

Optimization of Microwave-assisted Extraction of Flavonoids from *Cryptotaenia japonica Hassk* using Response Surface Methodology

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Abstract: The objective of the study was to optimize the processing of Microwave-Assisted Extraction (MAE) of flavonoids from *Cryptotaenia japonica Hassk*. The effects of processing parameters include ethanol proportion, extraction time, extraction temperature and ratio of liquid to solid on the flavonoids yield were determined and the optimal conditions for flavonoids were evaluated by means of Response Surface Methodology (RSM). The optimal conditions were detected and tri-dimensional response surfaces were plotted from the mathematical models. The F-test and p-value showed that all parameters had highly significant effects on the response value. The optimum extraction conditions were ethanol of 70% (v/v), extraction time of 15 min, extraction temperature of 75°C and liquid to solid ratio of 20 mL/g. Under these conditions, 27.01 mg/g total flavonoids were obtained by Microwave-Assisted Extraction (MAE), which are close to the predicted value (27.48 mg/g). MAE method was applied successfully to extract total flavonoids from *Cryptotaenia japonica Hassk*.

Keywords: *Cryptotaenia japonica Hassk*, flavonoids, microwave-assisted, Response Surface Methodology (RSM)

INTRODUCTION

Cryptotaenia japonica Hassk (Yajiaoban) is a valuable perennial herb and widely distributed in forests or shady places of China and Japan. *Cryptotaenia japonica Hassk* is a popular green food in west district of China and it also has been used as folk medicine to treat various ailments such as skin itch and traumatic injury (Yang and Xia, 2010). Its main chemical constituents include essential oil such as- (-) -and (+) - β -Selinenes, β -and (-) γ -elemenes (Cheng *et al.*, 2010, 2008). Recent studies showed it also includes flavonoids (Li *et al.*, 2006; Yao *et al.*, 2010).

Flavonoids are a group of plant phenolics, abundant in plant kingdom. Many studies found that flavonoids existed a wide range of pharmacological activities, which include antioxidant, antimicrobial, anti-inflammatory, anticancer, cardio protective, neuroprotective, antidiabetic, anti-osteoporotic, estrogenic, antiestrogenic, anxiolytic, analgesic and anti-allergic (Awaad *et al.*, 2012; Kang *et al.*, 2012; Kamiyama and Shibamoto, 2012; Calderon-Montano *et al.*, 2011; Cushnie and Lamb, 2011). In recent years, more and more investigators focus on flavonoids and efficient extraction technique is key issue for its widely application.

Microwave Assisted Extraction (MAE) is one of the important techniques for extracting functional

components from different plant matrices. MAE is based on absorption of microwave energy by molecules of polar chemical compounds and can heat the material homogeneous. Comparing with traditional extraction method, it has the advantage of strong penetrating force, high selectivity, high heating ability, less extraction time; reduce solvent consumption and energy requirements (Gao *et al.*, 2004; Kerem *et al.*, 2005). Especially, it also has been proved to improve the extraction rate for active components. Recently, many reports have been published on the application of MAE of secondary metabolites from plants (Routray and Orsat, 2012; Kong *et al.*, 2012; Kumar *et al.*, 2011; Merin *et al.*, 2010).

Response Surface Methodology (RSM) is widely employed for constructing and exploring estimated functional relationship between a response variable and design variables (Sharma *et al.*, 2009). Meanwhile successfully been used to model and optimize biochemical and biotechnological process related to food systems (Liyana-Pathirana and Shahidi, 2005). In this study, we used MAE to extract the total flavonoids from *Cryptotaenia japonica Hassk*. The effects of ethanol proportion, ratio of liquid to solid, extraction temperature and extraction time on total flavonoids yield were investigated and extraction conditions were optimized using response surface methodology by evaluating responses of total flavonoids yield.

MATERIALS AND METHODS

Materials and reagents: *Cryptotaenia japonica Hassk* was harvested in May from west region of Hunan province in China. All plant materials were washed and separated into root and edible parts. The latter were dried in an oven with 60°C and pulverized into an average size of 2 mm by a disintegrator and then kept in a dry place until use. Rutin was purchased from National Institutes for Food and Drug Control, sodium nitrite, sodium carbonate, aluminum nitrite, sodium hydroxide and ethanol were purchased from Sinopharm Chemical Reagent Co., Ltd. All of the chemicals and solvents are analytical grade.

Equipments and apparatus: Multifunctional microwave reaction/extraction apparatus (Shanghai xintuo analytical instruments Co., Ltd., Shanghai, China) was provided non pulse type variable frequency microwave power regulating function, can be worked in 0~1300 W range in any action/static setting storage. The emission frequency of the extractor was 2450 MHz. The temperature was measured using infrared detector, but the extraction power and extraction temperature cannot be set at the same time in this instrument, the rising and reduce of temperature is completed through the rise and fall of the microwave power controlled automatic. High speed refrigerated centrifuge (Hunan xiangyi centrifuge Co., Ltd., Changsha, China); 722s type visible spectrophotometer (Shanghai precision and scientific instrument Co., Ltd., Shanghai, China).

Determination of total flavonoids: The calibration curves were constructed using solutions of rutin at 0.1, 0.2, 0.4, 0.8 and 1.0 mg/L, respectively. Using the concentration of rut in standard solution (C) as abscissa and the Absorbency (A) as y-coordinate, the linear chart was constructed. The regression equation was $A = 1.0286C - 0.0161$ ($R = 0.9993$). The total flavonoids were measured according to colorimetric method with some modification (Zhu *et al.*, 2010). An aliquot of 1 mL of the sample solution was accurately removed in a volumetric flask (10 mL) by adding 0.6 mL of NaNO₂ (5%) solution, shaken up and then standing for 6 min. Secondly, 0.5 mL of the Al (NO₃)₃ (10%) solution was added to the volumetric flask, shaken and was left to stand for 6 min. Finally, 4.0 mL of the NaOH (4.3%) solution was added to the volumetric flask and followed by adding alcohol to the scale, shaken and left to stand for 15 min before determination. Using the sample solution without coloration as reference solution, 507 nm wavelengths were used to determine the content of flavonoids in the sample by ultraviolet-visible detector. Data was presented as mean for three replicate measurements. When extraction yield of flavonoids was calculated, the following formula can be used:

Table 1: Code and level of factors chosen for the trials

Independent variable	Level		
	-1	0	+1
Ethanol proportion (%)	70	80	90
Extraction time (%)	5	10	15
Extraction temperature (°C)	65	70	75
Ratio of liquid to solid (mL/g)	20	30	40

Table 2: Experimental designs using BBD and results

Experiment no	Ethanol, proportion (X ₁ , %)	Extraction time (X ₂ , min)	Extraction temperature (X ₃ , °C)	Ratio of liquid to solid (X ₄ , mL/g)	Yield of total flavonoids
1	1	-1	0	0	20.14
2	0	0	0	0	21.94
3	1	1	0	0	20.78
4	-1	1	0	0	24.41
5	-1	-1	0	0	21.40
6	0	0	-1	1	24.63
7	0	0	-1	-1	20.07
8	0	0	1	-1	26.04
9	0	0	1	1	25.44
10	-1	0	0	-1	17.82
11	0	-1	-1	0	21.62
12	0	1	-1	0	24.50
13	-1	0	0	1	24.79
14	1	0	0	-1	17.81
15	0	0	0	0	21.92
16	1	0	0	1	24.31
17	0	-1	1	0	22.79
18	0	1	1	0	25.49
19	0	1	0	-1	23.49
20	-1	0	-1	0	23.12
21	1	0	-1	0	20.03
22	1	0	1	0	22.12
23	-1	0	1	0	26.53
24	0	-1	0	1	23.50
25	0	-1	0	-1	18.31
26	0	1	0	1	25.14
27	0	0	0	0	23.33

Table 3: Analysis of Variance (ANOVA) for the experimental result

Source	S.S.	df	M.S.	F-value	p-value prob>F
Model	136.620	14	9.760	4.760	0.0051
X ₁	13.810	1	13.810	6.740	0.0234
X ₂	21.430	1	21.430	10.460	0.0072
X ₃	17.340	1	17.340	8.460	0.0131
X ₄	49.060	1	49.060	23.940	0.0004
X ₁ X ₂	1.410	1	1.410	0.690	0.4236
X ₁ X ₃	0.440	1	0.440	0.210	0.6529
X ₁ X ₄	0.058	1	0.058	0.028	0.8696
X ₂ X ₃	8.10E-003	1	8.10E-003	3.95E-003	0.9509
X ₂ X ₄	3.130	1	3.130	1.530	0.2398
X ₃ X ₄	6.660	1	6.660	3.250	0.0965
X ₁ ²	5.060	1	5.060	2.470	0.1422
X ₂ ²	0.025	1	0.025	0.012	0.9131
X ₃ ²	10.690	1	10.690	5.220	0.0413
X ₄ ²	9.83E-03	1	9.83E-03	4.79E-003	0.9459
Residual	24.590	12	2.050		
Lack of fit	23.300	10	2.330	3.600	0.2367
Pure error	1.290	2	0.650		
Cor total	161.210	26			

$$X = \frac{C \times V \times D}{m} \quad (1)$$

where,

- X (mg/g) = Extraction yield of flavoniods
- C (mg/mL) = Flavoniods content of test solution calculated by standard curve
- V (ml) = Volume of test solution
- D = Total dilution value
- m (g) = Mass of test sample

Optimization of the extraction procedure: There are many parameters affect the MAE efficacy, such as particle size of sample, extract cycle, liquid to solid ratio, extraction time, extraction temperature, extraction pressure and microwave power (Bucić-Kojić *et al.*, 2009; Spigno *et al.*, 2007). Based on the investigation on preliminary experiment results, appropriate ranges of extraction time, extraction temperature, ethanol proportion and ratio of liquid to solid were selected. The independent variables chosen were ethanol proportion (X₁), extraction time (X₂), extraction temperature (X₃) and liquid to solid ratio (X₄). A four-variables and three-level of BBD were adopted to optimize the extraction procedure, Three levels of each variable were coded as -1, 0 and +1 (Table 1). A total of 27 experimental runs with different combinations of four-factors were carried out. The experimental designs with the observed responses for total flavonoids yield are presented in Table 2. All the experiments were performed in random order and all experimental results were expressed as mean of five parallel measurements. Analysis of Variance (ANOVA) was performed by “Design expert 8.0.5.b” software (Table 3). p values<0.05 were regarded as significant difference and p values<0.01 as very significant. According to the analysis of variance, the regression coefficients of individual linear, quadratic and interaction terms were

determined. The regression coefficients were then used to generate dimensional and contour maps from the regression model.

RESULTS AND DISCUSSION

The investigation results on preliminary experiment: The effect of different ethanol proportion on the yield of extracting total flavonoids was shown in Fig. 1A. Different ethanol proportion was set at 60, 70, 80 and 90 and 100% (v/v), respectively and the other reaction conditions were set as follows: extraction time of 10 min, ratio of liquid to solid 30 mL/g. It showed that the yield of total flavonoids was greatly influenced by the ethanol proportion. When the ethanol proportion was lower than 80% (v/v), the yield of total yield was increased with the increase of ethanol proportion. When the ethanol proportion was higher than 80% (v/v), the yield was slowly decrease with the further increase of ethanol proportion and reach its minimum in the 100% (V/V), which might be attributed tothe change of solvent polarity with change of ethanol proportion. Furthermore, high concentration of ethanol may holding back the dissolution of polyphenolsandthen influencing the extraction rate (Yang *et al.*, 2009). Considering the limitation of the ethanol proportion, 70-90% (v/v) ethanol proportion was used to be optimal range in the Box-Behnken design experiments. The yield of total flavonoids was affected by different extraction time (1, 5, 10, 15 and 20 min, respectively) were shown in Fig. 1B. When other three factors (ethanol proportion, extraction temperature and ratio of liquid to solid) were fixed at 80% (v/v), 70°C and 30 mL/g. The results indicated that the yield of total flavonoids increased with the increase of MAE time in the beginning of extraction. The yield reached its maximum (25.4063 mg/g) in 10 min during the MAE process. When the extraction time was more than 10 min, the yield of total flavonoids would not increased

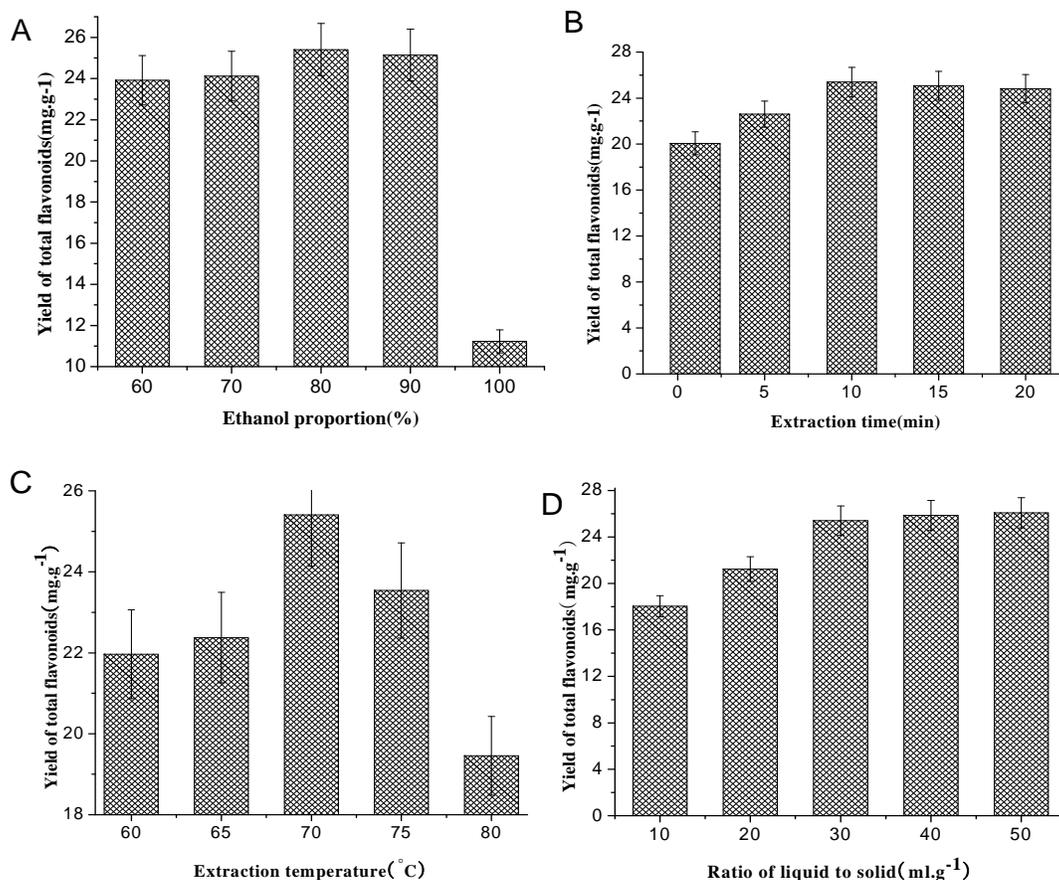


Fig. 1: Effect of ethanol proportion, extraction time, extraction temperature and ration of liquid to solid on the yield of total flavonoids

with the increased of extraction time, hence, the extraction time rangeto be optimized in the Box-Behnken design experiments from 5 to 10 min. The influence of extraction temperature on the yield of total flavonoids was shown in Fig. 1C. Different temperature were set at 60, 65, 70, 75 and 80°C, respectively when the other reaction conditions were set as follows: extraction time 10 min, ethanol proportion 80%, ration of liquid to solid 30 mL/g. As shown in Fig. 1C, the yield of total flavonoids was greatly influenced by the extraction temperature. When extraction temperature was lower than 70°C, the yield increased with the increase of extraction temperature. When temperature was higher than 70°C, the yield decreased with the further increase of temperature, the yield could reach its minimum 19.46 mg/g. These data suggest that a higher extraction temperature may lead to thermal degradation of total flavonoids. So the 70°C was chosen as the central point for the Box-Behnken design experiments. The yield of total flavonoids affected by different ratio of liquid to solid (10, 20, 30, 40 and 50 mL/g, respectively) can be seen in Fig. 1D, when the other three factors (extraction time, extraction temperature

and ethanol proportion) were fixed at 10 min, 70°C and 80% (v/v). The result illustrated the yield of total flavonoids were reached the higher value (25.41 mg/g) at the liquid to solid ratio of 30 mL/g and then it maintained a mild slope when the ratio of liquid to solid increased from 30 to 50 mL/g. As here was no significant difference between the yield at 40 mL/g and that at 50 mL/g. To save the solvent consumption, 20-40 mL/g were chosen as test level of the ratio of liquid to solid in the optimization experiments.

Statistical analysis and model fitting: The experimental data obtained from the 27-run-experiment were given in Table 2, the Analysis of Variance (AVONA) of total flavonoids yield is presented in Table 3, Using the designed experimental data (Table 2), the polynomial model for the flavonoids yield was regressed and shown as below (in term of coded factors):

$$Y (\text{Yield of total flavonoids}) = + 22.40 - 1.07X_1 + 1.34X_2 + 1.20X_3 + 2.02X_4 - 0.59X_1X_2 - 0.33X_1X_3 - 0.12 X_1X_4 - 0.045X_2X_3 - 0.89X_2X_4 - 1.29X_3X_4 - 0.97X_1^2 + 0.069 X_2^2 + 1.42 X_3^2 + 0.043X_4^2 \quad (2)$$

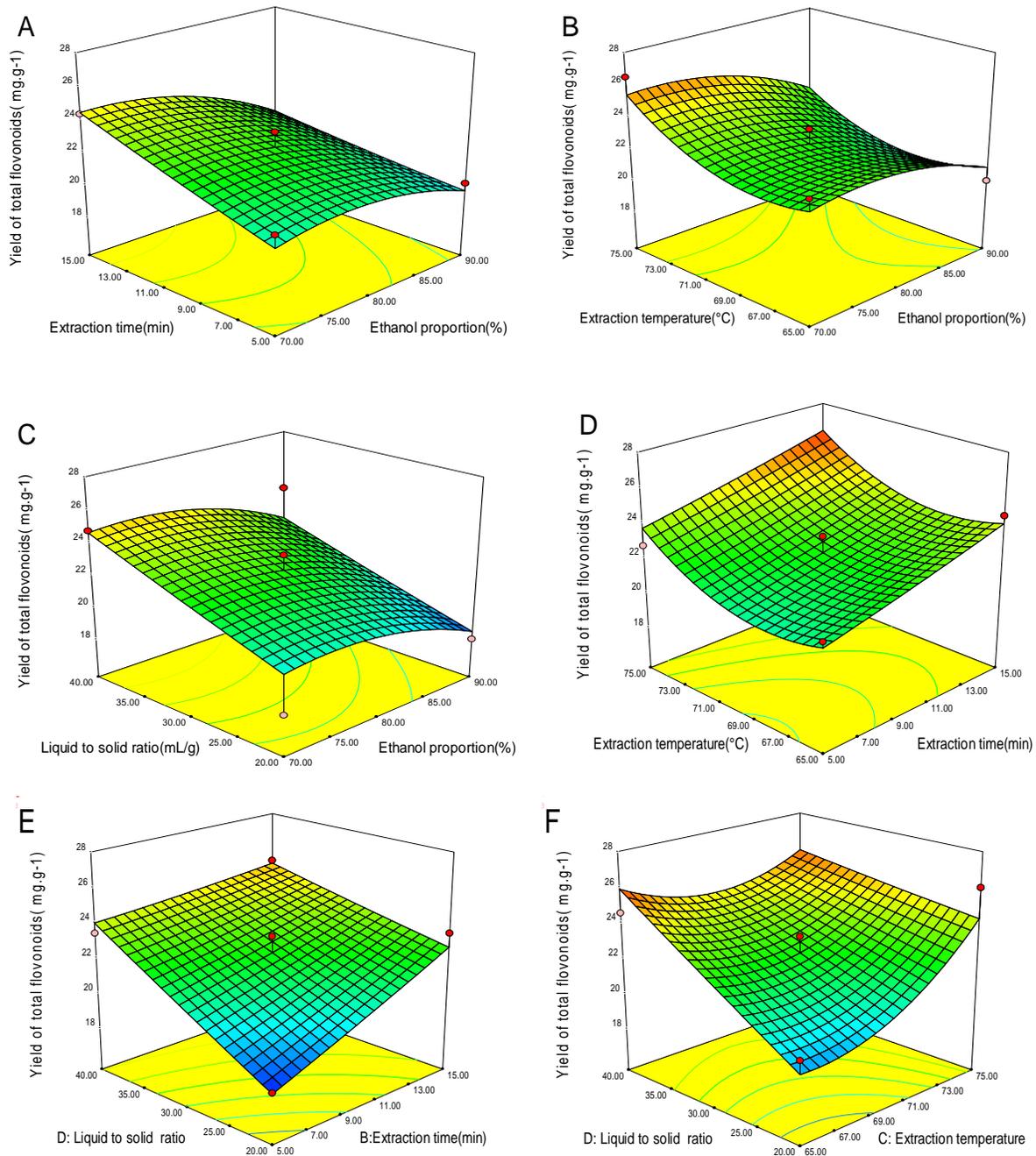


Fig. 2: Response surface plots showing the effects of variables on yield of total flavonoid

As shown in Table 3, the ANOVA response surface quadratic regression model showed that the model was highly significant ($p < 0.01$) with highly F-value (4.76), at the same time, the lack-of-fit statistics ($p = 0.2367$), which was used to test the adequacy of the model is not significant. No abnormality was obtained from the diagnoses of residuals. Thus, it can be concluded that the model was statistically sound and adequate to represent the relationship between the response and the independent variables.

The linear effects of each independent variable within the experimental range also can be deduced in Table 3. That total flavonoids yield was affected highly significantly by ratio of liquid to solid (X_4) ($p = 0.0004$), followed significantly by extraction time (X_2) ($p = 0.0072$), extraction temperature (X_3) ($p = 0.0131$), ethanol proportion (X_1) ($p = 0.0234$), whereas the interaction of all parameters were insignificant ($p > 0.1$).

The effects of the independent variables and their interaction on the yield of total flavonoids can also be

seen on three dimensional response surface curves and contour plots shown on the Fig. 2A to F. The plots were generated by keeping two of the variables constant, which indicated the changes in total flavonoids yield under different MAE conditions. The effects of extraction time and ethanol proportion on the yield of total flavonoids are shown in Fig. 2A. The other two factors, extraction temperature and liquid to solid ratio, were set at 70°C and 30 mL/g, it obviously that the yield increased significantly as the extraction time increased, it agree with the Table 3, as the linear effect ($p < 0.05$) was significant, from the profile of Fig. 2A, extraction time had a more significant effect on total flavonoids yield than ethanol proportion in the selected range. The interactive influences of the extraction temperature and ethanol proportion on yield of total flavonoids were presented in Fig. 2B. The other two variables extraction time and liquid to solid ratio were set at 10 min and 30 mL/g, respectively. The total flavonoids yield increased slowly as the temperature increase. During the initial extraction period (lower temperature), the total flavonoids yield increased with the ethanol proportion increased. At higher extraction temperature periods, the yield declined with an increase in ethanol proportion, likewise, the Fig. 2C showed the effect of ethanol proportion and liquid to solid ratio on total flavonoids yield when extraction temperature and extraction time, were held at 70°C and 10 min, respectively. The response surface plots indicate that the ethanol proportion and liquid to solid ratio had significant effects on total flavonoids yields. An increase in the extraction yield was noted with increased liquid to solid ratio no matter the ethanol proportion is lower or higher level, the total flavonoids yield slightly increased at the lower level of ethanol proportion and slightly reduced at the higher level of ethanol proportion along with liquid to solid ratio increase. In Fig. 2D, the influence of extraction temperature and extraction time on the total flavonoids yield are shown when other liquid to solid ratio and ethanol proportion were set 30 mL/g and 80°C, respectively, as shown in Fig. 2D, with the increased in extraction temperature, the total flavonoids yield increased slowly, similarly, total flavonoids yield steep increase with the extraction time increased. The Fig. 2E demonstrates the effect of extraction time and liquid to solid ratio on total flavonoids yield at a constant ethanol proportion of 80% and extraction time of 10 min. It that the effect of the liquid to solid ratio and extraction temperature on total flavonoids yield were linear, it was clear that with an increase of extraction time and liquid to solid ratio, the yield was gradually increased. As shown in Fig. 2F, an increase of the total flavonoids yield could be significantly achieved with the increase of liquid to solid ratio. Obviously, the output of total

Table 4: Comparison of MAE and maceration extraction

Extraction method	Maceration extraction	MAE
Ethanol proportion (%)	70	70
Extraction temperature (°C)	70	75
Extraction time (min)	90	15
Ration of liquid to solid (mL/g)	20	20
Yield of total flavonoids (mg/g)	21.69	27.01

flavonoids gradually increased followed the extraction temperature increasing from 65 to 75°C. Similarly, the total flavonoids yield was increased simultaneously with the increase in liquid to solid ratio.

Verification: The optimal conditions was obtained using model were 70.06% (v/v) ethanol proportion, extraction time of 15 min; extraction temperature of 75°C and ratio of liquid to solid of 20 mL/g. Considering the convenience of operation, the ethanol proportion was adjusted as 70% (v/v), the other factors were unchanged, 5 parallel experiments were performed under the optimal conditions, the total flavonoids yield (27.01 mg/g) was in close agreement with the predicated value (27.48 mg/g), these result indicates that the optimization was validation and reflecting the expected optimization.

Comparison of MAE and maceration extraction

method: Maceration extraction was performed in a water bath at in 70°C, 3.0 g sample powder were placed into a 250 mL glass flask with 60 mL 70% ethanol (v/v) and extracted for 90 min. The result was shown in Table 4. As Table 4 shown, the optimized MAE has produced 27.01 mg/g total flavonoids, which is higher than that of maceration extraction (21.69 mg/g), the MAE has advantage of reduction in extraction time, saving energy and increased extraction yield as compared with maceration extraction. These results indicated that the MAE was more suitable for extraction of total flavonoids from *Cryptotaenia japonica Hassk* than maceration extraction.

CONCLUSION

An optimized microwave-assisted extraction method of total flavonoids acids from *Cryptotaenia japonica Hassk* has been developed with RSM. This is the first report on the extraction of total flavonoids from *Cryptotaenia japonica Hassk* using MAE. RSM and desirability function enabled us to screen several significant extraction factors and find out the optimum extraction conditions in a quick and economical way. Total flavonoids can be efficiently extracted from *Cryptotaenia japonica Hassk* using MAE. The statistical testing and the 3-D response surface plots indicated that the changes of liquid to solid ratio,

extraction time and extraction temperature and ethanol proportion on the response Y had a significant effect on total flavonoids extraction yield. The actual extraction yield of total flavonoids was 27.01 mg/g under the optimization conditions at ethanol proportion of 70%, extraction time of 15 min, extraction temperature of 75°C and ratio of liquid to solid of 20 mL/g. The obtained yield was agreement well with the predicted values (27.48 mg/g). Compared with the maceration extraction technology, the optimized MAE can save time and energy and provide higher extraction yield of total flavonoids.

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