Ultrasound-assisted Extraction of Bayberry Tannin and Optimization Using Response Surface Methodology

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The extraction of bayberry tannins has potential to maximize the utilization of a forest waste. This study employed a four-level central composite design through response surface methodology to optimize the extraction of tannin from bayberry barks through ultrasound-assisted extraction (UAE). The effects of solute to solvent ratio (STSR), solvent concentration (SC), extraction time (ET), and sonication temperature (ST) on the total extraction yield of total condensed tannin (TCT yield) and total phenolic content (TPC) were investigated. The extracts were characterized with matrix-assisted laser desorption-time of flight mass spectrometer (MALDI-TOF MS), nuclear magnetic resonance (NMR), and gel permeation chromatography (GPC). The optimized condition was reached when the STSR and ST were set at 1:57.16 g/mL and 71.3%, when the ET and the ST was 39.1 min and 48.75 °C. In these conditions, the TCT yield and TPC reached their maximum values of 17.55% and 365.01 mg GAE/g, respectively. Furthermore, the polyflavonoids of bayberry tannin ranged from dimers to heptamers, which were only composed of proanthocyanidins (PC) containing galloy groups.

Keywords: Bayberry tannin; Ultrasound-assisted extraction; Optimization; Proanthocyanidin

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INTRODUCTION

Bayberry is a variety of the common medicinal plant used as a substitute for the treatment of gastrointestinal diseases in the field of traditional Asian medicine; it is a common economic and ecological plant in Asia (Chen *et al.* 2003). A small amount of bayberry bark is used in traditional Asian medicine, but most is used as a direct burning energy source in sawmills and pulp mills, which is wasteful (Feng *et al*. 2013). In addition, phenol is mostly used to prepare adhesives in wood industry, but it needs a substitute because of its toxicity and its origin from non-renewable resources (Cui *et al*. 2014). Tannin, which is present in bayberry bark and other extracts, has also been studied as an alternative to phenol to develop wood adhesives, due to its polyphenol components (Abdullah and Pizzi 2013). Because the specific structure of these polyphenol components is unknown, the means, mechanism, and dosage of substitution are not clear. Identifying the components of bayberry bark extract will promote further research using tannin instead of phenol and increase the potential of high-quality utilization of bayberry.

Conventional extraction methods such as solid-liquid extraction (SLE), supercritical fluid extraction (SFE), pressurized water extraction (PWE), microwave-assisted extraction (MAE), and ultrasound-assisted extraction (UAE) are applicable for the extraction of tannin (de Hoyos-Martínez *et al*. 2019). Amongst these methods, UAE is the

most efficient and economical one, achieving higher yields, lower solvent consumption, and shorter sonication time (Vilkhu *et al*. 2008). Based on the mechanism of the UAE technique, the ultrasonic cavitation may lead to the formation, growth, and collapse of numerous microbubbles in the solution. The mechanical vibrations caused by this process enter the plant matrix and burst the cell walls, allowing the solvent to penetrate more easily into the matrix, resulting in higher amounts of tannin extracts (Chemat *et al*. 2011; Ali *et al*. 2018). Furthermore, a higher extraction yield can result when using solvents with higher polarities. However, the precipitation produced by self-condensation of tannin in water will affect the extraction yield. Thus, water and methanol are usually selected as the solvents for tannin extractions (do Prado *et al*. 2014). To date, the literature lacks systematic studies of UAE technique for the extraction of tannins from the bayberry barks.

The application of ultrasonic extraction technology is promising, but it needs to be developed more fully to extract tannins from the bayberry barks. The main purpose of this research was to find the optimal process for ultrasound-assisted extraction of tannin from bayberry barks by studying the influence of the four variables of solute to solvent ratio (STSR), solvent concentration (SC), extraction time (ET), and sonication temperature (ST) on the total extraction yield of condensed tannin (TCT yield) and total phenolic content (TPC). Moreover, the experimental scheme was optimized with response surface methodology (RSM). Finally, this paper analyzed the main phenolic substances extracted from bayberry barks that with the highest TCT yield through matrix-assisted laser desorption-time of flight mass spectrometer (MALDI-TOF MS), nuclear magnetic resonance (NMR), and gel permeation chromatography analysis (GPC).

EXPERIMENTAL

Materials

The Biqi Chinese bayberry barks were bought from Xuelang Mountain, Jiangsu Province (China, 120°29'E, 31°59'N). The raw barks were washed, cleaned, and pre-treated by freeze-drying (Alpha 1-2 LDplus, Marin Christ, Osterode am Harz, Germany) for 2 days. The pre-treated barks were crushed into powder and passed through a 100-mesh sieve before use.

Chemicals and Reagents

Dimethyl sulfoxide-d6 (DMSO-d6) was purchased from Adamas (Shanghai, China). Folin-Ciocalteu reagent and gallic acid standard were purchased from Macklin (Shanghai, China). All other chemicals and solvents were of analytical grade; methanol, formaldehyde, hydrochloric acid (HCl), sodium carbonate (Na_2CO_3), pyridine, acetic anhydride, and HPLC-grade tetrahydrofuran (THF) were purchased from Sinopharm (Shanghai, China) and used without further pretreatment.

Constituent Extraction of the Bayberry Barks Using UAE

The selection of the proper solvent is very important for solvent extraction. In this study, the methanol was selected as the extraction solvent to obtain a higher TCT yield, since it has been reported that the extraction rates increased with the polarity of solvent (Markom *et al*. 2007; do Prado *et al*. 2014; Widyawati *et al*. 2014). Single factor experiments were introduced to investigate the effect of STSR (1:30, 1:40, 1:50, 1:60, and 1:70 g/mL), SC (50%, 60%, 70%, 80%, and 90% v/v), ET (15 min, 30 min, 45 min, 60

min, and 75 min) and ST (20 °C, 30 °C, 40 °C, 50 °C, and 60 °C) on the extraction constituents of the bayberry barks. These parameters and their respective scopes were generalized by other research (Dahmoune *et al*. 2014; Sousa *et al*. 2016; Ali *et al*. 2018).

Typically, when the ratio of solute to solvent was set at 1:50, 2 g of bayberry bark powder was added into 100 mL of 70% methanol. The mixture was ultra-sonicated for 45 min in an ultrasonic bath system (KQ-300DE, Kunshan, China) with a constant ultrasonic power of 300 W according to the experimental design. The reaction temperature was maintained at a fixed temperature (± 2 °C). After certain sonication time, the solution was filtered to remove the solid powder. After the solvent was removed by a vacuum rotary evaporator (RE-52AA, YaRong, China) at 45 °C, and the residual was freeze-dried, the bayberry bark extract was obtained.

Determination of TCT Yield

The modified Stiansy method was used to determine TCT yield (Yazaki and Hillis 1980). Briefly, 5 mL of formaldehyde solution (37 wt.%) and 5 mL concentrated hydrochloride (HCl) solution (36 wt.%) were mixed with 50 mL of tannin solution (0.004 g/mL) in a 250 mL conical flask. The mixture was heated at 120 °C with reflux for 60 min. After the mixture was cooled for 30 min, the condensed tannin content was collected after being filtered through a funnel and oven-dried at 120 °C for 2 h. The TCT yield was calculated using Eq. 1,

$$\text{TCT yield} = (m_1 \times Wd) / (m_2 \times Ws) \times 100\% \quad (1)$$

where m_1 represents the mass of condensed tannins after drying, m_2 represents the mass of the barberry tannin, and Wd and Ws were the mass weight of the extract and bayberry powder sample, respectively.

Determination of TPC

TPC was determined by the Folin-Ciocalteu method (Wen-Xian *et al*. 2011). In brief, 1 mL of sample solution (0.2 mg/mL) and 12 mL of distilled water were added to a 25 mL volumetric flask for reserve use. Next, 2 mL of Folin-Ciocalteu reagent and 10 mL of 10% Na_2CO_3 solution were added. The mixture was reacted at 50 °C for 60 min in the dark, and the TPC was determined by a UV spectrophotometer with the absorbance at 765 nm. TPC was expressed as mg gallic acid equivalents (GAE)/g fraction (Zhou *et al*. 2011).

Optimization of the results by RSM

A four-factor, five-level central composite design (CCD) was the most appropriate method to obtain the best UAE conditions for the tannin extracts from bayberry barks (Dahmoune *et al*. 2014). This method can provide an economical and effective experimental scheme when the experimental content involves four variables and the experimental quantity needs to be simplified. The range and level of the extraction variables are shown in Table 1. Table 3 shows the complete scheme of 30 experiments designed by CCD, as well as the experimental data of predicted and actual responses collected in terms of TCT yield and TPC. According to the independent variable, the variable $\underline{Y}$ was fitted into the quadratic model in the following Eq. 2,

$$Y = a_0 + \sum_{i=1}^{k} a_i X_i + \sum_{i=1}^{k} a_{ii} X_i^2 + \sum_j \sum_{i=2}^{k} a_{ij} x_i x_j \quad (2)$$

where Y is the predicted response, x_i and x_j represent the independent variables (i and j range from 1 to k), a_0 is a constant, a_i, a_{ii}, and a_{ij} are the regression coefficients of linear,

quadratic and interactive terms respectively, and *k* is the number of number of parameters (4 for current study).

Table 1. Experimental Domain for Central Composite Design

Factor	Factor levels				
	-2	-1	0	1	2
STSR[a] (X_1, g/mL)	1:30	1:40	1:50	1:60	1:70
SC[b] (X_2, %)	50	60	70	80	90
ET[c] (X_3, min)	20	30	40	50	60
ST[d] (X_4, °C)	30	40	50	60	70

[a] STSR= solute to solvent ratio.
[b] SC= solvent concentration.
[c] ET= extraction time.
[d] ST= sonication temperature.

Statistical Analysis

All experiments were carried out in triplicate to enable determination of the precision of experimental data. The results were analyzed with Design Expert software v. 10 (Stat-Ease, Minneapolis, MN, USA). Analysis of variance (ANOVA) was employed to analyze TCT yield and TPC. All the statistical analyses were carried out at ρ-values <0.05 significance level.

Purification of Bayberry Tannins

The bayberry bark extract was purified by gel permeation chromatography on a Sephadex LH-20 column (300 mm × 35 mm; Sigma, St. Louis, MO, USA). The sugar and glycosides were eluted with 40% methanol aqueous solution, and the purified tannins were obtained by eluting with 70% acetone. After rotary evaporated and freeze dried, purified bayberry tannins were used for further characterization (Yang *et al*. 2019).

MALDI-TOF MS Spectroscopy

Analyses were performed on a AB SCIEX MALDI-TOF/TOF 5800 (AB SCIEX, Framingham, MA, USA) equipped with a N_2 laser (337 nm). Samples were analyzed at a nitrogen laser wavelength of 337 nm and 3 ns per laser pulse. In the reflection mode, the acceleration voltage was 20.0 kV, and the reflection voltage was 23.0 kV. The substrate 2,5-dihydroxy benzoic acid (DHB, 10 mg/mL) was mixed with the sample (7.5 mg/mL) at a ratio of 1:3 (v:v), and an appropriate proportion of NaCl solution (2.6 mg/mL) was added to promote the single ion adduct ($[M+Na]^+$) (Hoong *et al*. 2010; Stringano *et al*. 2011). A total of 1.5 μL of the mixture was directly coated on the target and then subjected to MALDITOF MS analysis.

^{13}C-NMR and ^{1}H-NMR Analysis

The ^{13}C-NMR and ^{1}H-NMR spectra were recorded on Bruker Biospin AVANCE III 600MHz (^{1}H, 600.23 MHz, ^{13}C, 150.93 MHz; Karlsruhe, Germany). For analysis of ^{13}C-NMR and ^{1}H-NMR, the samples were all dissolved in DMSO-d6 (30 mg/mL) (^{1}H was 2.49 ppm, ^{13}C was 39.5 ppm) (Teng *et al*. 2016).

GPC Analysis

Due to the low solubility of the polymerization in THF, tannin was acetylated at room temperature with pyridinic anhydride (1:1, 2 mL) (Cadahía *et al*. 1996). After

overnight acetylation, the sample was dissolved in THF (2 mg/mL) for GPC analysis. An Agilent 1260 system (Palo Alto, CA, USA) with a diode array detector and a PLgel hybrid c-pillar (300 × 7.5 mm i.d., 5.0 µm) was used for molecular weight measurement. As a mobile phase, THF had a flow rate of 0.5 mL/min with a temperature of 35 °C. The analysis took 25 min. Calibration curves were obtained with polystyrene standards.

RESULTS AND DISCUSSION

Extraction Structure Analysis

Effect of extraction factors on TCT yield

It is necessary to analyze the impact of factors in order to achieve the most effective extraction and utilization of tannins. Furthermore, considering the interaction between the factors, compared to the four-factor three-level experimental design, the four-factor five-level design might be a more comprehensive choice. Therefore, the influence of different factors on TCT yield of tannins extracted from bayberry barks was studied by a four-factor, five-level CCD. The significance and coefficients of the established quadratic model are presented in Table 2, as well as the regression analysis of three responses. The STSR had a significant positive effect on TCT yield. In contrast, the interaction between STSR and ST, ET, and ST, and the quadratic influence of STSR, SC, ET, and ST had negative effects on TCT yield in different degrees. The other terms were not significant and were excluded from Eq. 3.

$$Y_1 = 17.3 + 0.81X_1 - 0.23X_2 - 0.29X_{34} - 0.65X_1^2 - 0.75X_2^2 - 0.35X_3^2 - 0.63X_4^2 \quad (3)$$

Table 2. Central Composite Design with Experimental and Predicted Responses

Term	Estimated regression coefficients			
	TCT yield[a]	ρ-value	TPC[b]	ρ-value
Intercept βo	17.30		358.45	
X_1	0.81	< 0.0001	16.02	<0.0001
X_2	-0.23	0.3356	-0.23	0.8916
X_3	-0.13	0.2610	-3.52	0.0546
X_4	0.048	0.6262	-0.58	0.7365
X_{12}	0.13	0.2363	4.31	0.0548
X_{13}	-0.007158	0.9443	4.47	0.0475
X_{14}	-0.22	0.0145	-4.29	0.0557
X_{23}	-0.084	0.9018	-0.3	0.8873
X_{24}	-0.17	0.1319	-5	0.0289
X_{34}	-0.29	0.0477	0.29	0.8906
X_1^2	-0.65	< 0.0001	-10.3	<0.0001
X_2^2	-0.75	< 0.0001	-13.41	<0.0001
X_3^2	-0.35	0.0008	-5.52	0.0033
X_4^2	-0.63	< 0.0001	-10.66	<0.0001
Model F-value	13.32	<0.0001	17.13	<0.0001
Mean	15.40		326.54	
C.V. %	3.37%		2.54%	
Adeq. precision	11.491		12.507	
R^2	0.9255		0.9411	
R_a^2	0.8560		0.8862	
R_p^2	0.6377		0.7117	

[a] TCT yield= total extraction yield of condensed tannin; [b] TPC= total phenolic content.

Table 3. Estimated Regression Coefficients and ANOVA for TCT Yield and TPC

Run	STSR (g/mL)	SC (%)	ET (min)	ST (°C)	TCT yield (%)		TPC (mg GAE/g DW)	
					Actual response	Predicted response	Actual response	Predicted response
1	40	60	50	60	14.94±0.27	14.59	316.93±0.56	308.40
2	40	60	30	60	15.38±0.31	15.24	329.41±0.42	323.21
3	50	70	40	50	17.87±0.29	17.30	355.53±0.27	358.45
4	60	60	30	40	16.08±0.15	15.60	337.46±0.36	329.42
5	40	60	30	40	14.04±0.36	13.77	303.20±0.29	306.35
6	40	80	30	40	13.60±0.17	13.58	307.61±0.39	307.86
7	30	70	40	50	12.95±0.20	13.08	287.40±0.31	285.21
8	40	80	30	60	14.14±0.19	14.35	303.03±0.17	304.71
9	50	90	40	50	14.38±0.28	13.84	306.47±0.42	304.33
10	50	70	20	50	16.08±0.15	16.16	340.10±0.38	343.41
11	50	70	40	50	17.43±0.42	17.30	357.87±0.25	358.45
12	50	70	40	70	14.92±0.13	14.89	304.56±0.35	314.67
13	40	80	50	60	13.17±0.26	13.36	283.56±0.41	288.71
14	60	80	30	40	15.79±0.34	15.91	345.37±0.29	348.17
15	40	80	50	40	13.72±0.18	13.75	293.43±0.36	290.70
16	50	70	60	50	15.21±0.07	15.65	323.99±0.45	329.31
17	50	70	40	30	14.14±0.16	14.70	318.46±0.18	316.98
18	60	80	30	60	15.66±0.32	15.80	333.74±0.21	327.84
19	50	70	40	50	17.03±0.26	17.30	368.97±0.33	358.45
20	60	60	30	60	16.44±0.30	16.18	332.08±0.24	329.09
21	70	70	40	50	15.92±0.11	16.32	338.47±0.36	349.28
22	50	70	40	50	17.61±0.21	17.30	349.90±0.32	358.45
23	60	60	50	60	15.78±0.18	15.50	335.31±0.29	332.16
24	60	80	50	60	14.75±0.31	14.78	338.60±0.41	329.71
25	50	70	40	50	16.77±0.09	17.30	361.59±0.19	358.45
26	50	70	40	50	17.07±0.10	17.30	356.86±0.26	358.45
27	40	60	50	40	14.72±0.19	14.28	287.40±0.31	290.39
28	50	50	40	50	13.70±0.42	14.76	294.49±0.39	305.27
29	60	60	50	40	16.52±0.28	16.08	338.73±0.33	331.32
30	60	80	50	40	16.20±0.17	16.05	345.58±0.29	348.88

The corresponding diagram of the interaction effect on TCT yield by changing two factors simultaneously is shown in Fig. 1. The positive linear influence of the STSR on TCT yield is clearly apparent in the 3D graph. The maximum TCT yield was 1:55 g/mL. Furthermore, the positive effect of SC is obvious in Fig. 1, as the properties of methanol and condensed tannin were similar (de Hoyos-Martínez *et al*. 2019). According to Mustafa and Turner (2011), the choice of solvent is based on the principle of "like dissolve like"; compounds are more easily dissolved in solvents having similar solubility properties. Therefore, the polarity of the condensed tannins in bayberry barks is closer to that of methanol. As a result, TCT yield increased with higher methanol concentrations, and reached its maximum at 70% methanol concentration. Regarding the interaction between ET and ST, the decrease of TCTC is due to the thermal degradation of condensed tannin caused by excessive temperature (Vergara-Salinas *et al*. 2013). According to ANOVA (Table 2), the established model was significant with a Model F-value of 13.32. The high value of the coefficient of determination (R^2 = 0.9255) confirms that the derived model is valid and describes the relationship between variables and responses accurately. Moreover, the value of adjusted correlation coefficient (R_a^2 = 0.8560) is close to R^2, confirming the high significance of the model. This result implies the sufficiency of the model in the prediction relationship in development through a high predicted correlation coefficient (R_p^2 = 0.6377) (Maran *et al*. 2013). The coefficient of variation is 3.37% (CV< 10%), indicating that there was a small deviation between the experimental and predicted values (He *et al*. 2016). The adequate precision of 11.491 shows that the model had a satisfactory fitness.

Effect of extraction factors on TPC

As shown by the linear terms of the equation in Table 2, STSR had a very significant effect on TPC. There was a specific negative effect on the quadratic terms for all four extraction parameters. The interaction between STSR and ET showed a slight positive effect, while the interaction between SC and ST had a moderately significant passive effect. All the rest of the terms were non-significant and thus omitted from Eq. 4.

$$Y_2 = 358.45 + 16.02X_1 + 4.47X_{13} - 5X_{24} - 10.3X_1^2 - 13.41X_2^2 - 5.52X_3^2 - 10.66X_4^2 \quad (4)$$

Similar to the results of TCT yield, STSR had an obvious positive effect, while the interaction of SC and ST had a moderately negative effect on TPC, as shown in Fig. 2. The initial increase in TPC may be due to the decrease in molecular interactions within the solvent caused by high temperatures, resulting in the enhanced solubility. Moreover, the thermal effect reduced solvent viscosity, which led to the increased solubility of the solvent in the plant matrix (Wang *et al*. 2013; Moorthy *et al*. 2016; Xu *et al*. 2017). However, high temperature and long time will lead to thermal degradation of the phenolic compounds (Vergara-Salinas *et al*. 2013), which explains the negative effect of the interaction between SC and ST on the response. These results are consistent with current research; as the temperature increased, TPC also dropped to a similar degree.

The established model was significant (ρ-value< 0.0001) at a Model F-value of 16.02. High values of R^2 (0.9411), R_a^2 (0.8862), and R_p^2 (0.7117) indicate a high correlation. Moreover, values of CV (2.54) and adeq. precision (12.507) further show that the established model is good for TPC.

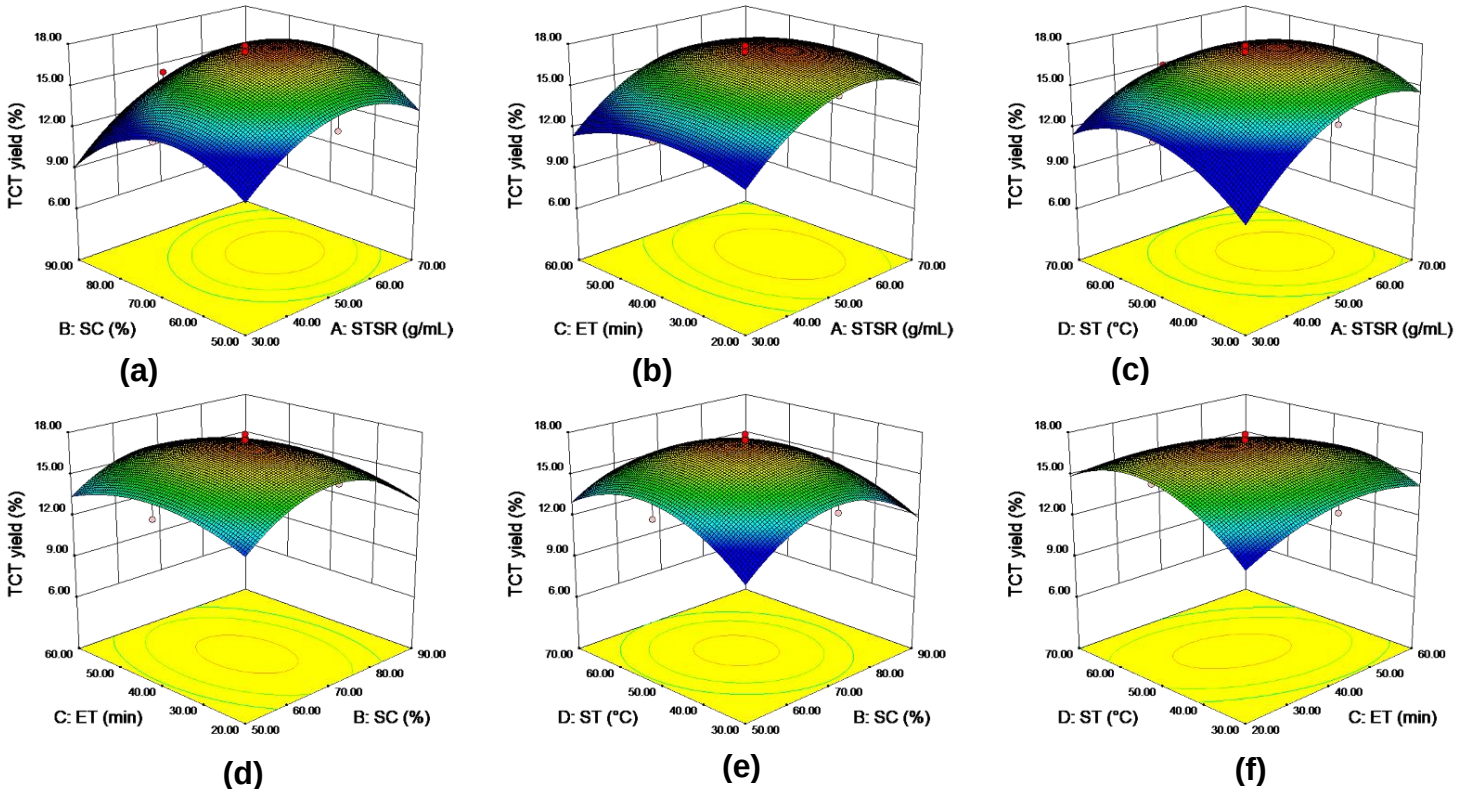

Fig. 1. Response surface plot for extract of bayberry barks (a) STSR and SC on TCT yield, (b) STSR and ET on TCT yield, (c) STSR and ST on TCT yield, (d) SC and TCT yield, (e) SC and ST on TCT yield, and (f) ET and ST on TCT yield

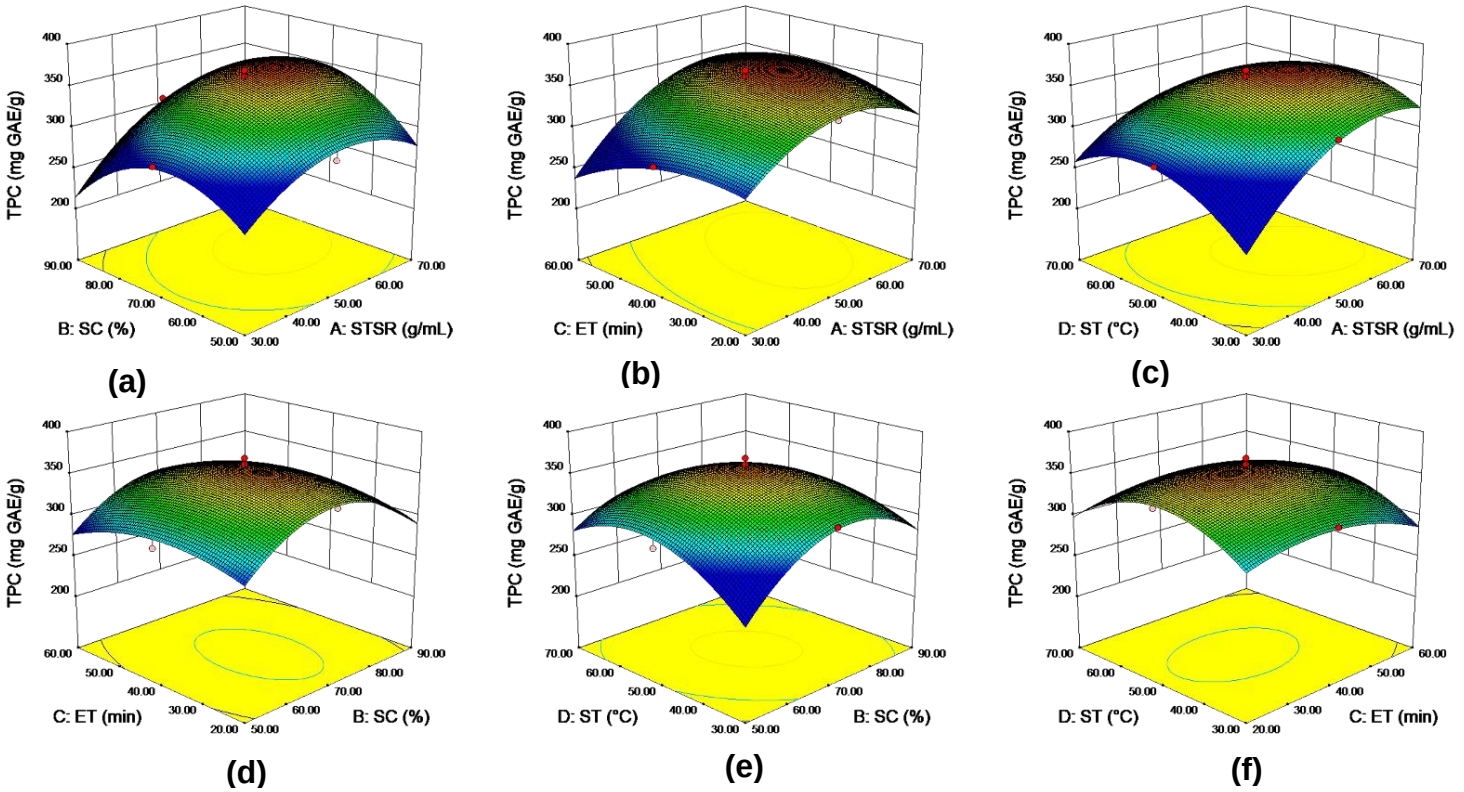

Fig. 2. Response surface plot for extract of bayberry barks (a) STSR and SC on TPC (b) STSR and ET on TPC (c) STSR and ST on TPC (d) SC and ET on TPC (e) SC and ST on TPC (f) ET and ST on TPC

Validation of the experimental model

To verify the established model, the optimal conditions of UAE to obtain maximum TCT yield and TPC were determined and used for experimental verification. The optimized condition was determined with ratio of 1:57.16 g/mL at 71.3% SC with ET of 39.1 min at a temperature of 48.8 °C. The predicted values of TCT yield (17.55%) and TPC (365.01 mg GAE/g) obtained from the experimental model (Table 4) were within 95% mean confidence intervals, suggesting the obtained models can accurately predict the process of extracting condensed tannins from bayberry barks by UAE.

Table 4. Predicted and Obtained Response Values and Confidences

Response	Predicted response	95% PI low	Obtained response	95% PI high
TCT yield (%)	17.55	16.36	17.26 ± 0.37	18.74
TPC (mg GAE/g DW)	365.01	346.04	369.52 ± 0.15	383.98

Extraction Structure Analysis

MALDI-TOF MS analysis

The MALDI-TOF MS spectra has been widely used in the study of the complexity of condensed tannins. It provides the structural information of flavon-3-ol subunits. As shown in Fig. 3, the repeated patterns of peaks were observed in MALDI-TOF MS spectra, ranging from 750 to 2500 Da. The fixed interval between two adjacent ion peaks with the strongest signals is 152 Da. According to Li *et al*. (2010a, b) and Oo *et al*. (2008), the fixed interval of 152 Da revealed the existence of galloyl, while intervals of 304 Da and 456 Da revealed the existence of (epi)gallocatechin and (epi)gallocatechin gallate subunits, respectively (Fig. 4). In addition, no difference of 16 Da was observed between these peaks (Fig. 3), indicating that the polyflavonoids of bayberry tannin were composed of PC (de Souza Mesquita *et al*. 2019).

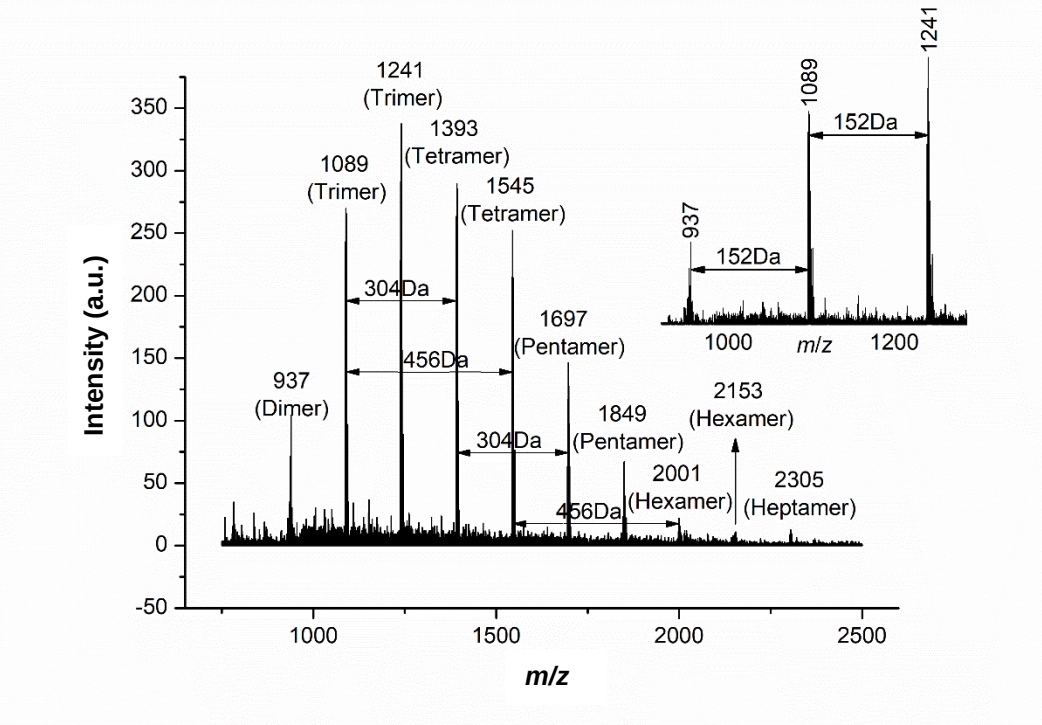

Fig. 3. MALDI-TOF MS spectra of bayberry tannin

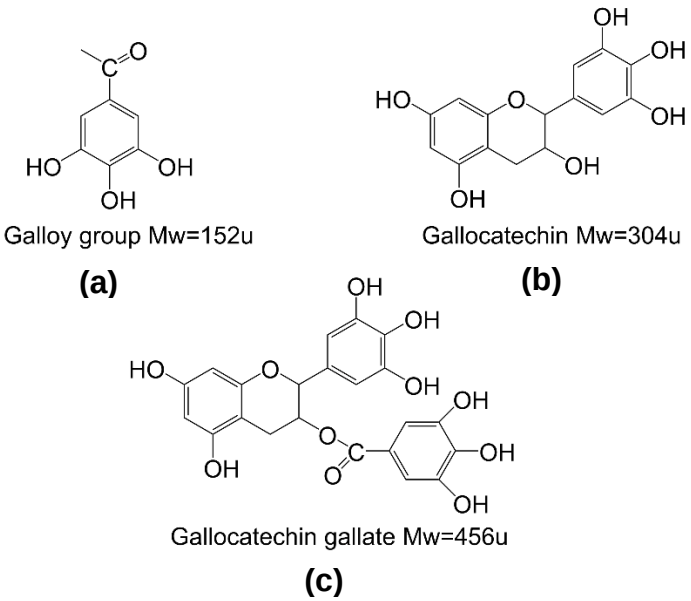

Fig. 4. Structure of the (a) galloyl group, (b) gallocatechin, and (c) gallocatechin gallate

The theoretical polymerization degree of barberry tannin was calculated by Eq. 5,

$$[M + Na]^{+} = 23.0+2.0+152.0a+304.0b \quad (5)$$

where *m*/*z* 23.0 and 2.0 presented the molar mass of the sodium, and terminal hydrogen, respectively. The values 152.0 and 304.0 represented the molar mass of the galloyl and gallocatechin, meanwhile *a* and *b* was the number of these subunits in each polymer, respectively (Teng *et al*. 2016).

As shown in Table 5, the polyflavonoids of bayberry tannin range from dimers to heptamers and were all constituted by same flavon-3-ol subunits.

Table 5. MALDI-TOF MS of Condensed Tannins from Bayberry Barks

	G[a]	GC/EGC[b]	Calculated	Experimental
Dimer	2	2	937	937
	0	3	937	937
Trimer	1	3	1089	1089
	0	4	1241	1241
Tetramer	1	4	1393	1393
	0	5	1545	1545
Pentamer	1	5	1697	1697
	0	6	1849	1849
Hexamer	1	6	2001	2001
	0	7	2153	2153
Heptamer	1	7	2305	2305

[a] G is the galloyl (152 Da).
[b] GC/EGC is the Gallo catechin/Epigallo catechin (304 Da).

^{13}C NMR and ^{1}H NMR spectra

Figure 6A represents ^{13}C NMR spectra of bayberry tannin. There was an obvious resonance at 146 ppm, which belongs to C3' and C5' (B ring) of prodelphinidin units (Fig. 5), indicating that the polyflavonoids of bayberry tannin were only composed of PC (Teng *et al*. 2016). In addition, resonance at 164.7 ppm was ascribed to C9 in PC, and C5, C7,

and C8a had their chemical shift around 156 to 160 ppm. The 139 ppm and 120.1 ppm signals were designated to the C4" and C1" respectively, while 109.1 ppm was designated to C2" and C6", confirming the existence of galloy groups. Figure 6A showed resonances at 134.7 ppm and132.8 ppm attributed to C4' and C1' respectively, while a resonance at 108.4 ppm to C2' and C6'. The chemical shift of C4a was 99.2 ppm, and that of C6 and C8 was 94 ppm. Furthermore, 71 ppm and 29.1 ppm were resonances of C3 (Czochanska *et al*. 1980) in extension units and C4 in terminal units.

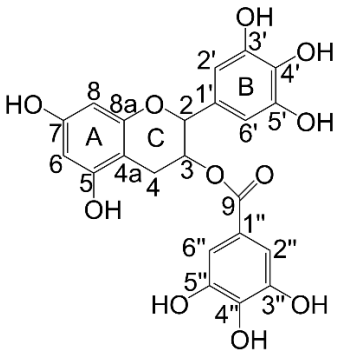

Fig. 5. The localizations of carbon and hydrogen in condensed tannin structure

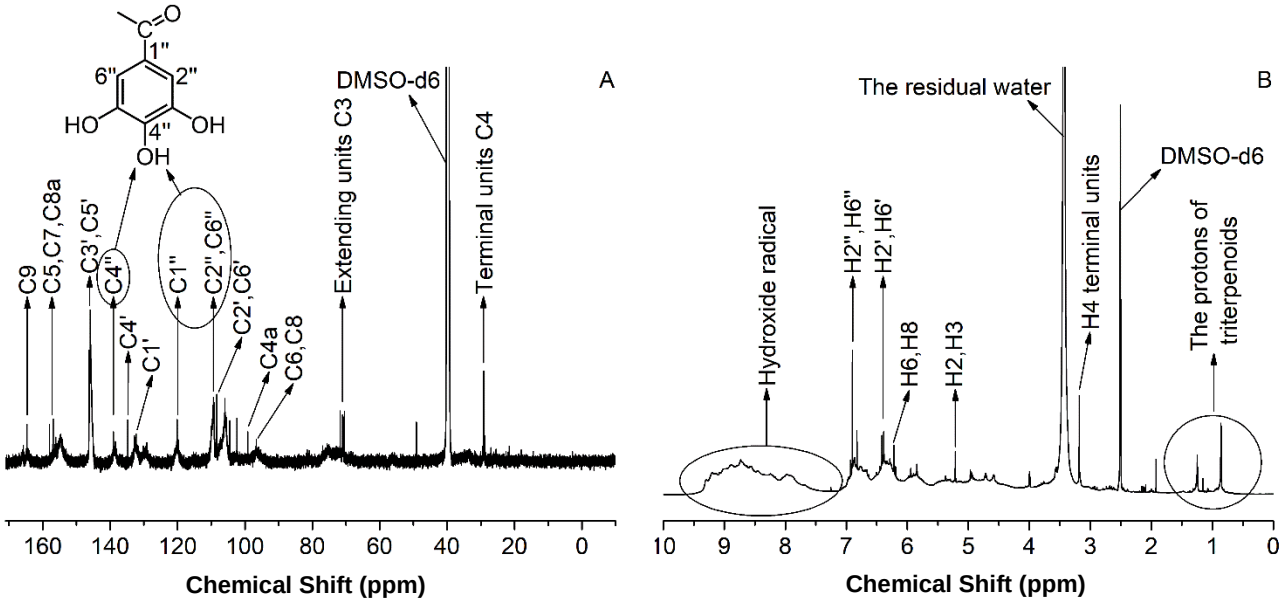

Fig. 6. ^{13}C NMR and ^{1}H NMR spectrum of bayberry tannin.

The ^{1}H NMR spectra of bayberry tannin is shown in Fig. 6B. Resonances at 7 to 10 ppm were attributed to the proton peaks of the hydroxyl groups of PC with different degrees of polymerization. The sharp peak of 6.9 ppm corresponds to the resonance of H2"and H6" protons in C ring. H2', H6' and H6, H8 had their chemical shift at 6.4 ppm and 6.22 ppm respectively. In addition, the chemical shift of H2 and H3 was 99.2 ppm, and that of H4 in terminal units was 3.2 ppm. Figure 6B showed the resonances between 0.5 to 1.5 ppm attributed to the protons of triterpenoids remaining after extraction (Muccilli *et al*. 2017).

GPC analysis

GPC is an effective method to calculate the average molecular weight of condensed tannin, and it can also be used to analyze the molecular weight distribution simultaneously (Teng *et al*. 2016). Based on previous studies, the retention time of condensed tannin was 14 to 17.5 min, while a series of hybrid peaks appears later due to the solvent THF (Yang *et al*. 2020).

As tannins are acetylated, the number of acetyl groups (CH_3CO-) needs to be removed to calculate the molecular weight distribution. Figure 7 shows that the retention time of bayberry tannin was 15.9 to 17.3 min, and its weight-average molecular weight (Mw) and numerical-average molecular weight (Mn) were 1442.5 and 1192.1 respectively. According to the MALDI-TOF MS, ^{13}C NMR and ^{1}H NMR analysis, the polymerization degree of bayberry tannin was 3.2 if gallocatechin gallate (456 Da) was taken as the monomer unit, while the value was shown as 4.7 in line with the gallocatechin (304 Da) unit.

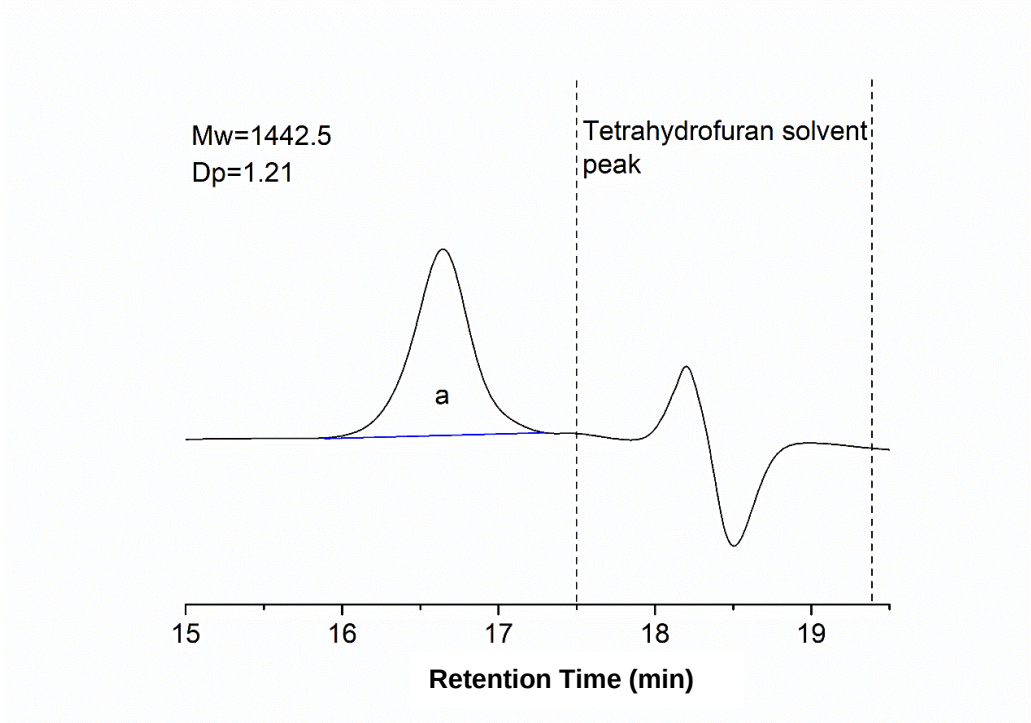

Fig. 7. GPC chromatograms of bayberry tannin

CONCLUSIONS

1. By studying the effects of four extraction factors: STSR, SC, ET, and ST through CCD, this research successfully extracted tannin from 100-mesh bayberry barks with UAE. The optimized condition was determined with ratio of 1:57.2 g/mL at 71.3% SC with ET of 39.1 min at a temperature of 48.75 °C. Under the optimized conditions, TCT yield and TPC reached their maximum values of 17.55% and 365.0 mg GAE/g, respectively. The statistical analysis of the high correlation coefficients confirms the validity of the stablished model. The verification of the optimized conditions shows that the experimental value is within the 95% prediction interval and the deviation is small.

2. Furthermore, the monomer unit of the polyflavonoids of bayberry tannin was identified as PC *via* MALDI-TOF, ^{13}C NMR, and ^{1}H NMR, which ranged from dimers to heptamers. The molecular weight distribution of bayberry tannin (Mw=1442.5, DP=1.21) was determined by GPC, and the degree of polymerization was deduced to 3.2 and 4.7, if gallocatechin gallate and gallocatechin was taken as the subunit respectively.

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