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Extraction, Partial Identification and Bioactivities of Total Flavonoids from *Carex meyeriana* Kunth

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Abstract: To date, knowledge associated with extraction, chemical constituents and bioactivities of Total Flavonoids from *Carex meyeriana* Kunth (TFCMK) that remains unclear. Therefore, this paper was designed to optimize the ethanol reflux extraction method by response surface methodology and determine the chemical constituents by Liquid Phase-Mass Spectrometry (HPLC-MS); additionally, the bioactivities including antioxidant, antibacterial and hemolysis test were also explored. The maximum TFCMK extraction yield of 6.248%±0.016% was obtained under the optimal conditions as follows: Ethanol concentration 50%, ratio of liquid-to-solid 31 mL/g, extraction time 91 min and extraction temperature 71°C. Meanwhile, 9 compounds of TFCMK were identified by using LP-MS for the first time. In addition, TFCMK showed higher antioxidant activity compared with vitamin C, as evidenced by the lower EC_{50} or IC_{50} of TFCMK (reducing power assay: 0.125±0.025 mg/mL, DPPH radicals: 0.28± 0.031, $ABTS^{+}$ radicals:0.012 ± 0.005, hydroxyl radicals: 0.028±0.008 mg/mL; The EC_{50} or IC_{50} of VC 0.156±0.012, 0.059±0.019, 0.068±0.024 and 0.032±0.007 mg/mL, respectively.). Broth microdilution assays had demonstrated that the minimal inhibitory concentrations (MICs) of TFCMK on Bacillus pumilus, *Bacillus subtilis* and *Escherichia coli* were 0.625±0.0032, 1.250±0.0216 and 5.000±0.0205 mg/mL, respectively. Besides, the hemolysis rate (less than 5%) *in vitro* implied that TFCMK possessed a good blood compatibility. In summary, this paper provides evidences that TFCMK might be applied as an antioxidant and antibacterial agent in the pharmaceutical and chemical industries.

Keywords: *Carex meyeriana* Kunth, Total Flavonoids, Extraction, Antioxidant, Antibacterial, Hemolysis Test, Response Surface Methodology, Liquid Phase-Mass Spectrometry

Introduction

Carex meyeriana Kunth (CMK), a perennial herb belonging to a genus *Cares* species in the family *Cyperaceae*, is widely distributed in China including Heilongjiang, Jilin, Inner Mongolia and Sichuan (Reznicek, 1990) and other countries such as Far East Russia, Mongolia, Korea and Japan (Yang *et al*., 2017a). The shoe pads, waist supports, mattress and other daily necessities made of CMK as the main material can keep warm and actively absorb moisture and they are hard to self-corrode (Luo and Zhang, 2008). CMK is a valuable germplasm resource and CMK together with the *Panax ginseng C.A. Mey* and pelt of marten is called "three treasures in Northeast China" (Yun *et al*., 2016). In our previous study, polysaccharides (Hu *et al*., 2018) and essential oils (Cui *et al*., 2018) of CMK were explored in the details. However, there has been no report on its flavonoids and their extraction process and chemical composition, especially bioactivities.

The traditional ethanol reflux extraction method (Yang *et al*., 2016) is nondestructive to the target product and featured by less impurities, mildew proofness and easy preservation. In order to improve the extraction yield of flavonoids, Response Surface Methodology (RSM) has been widely employed in

various extraction processes to determine the best experimental parameters, evidenced by the true limit state surface and statistical significance of the independent variables (Wang *et al*., 2017). Time-of-Flight (TOF)-MS and Liquid Chromatography (LC) can be used to analyze the quality of complex compounds and to better separate flavonoids (Dong *et al*., 2017). The antioxidant activity of flavonoids is usually evaluated through the reduction ability of Fe^{2+}, scavenge DPPH free radicals, $ABTS^{+}$ free radicals and hydroxyl free radicals.

The optimum extraction process of TFCMK by ethanol reflux was determined through RSM and the chemical constituents were studied by Liquid Chromatography-Mass Spectrometry (LC-MS); more importantly, the antioxidant, antibacterial activity and lysis erythrocytes *in vitro* were investigated. Taken together, this paper is the first time to systematically explore the basic research of TFCMK, thereby providing a scientific basis for the development and utilization of TFCMK.

Materials and Methods

Materials

Carex meyeriana Kunth (CMK) samples were collected from a wetland in the northern suburbs of Jiamusi, Northeast China (45°56′N, 129°29′E). The ground part of CMK was identified by Prof. Guangshu Wang (Jilin University, China). The herbarium samples (Voucher No. TFCMK -04) were deposited at School of Chemistry and Pharmaceutical Engineering, Jilin Institute of Chemical Technology, Jilin, China. TFCMK were placed in a semi-dark room for natural drying. Diphenyl Picrylhydrazinyl (DPPH) and 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (both Aladdin, St. Shanghai, China) and L-Ascorbic acid (VC, Hushi, St. Shanghai, China) were used. All reagents and solvents were the analytical grade. All the bacterial strains including *Bacillus subtilis* ATCC 6633, *Bacillus pumilus* ATCC 700814 and *Escherichia coli* ATCC 25922 were purchased from Beijing Zhongke Quality Inspection Biotechnology Co. Ltd. The hemolysis of TFCMK was assessed by evaluating the lysis of human Red Blood Cells (hRBCs) obtained from the General Hospital of Jilin Chemical Group Corporation (Jilin, Jilin, China).

Methods

Extraction Experiment

Five gram of the CMK crushed was placed in a 250 mL round-bottom flask together with the extracting solvent, followed by extraction at appropriate ethanol concentration, liquid-to-material ratio, extraction time and temperature. After that, the samples were filtered and dried on a freeze dryer.

Determination of Total Flavonoid Content

The content of total flavonoids was determined by sodium nitrite chromogenic method (Liu *et al*., 2012). The standard reserve solutions of rutin were accurately prepared at 510 nm on an ultraviolet spectrophotometer. Results showed the linear equation was Y = 14.548x+0.0013 (R^2 = 0.9997) in the linear range of 0-0.06 mg/mL. The extraction rate of TFCMK was calculated as follows:

$$Extraction\ rate\ of\ TFCMK = \left[\left(C \times V \times 10^{-3}\right) / M\right] \times 100\% \quad (1)$$

where, C, V and M are the concentration of TFCMK (mg/mL), dilution multiple (mL) and quality of TFCMK (g), respectively.

Optimization of Extraction Technology by BBD-RSM

According to RSM and Box-Behnken central composite experiment Design (BBD), the four factors of ethanol concentration (X_1, %), liquid-to-material ratio(X_2, mL/g), extraction time (X_3, min) and extraction temperature (X_4, °C) were optimized and defined as three horizontal levels of high (+1), medium (0) and low (-1) (Table 1). The factors were coded as follows (Mou *et al*., 2017):

$$X_i = (x_i - x_0) / \Delta x_i \quad (2)$$

where, Xi and x_i are the coded and actual values of the independent variable, respectively, x_0 is the actual value of the independent variable at the central point and Δx_i is the step of the variable.

With the extraction rate of TFCMK as the response value, the BBD experiment was designed on Design-Expert V 8.0.6.1 Trial. The four-factor and three-level response surface analysis was established involving 24 groups of factorial experiments and 5 groups of center experiments (Table 2).

The quadratic polynomial regression equation for optimization was fitted as follows (Chen *et al*., 2012):

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i<j}^{n} \beta_{ij} X_i X_j + \varepsilon \quad (3)$$

where, Y is the predicted extraction rate of TFCMK and β_0 is the constant coefficient; the first-order coefficients of β_i, β_{ii} and β_{ij} are X_i; the second-order coefficients and the interaction influence are the experimental errors (random).

Chemical Composition Analysis of TFCMK by HPLC–ESI-Q-TOF MS/MS

The flavonoids were dissolved in a small amount of acetonitrile into a reserve solution at a certain concentration.

Table 1: Independent variables and levels used for box–behnken design

			Levels and range		
Factors	Units	Code	-1	0	+1
Ethanol concentration	v/v	X_1	40	50	60
Liquid-solid ratio	mL/g	X_2	20	30	40
Extraction time	min	X_3	60	90	120
Extraction temperature	°C	X_4	60	70	80

Table 2: Design and results of responsse surface experiment

No.	X_1	X_2	X_3	X_4	Y%
1	-1	-1	0	0	1.46
2	1	-1	0	0	1.84
3	-1	1	0	0	2.70
4	1	1	0	0	1.30
5	0	0	-1	-1	2.06
6	0	0	1	-1	1.90
7	0	0	-1	1	2.95
8	0	0	1	1	2.63
9	-1	0	0	-1	2.62
10	1	0	0	-1	2.12
11	-1	0	0	1	2.66
12	1	0	0	1	2.12
13	0	-1	-1	0	1.43
14	0	1	-1	0	2.19
15	0	-1	1	0	2.14
16	0	1	1	0	2.77
17	-1	0	-1	0	2.56
18	1	0	-1	0	2.01
19	-1	0	1	0	2.51
20	1	0	1	0	2.39
21	0	-1	0	-1	2.45
22	0	1	0	-1	2.46
23	0	-1	0	1	2.29
24	0	1	0	1	2.44
25*	0	0	0	0	6.08
26*	0	0	0	0	6.03
27*	0	0	0	0	6.12
28*	0	0	0	0	6.58
29*	0	0	0	0	6.36

*Five replicates of central point

HPLC conditions were as follows: Symmetry® C18 (5μm, 4.6×250mm); injection volume of 5 μL; mobile phase containing 0.1% formic Acid (A) and acetonitrile (B). The elution gradient of the mobile phase was as follows: 0-25 min, 85-65%A; 25-35 min, 60-45%A; 35-50 min, 45-20%A; 50-55 min, 20-0%A; 55-60 min, 0%A; 60-60.1 min, 0-15%A; 60.1-66 min, 15%A.

The MS conditions in negative mode were as follows: injection voltage of the ESI source at 4000 V, dry gas volume flow rate at 1 L/min, atomization gas pressure 30 psi, auxiliary gas pressure 60 psi, interface continuous heating and a TOF-MS detector (Agilent Technologies, Santa Clara, CA, USA). The MS ion source was operated at 300°C and a scan mass range of m/z 100-1500.

Antioxidant Activity

Four systems were used to evaluate antioxidant activity of TFCMK *in vitro* and VC was served as the positive control. The reducing power was determined following a reported protocol with TFCMK and VC solutions (0.004, 0.008, 0.016, 0.032, 0.068 mg/mL) (Mendes *et al*., 2011). The results were expressed as the effective concentration at 0.5 absorbance unit (EC_{50}).

The DPPH· scavenging rate was determined by using an existing protocol with TFCMK solutions (0.004, 0.008, 0.016, 0.032, 0.068 mg/mL) and VC solutions (0.001, 0.003, 0.005, 0.009, 0.012 mg/mL) (Wang *et al*., 2016). The IC_{50} was expressed as the inhibition concentration at 50% clearance rate.

The $ABTS^+$ scavenging rate was determined a with same as the above TFCMK and VC solutions (0.001, 0.025, 0.005, 0.075, 0.01 mg/mL) (Moo-Huchin *et al*., 2015).

The hydroxyl radical scavenging was determined following a protocol with TFCMK solutions (0.06, 0.13, 0.25, 0.50, 0.75 mg/mL) and VC solutions (0.1, 0.2, 0.4, 0.6, 0.8 mg/mL) (Hifney *et al*., 2016).

Antibacterial Activities

The Minimum Inhibitory Concentration (MIC), defined as the lowest concentration of TFCMK to inhibit bacterial distinct growth, was detected by a broth microdilution method (Li *et al*., 2017) with some modifications. *B. subtilis*, *B. pumilus* and *E. coli* strains were cultured in 96-well plates. The concentration decreased by 2-fold to dilute the TFCMK into a gradient of 0.625-5.000 mg/mL. Each well was added with the standard suspension of one of the strains (10^8 CFU/mL) and the final anhydrous ethanol in each well was used to dissolve the TFCMK, followed by incubation at 37°C for 24 h. Chloramphenicol (0.800-5.000 mg/mL) was used as a positive control and the solution containing anhydrous ethanol without TFCMK was used as a negative control. All assays were repeated in triplicate.

Hemolysis Rate

The hemolysis rate of TFCMK assay was determined by a protocol (Chen, 2016).

The series of flavonoid solutions equivalent to the concentrations in the TFCMK solutions (0.10, 0.25, 0.50, 1.00, 1.50, 2.00 mg/mL) were used as the experimental group, distilled water and normal saline were used as positive and negative controls respectively. The hemolysis rate of TFCMK was calculated as follows:

$$Hemolysis\ rate(\%) = \left[\left(A_s - A_c\right)/\left(A_p - A_c\right)\right]\times 100\% \qquad (4)$$

where, A_s, A_c and A_p are the absorbance of the test sample, negative control and positive control, respectively.

Statistical Analysis

The experimental data were statistically tested by Analysis of Variance (ANOVA) by IBM SPSS 24 and expressed as mean ± standard error. The significant data at $p<0.05$ were calculated by Duncan's multiple range tests.

Results and Discussion

Single-Factor Analysis

Effects of Ethanol Concentration on Extraction Rate of TFCMK

The effects of ethanol concentration (30%, 40%, 50%, 60%, 70%) were investigated under the CMK dosage of 5 g, temperature at 50°C, liquid-to-solid ratio of 20 mL/g and extraction time of 60 min. The results are shown in Fig. 1a. The extraction rate of TFCMK were increased with the rise of ethanol concentration and maximized at 50%. Because as the ethanol concentration increased, the polarity of the solvent decreased and the solubility of TFCMK was intensified, which were beneficial to the leaching of flavonoids. Whereas, at higher ethanol concentration, some higher polarity flavonoids may be precipitate due to poor solubility, which in turn affected the flavonoid extraction (Yang *et al*., 2017b).

Effects of Liquid-to-Solid Ratio on Extraction Rate of TFCMK

The effects of liquid-to-solid ratio (10, 20, 30, 40, 50 mL/g) were investigated under CMK dosage of 5 g, temperature at 50°C, extraction time of 60 min and ethanol concentration of 50%. The extraction rate maximized at the liquid-to-solid ratio of 30 mL/g (Fig.1b). because with the continuous increase of solvent ethanol, the concentration gradient between the solid and solvent was considered as mainly the driving force for mass, so that the flavonoid substances were dissolved more effectively (Xie *et al*., 2017). However, with above the liquid-to-solid ratio of 30 mL/g, the content of flavonoids was limited and more impurities were extracted. As a result, the extraction rate decreased.

Effects of Extraction Time on Extraction Rate of TFCMK

The effects of extraction time (30, 60, 90, 120, 150 min) were investigated under the CMK dosge of 5 g, temperature at 50°C, liquid-to-solid ratio of 30 mL/g and ethanol concentration of 50%. With the prolongation of extraction time, the extraction rate of TFCMK increased continuously and maximized at 90 min (Fig.1c). Because as the extraction time was prolonged, the plant cell walls were destroyed and the release quantity of flavonoids increased, which led to an increase in the extraction rate of TFCMK. However, when the extraction time was too long, promoting the degradation of polyphenols, thereby reducing the flavonoid extraction (Åahin *et al*., 2017).

Effects of Extraction Temperature on Extraction Rate of TFCMK

The effects of extraction temperature (40, 50, 60, 70, 80°C) were investigated under the CMK dosage of 5 g, liquid-to-solid ratio at 30 mL/g, extraction time of 90 min and ethanol concentration of 50%. With the rise of extraction temperature, the extraction rate of TFCMK increased and maximized at 60°C (Fig. 1d). At above 60°C, the extraction rate began to decline. Due to the fact that low temperature dose not facilitate the diffusion of molecules, leading to the incompletion of extraction, while high temperature is prone to cause the degradation of bioactive constituents, both of which can induce the decrease of extraction yield (Ya *et al*., 2017).

Optimization of Extraction Factors with RSM

Model Fitting Analysis

Based on the experimental data of Table 2, a second-order polynomial model (Equation 8) was used to predict the response value Y (extraction rate/%):

$$Y = 6.23\text{-}0.23\times X_1 + 0.19\times X_2 + 0.096\times X_3 + 0.12\times X_4 - 0.45^{'}X_1\times X_2 + 0.11\times X_1\times X_3 - 0.012\times X_1\times X_4 - 0.031\times X_3\times X_4 + 0.032\times X_2\times X_4 - 0.038\times X_3\times X_4 - 2.08\times X_1^2 - 2.18\times X_2^2 - 1.92\times X_3^2 - 1.78\times X_4^2 \quad (5)$$

where, X_1, X_2, X_3 and X_4 are the coded variables for the ethanol concentration (%), liquid-to-solid ratio (mL/g), extraction time (min) and extraction temperature (°C), respectively.

Fitting Model

ANOVA confirms the response surface quadratic model has certain sufficiency and adaptability. The high F value (42.016) and low P value (<0.0001) (Table 3) prove that the model has obvious significance and is very ideal. Comparison of P values showed that the order of single factors affecting the model was ethanol concentration (X_1)> liquid-to-solid ratio (X_2)> extraction temperature (X_4)> extraction time (X_3). The coefficient of determination R^2 = 0.9768 suggests the model is reliable and can be used to interpret 97.68% of the data. According to the adjustment of the model, the adj-R^2 = 0.9535 indicates this model can predict most of the extracted variation (>96%). The lack of fit (p = 0.1779) suggests the model is insignificant. Thus, the experimental results are obviously correlated with the theoretical values derived from the fitting of the corresponding polynomials. The model prediction is reliable and repeatable in the ranges of the parameters allowed. The ANOVA shows the model precision is 19.340>4 and the coefficient of variation is 11.36% <30%, indicating the model has high reproducibility, high accuracy and reliability (Qu *et al*., 2016). The model is suitable for analysis and prediction of the extraction results of TFCMK.

Response Surface Analysis

The specific response target and the corresponding independent variables were investigated via graphic analysis of response surface, which consists of 3D response surface plots that clearly reflect the impact of various factors on the response target. The 3D response surface plots comprehensively reflect the whole region of predictive behavior (AnnGiovannitti-Jensen and Myers, 1989) and can be obtained by finding the interaction of various factors in the reaction process. The effect of each factor on the extraction rate of TFCMK can be judged from the flexibility of the response surface plots (Fig. 2).

Firstly, the increase of ethanol concentration and the liquid-to-solid ratio, the extraction rate rises significantly and the result reversed if the ethanol concentration continues to increase (Fig. 2a). Furthermore, the interaction of these two factors significantly affects the extraction rate of TFCMK. Secondly, with the increase of ethanol concentration combined with the intermediate extraction time and extraction temperature, the change of extraction rate is relatively gentle (Fig. 2b and c), indicating the interaction of ethanol concentration with extraction time or extraction temperature little affects the extraction rate.

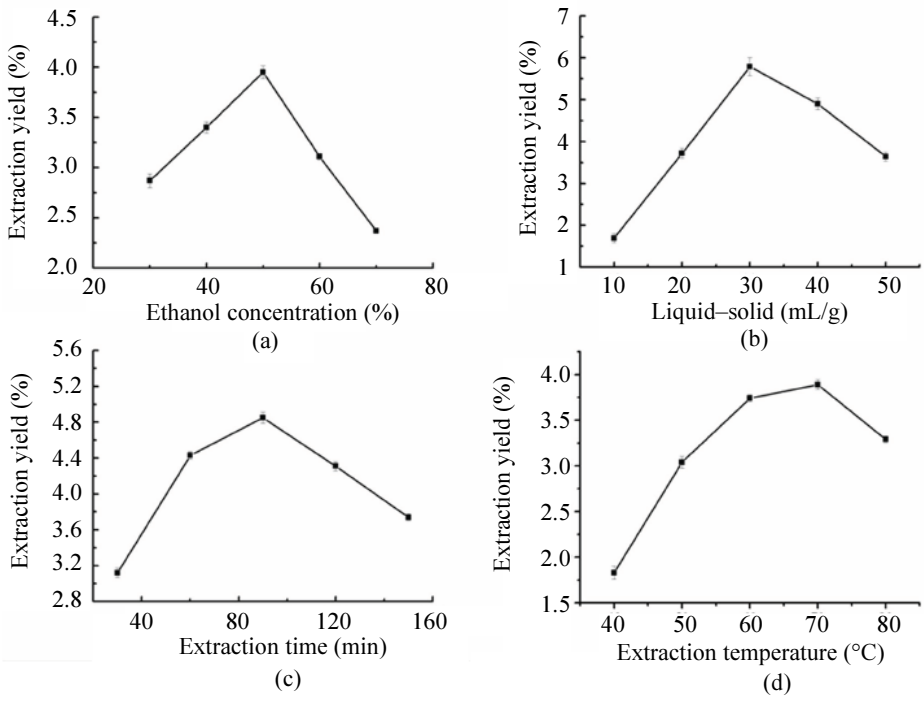

Fig. 1: Effects of (a) Ethanol concentration, (b) liquid–solid ratio, (c) extraction time and (d) extraction temperature on extraction rate of TFCMK. Data were shown as mean ± SD (n = 3)

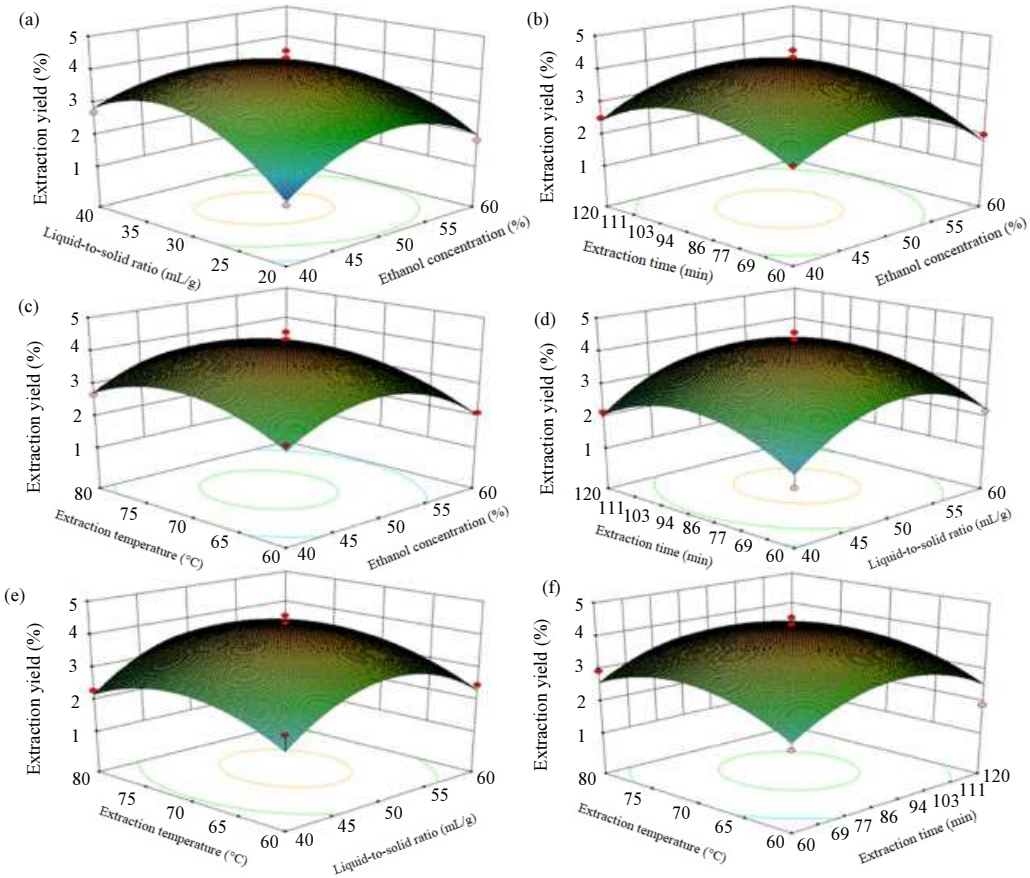

Fig. 2: Response surface of the effects of independent variables on the extraction yield of TFCMK. Extraction yield vs. (a) X_1 and X_2, (b) X_1 and X_3, (c) X_1 and X_4, (d) X_2 and X_3, (e) X_2 and X_4 and (f) X_3 and X_4. Note: X_1: Ethanol concentration (%), X_2: liquid- to-solid ratio (mL/g), X_3: Extraction time (min), X_4: Extraction temperature (°C)

Table 3: ANOVA for the response surface quadratic model

Source	Sum of squares	Degree of freedom	Mean square	F-value	P-value	Remarks
Model	68.6520	14.000	4.904	42.016	<0.0001	**
X_1	0.6320	1.000	0.632	5.416	0.0355	*
X_2	0.4240	1.000	0.424	3.632	0.0774	not significant
X_3	0.1110	1.000	0.111	0.948	0.3467	not significant
X_4	0.1840	1.000	0.184	1.579	0.2295	not significant
X_1 X_2	0.7940	1.000	0.794	6.807	0.0206	*
X_1 X_3	0.0470	1.000	0.047	0.402	0.5364	not significant
X_1 X_4	0.0010	1.000	0.001	0.005	0.9448	not significant
X_2 X_3	0.0040	1.000	0.004	0.033	0.8589	not significant
X_2 X_4	0.0040	1.000	0.004	0.035	0.8537	not significant
X_3 X_4	0.0060	1.000	0.006	0.049	0.8280	not significant
X_1^2	30.9110	1.000	30.911	240.820	<0.0001	**
X_2^2	24.0200	1.000	24.020	264.850	<0.0001	**
X_3^2	20.5520	1.000	20.552	205.812	<0.0001	**
X_4^2	68.6520	14.000	4.904	176.093	<0.0001	**
Residual	1.6300	14.000	0.120			not significant
Lack of Fit	1.4200	10.000	0.140	2.670	0.1779	not significant
Pure error	0.2100	4.000	0.530			
Cor Total	70.2900	28.000				
R-Squared	0.9768					
Adj R-Squared	0.9535					
Pred R-Squared	0.8788					
C.V. %	11.6400					
Adeq Precision	19.3400					

**: Indicate highly significant (p<0.01), *: Indicate significant (p<0.05)

Thirdly, the increase of liquid- to-solid ratio, the variation of extraction rate is not obvious and its interaction with the extraction time or extraction temperature does not obviously affect the response value (Fig. 2d and e). Finally, the increasing extraction time combined with the optimal extraction temperature, the response value is not obvious and has no significant effect on the model (Fig. 2f). To sum up, these results are consistent with the ANOVA.

Verification of the Models

The optimum extraction parameters (encoding extraction parameters: 0.060, 0.049, 0.022, 0.035) were obtained on software through further analysis of the fitting linear equation. Ethanol concentration 49.40%, liquid-to-material ratio 30.49 mL/g, extraction time 90.67 min and extraction temperature 70.35°C were obtained from Design-Expert software, where the theoretical extraction rate of TFCMK was 6.248%. However, considering the operability in actual production, the optimum extraction parameters can be modified as follows: ethanol concentration is 50%, liquid-to-material ratio is 31 mL/g, extraction time is 91 min and extraction temperature is 70°C. The extraction rate of TFCMK is 6.240% ±0.04% from three repeated experiments under the optimum extraction conditions. The final experimental results are basically consistent with the predicted values on the software (relative error = 0.128%<2%), indicating our model is suitable for the extraction of TFCMK and theoretically underlies the future development of CMK.

Chemical Composition Analysis of TFCMK by LC–MS/MS

The LC chromatogram at 254 nm and the total ion chromatogram (Fig. 3) were analyzed (Table 4). The compound 1 (t_R = 6.875 min) was identified as Catechin with the literature (Liu *et al*., 2009). The molecular formula is $C_{26}H_{28}O_{14}$. The molecular ion peak $[M\text{-}H]^-$ was at m/z 289.0717. Other fragment ions were m/z 245 $[(M - H)-44]^-$ and m/z 203 $[(M - H)-44-42]^-$ that indicated the loss of the $C_2H_2O^-$ and $-COO^-$ groups, respectively (Henri *et al*., 2018).

The compound 2 (t_R = 7.905 min) was identified as Kaempferol-3, 7-di-*O*-glucoside with the literature (Francescato *et al*., 2013). The molecular formula is $C_{27}H_{30}O_{17}$. The molecular ion $[M–H]^-$ at m/z 609.1461 and the base ion fragment at m/z 447 $[(M–H)–162]^-$ the exhibiting the loss of a glucose residue at the 7-*O* position, the other ion fragment at 285 $[(M–H)–162–162]^-$, indicating a di-*O*-glycoside.

The compound 3 (t_R = 8.534 min) was identified as 6-C-arabinosyl-8-C-glucoside (isoschaftoside) with literature (Lai *et al*., 2016). The molecular formula is $C_{26}H_{28}O_{14}$. The molecular ion $[M–H]^-$ were m/z 563.1406 and the base ion fragments at m/z503 $[M–H– 60]^-$. Other fragment ions were m/z 473 $[M–H–90]^-$, 383$[M–H–180]^-$(A [aglycone: Apigenin M–H–270)]+113), 353$[M–H–210]^-$(A+83) (Zhu *et al*., 2017).

The compound 4 (t_R = 9.049 min) was identified as orientin by comparison with literature (Tahir *et al*., 2012). The molecular formula is $C_{21}H_{20}O_{11}$. The molecular ion $[M\text{-}H]^-$ at m/z 447.0959 and the base fragment ions at m/z 327$[M–H–120]^-$. Other fragment ions were m/z 357$[M–H–90]^-$, 297$[M– H– 150]^-$, 285$[M–H–162]^-$.

The compound 5 (t_R = 9.153 min) was identified as luteolin glucoside with literature (Sánchezrabaneda *et al*., 2003), showed a ion molecular $[M–H]^-$ at m/z 447.0928 and the base molecular fragment m/z 285 $[M–H–glucosyl]^-$. The molecular formula is $C_{27}H_{30}O_{16}$. This compound it was distinctly identified as luteolin glucoside, according to its mass spectrum fragment ($[M– H]^-$ at m/z 447 and yielded to m/z 285) and thus this compound was tentatively identified as luteolin glucoside.

The compound 6 (t_R = 11.143 min) was identified as rutin with literature (Chen *et al*., 2015). The molecular formula is $C_{27}H_{30}O_{16}$. The molecular ion $[M–H]^-$ at m/z 609.1452 and the base ion fragment at m/z 301$[M–H–90]^-$ (Ćirić *et al*., 2012), exhibited the loss of their glycosyl groups and corresponding to quercetin aglycone.

The compound 7 (t_R = 13.704 min) was identified as luteolin-7-O-β-D-glucuronide with literature (Ćirić *et al*., 2012). The molecular formula is $C_{21}H_{18}O_{12}$. The molecular ion $[M–H]^-$ at m/z 461.0733 (Li *et al*., 2017) and the base ion fragment at m/z 285, exhibited the flavone *O*-glycosides.

The compound 8 (t_R = 16.329 min) was identified as isochlorogenic acid B with literature (Yin *et al*., 2013). The molecular formula is $C_{25}H_24O_{12}$. While ion peak $[M–H–163]^-$ at m/z 515.1207, the first cracking reaction occurs when the caffeoyl fragment at m/z 163 is lost and the base ion fragment at m/z 353 $[M–H–163\text{-}192]^-$ (Li *et al*., 2017), On this basis, some fragments of quinic acid group were separated again, the fragments at m/z 192 were lost and the fragments of caffeoyl group remained, resulting in ion fragments at m/z 161. It indicated that two caffeoyl groups existed in the chemical structure.

The compound 9 (t_R = 22.369 min) was identified as luteolin with literature (Toplan *et al*., 2017). The molecular formula is $C_{15}H_{10}O_6$. The molecular ion peak $[M–H–28]^-$ at m/z 285.0392 and 257 were obtained by the loss of –CO from the fragment ion peak at m/z 257 and 229. The fragment ions m/z 151 and 133 were obtained by RDA cleavage of fragment ions m/z 285. It is presumed that the compound was luteolin and the base ion fragments at m/z 199. Other ions fragment were m/z 175 and 151. In addition base peak at m/z 133 was obtained by the RDA cleavage of the structure.

The compounds of 10-12 in Table 4 were found to contain m/z 285 $[M-H]^-$ by MS/MS fragments, it is speculated that they belongs to such luteolin flavonoids. At present, there are no reports of using LC-MS/MS to analyze the flavonoids of TFCMK and with many isomers. The two peaks at t_R = 23.560 min were not separated so that they could not be resolved. Therefore, the analytical constituents was need to be improved.

Antioxidant Activity

Antioxidants can prevent hydroxyl radicals from destroying biological macromolecules. The ability to scavenge hydroxyl radicals is directly related to the antioxidant capacity (Cacciuttolo *et al*., 1993). The antioxidant experiment results of the four systems includes the reducing power scavenging activity, DPPH· scavenging activity, $ABTS^+$ and Hydroxyl radicals of both TFCMK and VC were enhanced with the increasing concentration (Fig. 4a-d). The EC_{50} or IC_{50} values of TFCMK were 0.125 ±0.025, 0.28±0.031, 0.012±0.005 and 0.028±0.008 mg/mL and the EC_{50} or IC_{50} values of VC 0.156±0.012, 0.059±0.019, 0.068±0.024 and 0.032±0.007 mg/mL, respectively. Results suggest TFCMK have significant antioxidant activity, which showed stronger in the system of reducing power and scavenging hydroxyl radicals. Firstly, the possible reason mainly is the phenolic hydroxyl structures of flavonoids play an antioxidant role through the reduction of phenolic hydroxyl. All the 9 compounds have phenolic hydroxyl groups and benzene ring structure. The reaction of phenolic hydroxyl with free radicals produces a stable structure and terminates the free radical chain reaction. Phenolic hydroxyl radicals are mainly produced by ionizing hydrogen atoms, neutralizing oxygen free radicals and combining with ionized flavonoids to form dimers, which prevent reverse binding and thus scavenge radicals (Boulanouar *et al*., 2013; Karmakar *et al*., 2013). Secondly, the effect of double bonds in the structural nucleus of flavonoids is also crucial to the oxidation resistance. Theoretically, double bonds act as electron delocalization and lengthen the conjugation system, which is conducive to the formation of more stable free radicals after the electron loss in flavonoid nuclei and then interrupts the chain reaction (Acheampong *et al*., 2016).

Antibacterial Activity

TFCMK showed significant antibacterial activity against *B. pumilus*, *B. subtilis* and, *E. coli* according to the MIC with in 0.625−5.000 mg/mL (Table 5). Among them, the MIC of *B pumilus* was 0.625 mg/mL, which exhibited a higher inhibition effect, as evidenced by the MIC of Chloramphenicol was less than 0.8 mg/mL. The cooperation effects of the above 9 compounds can not be excluded. The a ntibacterial mechanism of TFCMK may be through its own hydrophobic (Zhou *et al*., 2018) in teraction with the lipid bilayer of the bacterial cell membrane and it can affect the stability of the cell membrane to achieve bactericidal effect (Taylor, 2013; Veloza and Mantillamuriel, 2014). The antimicrobial effect of flavonoids is mainly due to the destruction of bacterial cell membrane by their hydrophobic benzene ring, thus achieving the purpose of sterilization. The antibacterial l mechanisms of TFCMK need further research. With the widespread application and abuse of antibiotics, more and more drug-resistant strains are found in clinic. The development of natural plant antibacterial agent with the lower MIC has become a new method to solve the problem of bacterial resistance.

Hemolysis Analysis

To test whether TFCMK possessed a blood compatibility, the hemolysis test which was used to detect degree or proportion of damage to red blood cells, was carried out (Zhang *et al*., 2017). According to the International Organization for Standardization (ISO), the hemolysis rate ≤5% suggests the sample meets the hemolysis requirement of medical treatment and the hemolysis rate >5% indicates the occurrence of hemolysis. The results are shown in Fig. 5. In the concentration range of 0.1-2.0 mg/mL, the hemolysis rate declines, but is less than 5%, indicating TFCMK meets the hemolysis requirement of medical treatment in this concentration range (Sobrinho *et al*., 2016). Thus, it is affirmed that TFCMK can be applied for pharmaceutical and chemical industries.

Table 4: Chemical constituents of TFCMK

No.	*tR* (min)	$[M-H]^-$ (m/z)	Error(ppm)	Molecular formula	Ion fragment	Compound
1	6.8750	289.0717	-1.26	$C_{15}H_{14}O_6$	245, 203, 188, 187, 161, 137, 125, 109, 57, 45	Catechin
2	7.9050	609.1461	-0.70	$C_{27}H_{30}O_{16}$	609, 571, 539, 489, 447, 327, 285, 227, 151	Kaempferol-3,7-di-O-glucoside
3	8.5340	563.1406	1.550	$C_{26}H_{28}O_{14}$	545, 503, 473, 443, 413, 383, 353, 325, 297, 59	6-C-Arabinosyl-8-C-glucoside (isoschaftoside)
4	9.0490	447.0902	2.640	$C_{21}H_{20}O_{11}$	357, 339, 327, 297, 285, 269, 175, 151, 41	Orientin
5	9.1530	447.0928	2.640	$C_{21}H_{20}O_{11}$	357, 339, 327, 297, 285, 269, 175, 151, 41	Luteolin glucoside
6	11.143	609.1452	3.840	$C_{27}H_{30}O_{16}$	573, 503, 461, 387, 301, 285, 257, 181, 179, 151	Rutin
7	13.704	461.0733	-1.77	$C_{21}H_{18}O_{12}$	374, 327, 357, 301, 285, 269, 241, 203, 179, 130, 85, 56	Luteolin-7-O-ß-d-glucuronide
8	16.329	515.1207	-4.22	$C_{25}H_{24}O_{12}$	405, 353, 265, 173, 135, 44	Isochlorogenic acid B
9	22.369	285.0392	4.740	$C_{15}H_{10}O_6$	243, 199, 175, 151, 133, 121, 107, 65, 41	Luteolin
10	11.400	593.1491	1.700	$C_{27}H_{30}O_{15}$	573, 447, 387, 285, 269, 165	Unknow
11	12.592	447.0895	4.500	$C_{21}H_{20}O_{11}$	125, 151, 178, 271, 285, 300, 327	Unknow
12	16.877	505.1972	2.410	$C_{23}H_{22}O_{13}$	434, 315, 285, 227, 134, 44	Unknow

Table 5: Minimal Inhibitory Concentrations (MIC) of TFCMK

Sample	Unit	*B. pumilus*	*B. subtilis*	*E. coli*
TFCMK	mg/mL	0.625	1.250	5.000
Chloramphenicol	mg/mL	<0.800	<0.800	<0.800

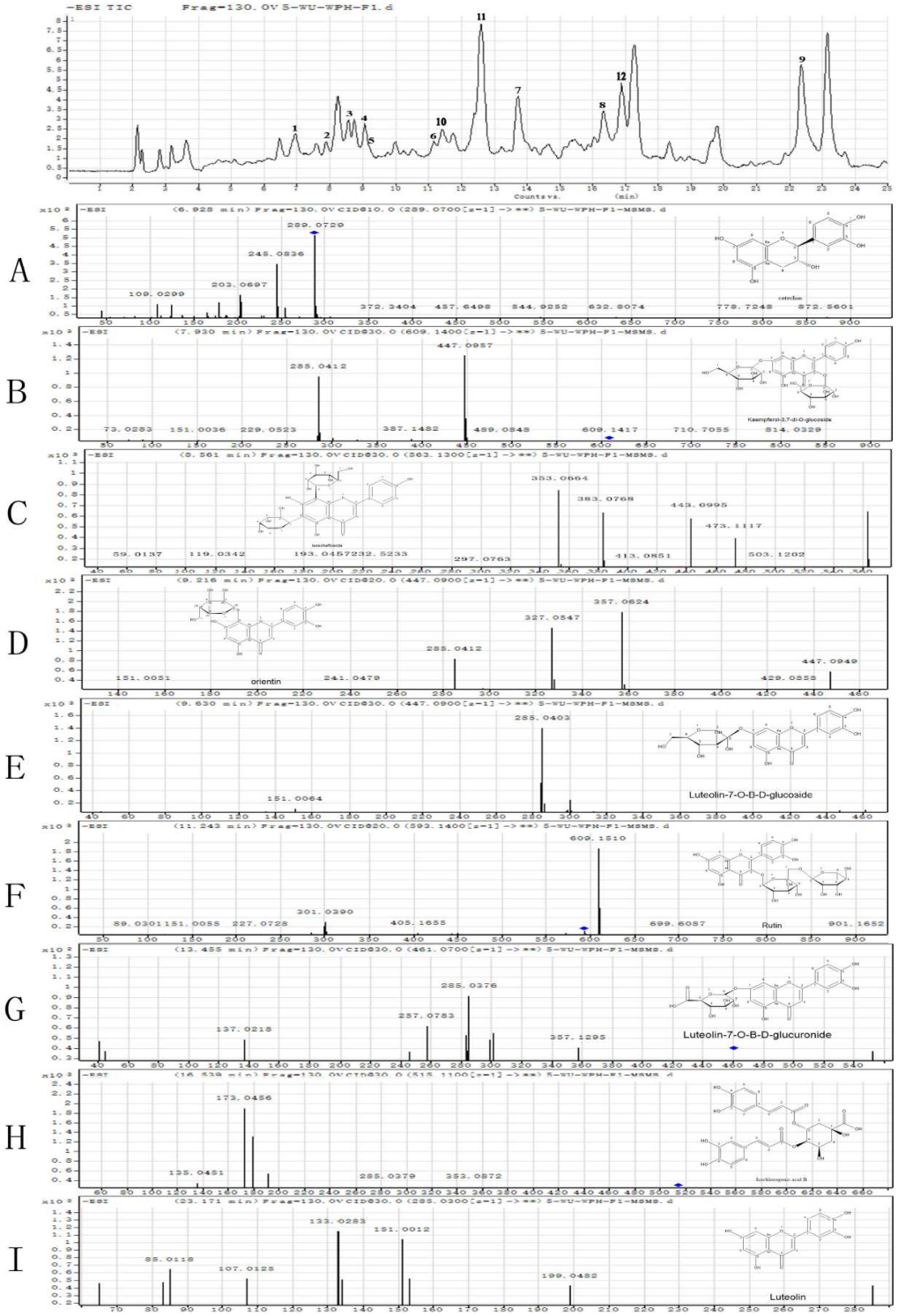

Fig. 3: ESI total ion flow diagram and chemical structures of A-I

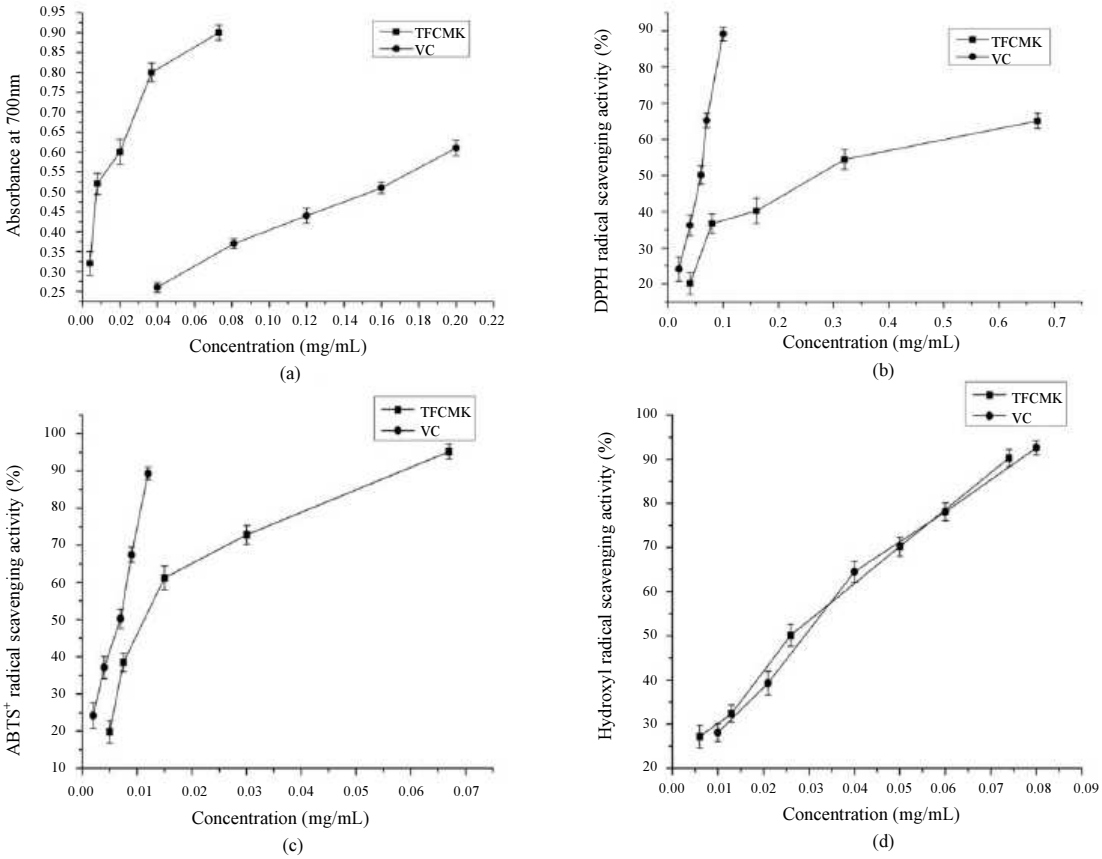

Fig. 4: Antioxidant activities of TFCMK. (a) Reducing power assay; (b) DPPH·, (c) ABTS+ and (d) Hydroxyl radical scavenging assays

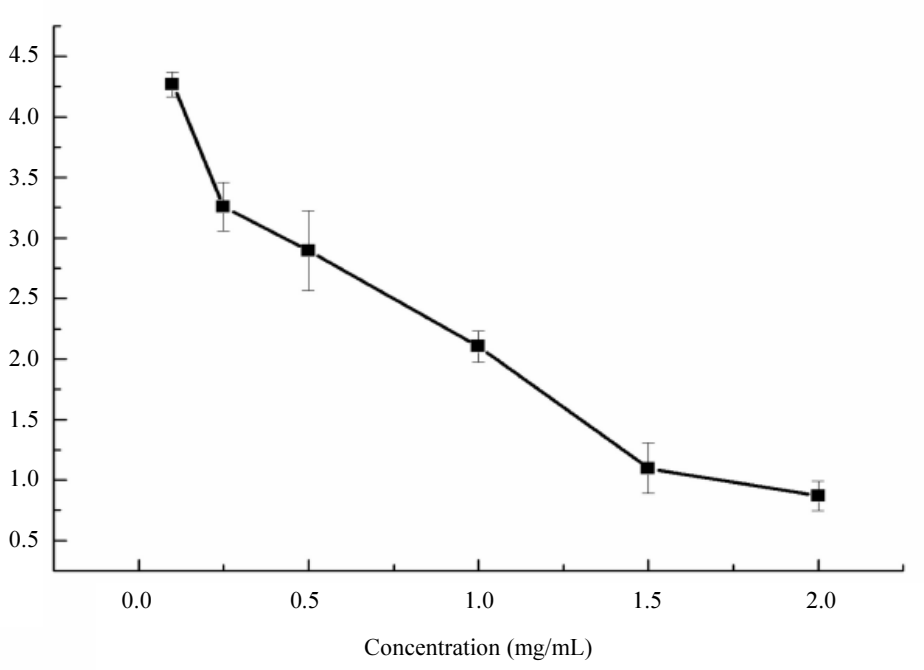

Fig. 5: Hemolysis rate of flavonoids from TFCMK

Conclusion

The maximum extraction yield of TFCMK was observed under the optimal condition and the actual yield reached at 6.24%±0.04%. Meanwhile, 9 compounds of TFCMK firstly identified by LC-MS/MS. In addition, TFCMK possessed a higher antioxidant activity and antibacterial activity, quantified by the IC_{50} and MICs; *in vitro* assay, hemolysis rate was less than 5%. The above data indicates that TFCMK might be applied as an antioxidant and antibacterial agent in the pharmaceutical and chemical industries. Herein, this study underlies further research on CMK in the future.

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Author's Contributions

Hongbiao Wang and Penghui Wang: The antioxidant, antimicrobial and hemolytic experiments were studied and analyzed in detail.

Xiudong Yang: The revision and proofreading of the manscript was completed.

Xianhe Cheng and Chun Liu: The chemical composition of TFCMK by LC-MS/MS was analyzed.

Mengru Dong, Ziqiang Yan and Yuejiao Guo: Response Surface Methodology (RSM) was mainly explored.

Hongli Zhou: The design route and experimental guidance of this manuscript were guided.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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