the sample cell and is good for leaching the active ingredients[16]. However, when the microwave power was more than 160W, the yields showed a declining trend. As shown in Figure 1, high microwave irradiation power does not result in high yields of bioactive ingredients. It was speculated that the superfluous energy offered by high irradiation power disturbed the molecular interactions. It leads to partial carbonize of samples as internal overheating and the isomerization or degradation of polyphenols, flavonoids, triterpenoid and VitaminC. Among them, the change trend of VC is the most obvious, and microwave power is the most significant factor for VC; the change trend of triterpenoid is gentle during the process; the yield of polyphenols and flavonoids increases again when microwave power is 320W, and it is possiblity that the increasing microwave power breaks the cell which is broken incompletely. But they didn't reach the maximum value. Therefore, it was appropriate to select 160W as the practical microwave irradiation power.

Effect of microwave time on yield

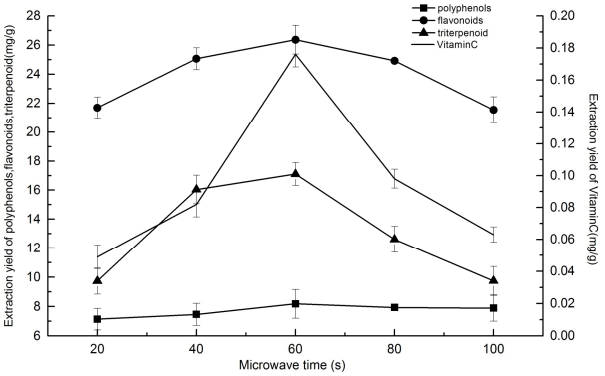

Fig.2 Effect of microwave treatment time on yield

Microwave irradiation is able to generate sufficient energy in a short time. Thus, microwave irradiation time should be controlled properly. The rest of the variables were a solid-liquid ratio of 1:40 g/mL and microwave irradiation power of 160W. The yields of bioactive substances after various extractions are shown in Figure 2. It was observed that 60s of irradiation time can increase the yields and prolonged irradiation led to a reduction in yields. The highest yield was obtained after 160W for 60s's extraction. Because the longer time will increase that microwave energy of the moisture from the substrate accumulates. It's more obvious to destruct the cell wall. So that the yield of the four active substances increases[17]. Time played an important role on the yields. When

microwave irradiation time increases from 60 to 100s, the yields of four target analytes decrease dramatically. Different bioactive substance present different trend. This phenomenon is relevant with their molecular structure. The nature of the material is decided by the structure of the material. Long time of microwave radiation damages the structure of molecules, decreases the yield, which is bad for the extraction of active substances. Thus, 60s was deemed as the optimal microwave irradiation time.

Effect of solid-liquid ratio on yield

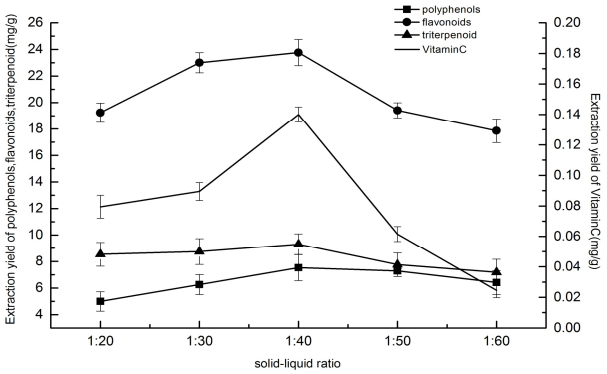

Fig.3 Effect of solid-liquid ratio on yield

Solid-liquid ratio is an important factor in separation technique. To avoid inadequate extraction, the effect of solid-liquid ratio on the yields of polyphenols, flavonoids, triterpenoid and VitaminC were studied with different solid-liquid ratios (1:20, 1:30, 1:40, 1:50, 1:60 g/mL) under a microwave irradiation power of 160W and an extraction time of 60s. In Figure 3, the yields of bioactive substances increase dramatically with the increasing of solid-liquid ratios from 1:20 to 1:40 g/mL; the yields is the highest when solid-liquid ratio is 1:40 g/mL; while solid-liquid ratios are higher than 1:40g/mL, high solid-liquid ratios bring a decrease of their yields. Suitable solid-liquid ratio can make Clinacanthus nutans dissolve completely and avoid excessive solvent dispersing microwave energy. Among them, triterpenoid and polyphenols change gently during this process; the yield of VC and flavonoids decreases obviously after 1:40. Thus, taking the consumption of solvent into account, the solid-liquid ratio in present test was identified as 1:40 g/mL.

Results and discussion of orthogonal test

Orthogonal experimental design was employed to optimize the MAE process of polyphenols, flavonoids, triterpenoid and VitaminC, and results are shown in Table 2. It shows that solid-liquid ratio is the most significant factor while extraction time is the insignificant for the polyphenols yield, in an order of C>A>B, and the optimal level is $A_3B_3C_3$; microwave irradiation power is the most significant factor while solid-liquid ratio is the insignificant for the flavonoids yield, in an order of A>B>C, and the optimal level is $A_2B_3C_2$; extraction time is the most significant factor while microwave irradiation power is the insignificant for the triterpenoid yield, in an order B>C>A, and the optimal level is $A_2B_1C_3$; extraction time is the most significant factor while solid-liquid ratio is the insignificant for the VitaminC yield, in an order of B>A>C, and the optimal level is $A_2B_1C_3$. Considering that microwave power is the most significant influence on flavonoids, so the best microwave power 160W of flavonoids is elected as the optimal microwave power; microwave time is the most significant influence on triterpenoid and VitaminC, so the best microwave time 50s of triterpenoid and VitaminC is elected as the optimal microwave time; solid-liquid ratio is the most significant influence on polyphenols, so the best solid-liquid ratio 1:45 mg/mL of polyphenols is elected as the optimal solid-liquid ratio. According to this experimental design, the optimum conditions for MAE of polyphenols, flavonoids, triterpenoid and VitaminC from Clinacanthus nutans are that microwave irradiation power of 160W, microwave time of 50s and solid-liquid ratio of 1:45 g/mL.

Confirmation experiments were carried out at these best conditions, and the yields of polyphenols, flavonoids, triterpenoid and VitaminC were 8.382mg/g, 24.928mg/g, 14.058mg/g and 0.129mg/g, which were in good agreement with the predicted.

No.	A	B	C	Polyphenols (mg/g)	flavonoids (mg/g)	triterpenoid (mg/g)	VitaminC (mg/g)
1	1	1	1	6.768	23.408	11.560	0.057
2	1	2	2	7.317	21.813	11.455	0.054
3	1	3	3	8.116	22.189	10.970	0.045
4	2	1	2	7.430	24.088	11.822	0.094
5	2	2	3	7.973	24.573	9.669	0.076
6	2	3	1	6.824	25.343	12.642	0.067
7	3	1	3	8.150	24.099	13.952	0.132
8	3	2	1	6.875	21.352	10.068	0.049
9	3	3	2	7.414	24.999	9.517	0.039
polyphenols (mg/g)	7.400	7.449	6.822	order		the optimal level	
	7.409	7.388	7.387				
	7.480	7.451	8.080	C>A>B		$A_3B_3C_3$	
	0.080	0.063	1.258				
flavonoids (mg/g)	22.470	23.865	23.368	order		the optimal level	
	24.668	22.579	23.633				
	23.483	24.177	23.620	A>B>C		$A_2B_3C_2$	
	2.198	1.598	0.265				
triterpenoid (mg/g)	11.328	12.445	11.423	order		the optimal level	
	11.378	10.397	10.931				
	11.179	11.043	11.530	B>C>A		$A_2B_1C_3$	
	0.199	2.048	0.599				
VitaminC (mg/g)	0.052	0.094	0.058	order		the optimal level	
	0.079	0.060	0.062				
	0.073	0.050	0.084	B>A>C		$A_2B_1C_3$	
	0.027	0.044	0.026				

Table 2 Results of orthogonal test

CONCLUSION

This study focused on the optimization of MAE extraction process for bioactive substances (polyphenols, flavonoids, triterpenoid, VitaminC) from Clinacanthus nutans. The maximum extraction yields were obtained for 160W as irradiation power for 50s as processing time, higher for that obtained with a conventional method. Analyse the result of single factor test by Origin9.0. The study shows that: order of the polyphenols yield is solid-liquid ratio>microwave irradiation power>microwave time; order of the flavonoids yield is microwave irradiation power>microwave time>solid-liquid ratio; order of the triterpenoid yield is microwave time>solid-liquid ratio>microwave irradiation power; order of the VC yield is microwave time>microwave irradiation power> solid- liquid ratio.

According to the above experiments, the optimum MAE conditions were determined to be: microwave irradiation time of 50s, microwave irradiation power of 160W and solid-liquid ratio of 1:45 g/mL. It's desirable to extract the active substances from Clinacanthus nutans under the conditions. Further investigation on the antioxidant and antimicrobial activity of the recovered

phytocompounds obtained by microwave pre-treatment is essential to determine the bioactive activity of the extracts.

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