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Optimization of Cellulase-Assisted Extraction of Total Flavonoids from Corn Bract and Evaluation of Antioxidant and Antibacterial Aactivities

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Abstract: Corn bract is a corn-based agricultural waste material that distributed worldwide. In present work, response surface methodology was applied to optimize the cellulase-assisted extraction of Total Flavonoids from Corn Bract (TFCB), the *in vitro* antioxidant and antibacterial as well as the *in vivo* antioxidant activities of TFCB were investigated. Results showed that the optimal conditions for TFCB extraction were as follows: Amount of cellulase, 0.4% (w/w); incubation time, 2 h; liquid-to-solid ratio, 35: 1 mL/g; ethanol concentration, 71% (v/v); TFCB yield was 1.284±0.01%, which was 25.39% higher than that of heat reflux extraction. In addition, when compared with vitamin C, TFCB showed weaker *in vitro* free radical-scavenging capacities, but stronger antioxidant activities in mice. Moreover, TFCB also exerted certain inhibitory effects against Gram-positive bacteria. This study will provide an evidence for the potential of comprehensive utilization of discarded corn bract.

Keywords: Corn Bract, Total Flavonoids, Cellulase-Assisted Extraction, Antioxidant Activity, Antibacterial Activity

Introduction

Corn (*Zea mays* L.), a member of the family Poaceae, is one of the most abundant crops cultivated worldwide (Barth, 2008; NWE, 2018). In addition to dietary property, some parts of corn have been utilized as therapeutic agents. Corn silk (*Stigma maydis*), the stigma and style of corn, is the most famous corn-based traditional medicine (Hasanudin *et al*., 2012). It consists of various bioactive constituents including flavonoids, tannins, steroids, alkaloids, polysaccharides, as well as vitamins (Peng *et al*., 2016) and possesses numerous pharmacological activities such as anti-diabetic, antioxidant, anti-tumor, anticoagulant, diuretic, etc (Zhao *et al*., 2017). Although the medicinal value of corn silk has been well studied and applied, regarding corn bract, the subtending leaf surrounding corn kernels (Fig. 1), there is little information on its active components, most of corn bracts are discarded as agricultural waste materials and open burning is a frequently-used disposal method (Luo *et al*., 2017).

Fig. 1: The appearance of corn bract

Flavonoids are a kind of compounds widely distributed in plants as secondary metabolites (Zakaryan *et al*., 2017). An increasing number of studies have reported the extraction methods of flavonoids from different plant sources (Liu *et al*., 2016; Jing *et al*., 2016; Wang *et al*., 2017; Yang *et al*., 2017; Yu *et al*., 2017). Enzyme-assisted extraction is established as an emerging technique to accelerate the release of bioactive components from plant materials, due to its advantages of high extraction yield, environmental compatibility,

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low energy requirement and simplified manipulation (Chen *et al*., 2011). Cellulase is one of the common used hydrolytic enzymes to hydrolyze and decompose the components of cell wall and enhance the release of intracellular constituents (Fu *et al*., 2008). Furthermore, cellulase has been proved to be efficient for the extraction of flavonoids from different kinds of plant materials including *Illicium verum* (Huang *et al*., 2016), *Ampelopsis grossedentata* (Gao *et al*., 2016), *Larix gmelini* (Wang *et al*., 2011) and *Geranium sibiricum* L. (Yang *et al*., 2010).

Reactive Oxygen Species (ROS)-induced oxidative stress is related to many kinds of diseases (Mao *et al*., 2017). Excess ROS can exert damaging effects on DNA, proteins and lipids, leading to cell aging and death (Lee and Wei, 2001; Martinez-Useros *et al*., 2017). It is therefore important to supplement exogenous antioxidants when facing oxidative stress, due to the fact that the levels or activities of endogenous antioxidants are usually lower than that required for the scavenging of free radicals (Szuroczki *et al*., 2016). Plant-derived flavonoids have multifold activities and the best described pharmacological activity of flavonoids is to act as potent natural-based antioxidant (Sarian *et al*., 2017). Moreover, a growing number of articles have demonstrated that antioxidant flavonoids always possess antimicrobial activities *in vitro* (Erasto *et al*., 2004; Liu *et al*., 2010; Hong *et al*., 2014; de Camargo *et al*., 2017).

In present investigation, with the aim of exploring the applicability of Cellulase-Assisted Extraction (CAE) for the preparation of Total Flavonoids from Corn Bract (TFCB), as well as the antioxidant and antibacterial potentials of TFCB. The Response Surface Methodology (RSM) coupled with Box-Behnken Design (BBD) was employed to optimize the process parameters of CAE for TFCB. Then, the *in vitro* antioxidant and antibacterial activities of TFCB were evaluated via different models and bacterial strains. Moreover, the *in vivo* antioxidant activities of TFCB were performed in an ethanol-induced oxidative stress mice model to further confirm its effects. The work described herein could be helpful for the comprehensive exploration and utilization of corn bract.

Materials and Methods

Materials

The dried corn bracts were harvested from the suburbs of Jilin City, China and authenticated by Prof. Guangshu Wang, School of Pharmaceutical Sciences, Jilin University (Jilin, Changchun, China). A voucher specimen (No. CB-007) was preserved in School of Biology and Food Engineering, Changshu Institute of Technology (Jiangsu, Changshu, China). Prior to experiment, corn bracts were grinded into powders and sieved to 60-mesh.

Cellulase (10000 U/g) was obtained from Macklin Biochemical Co., Ltd. (Shanghai, China). Rutin was purchased from Winherb Medical Technology Co., Ltd. (Shanghai, China). Vitamin C (VC) was from Puripharm Co., Ltd. (Zhejiang, Huzhou, China). Chemicals used for the in vitro antioxidant evaluation including 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH), Phenazine Methosulphate (PMS), 1, 10-phenanthroline, Nitroblue Tetrazolium (NBT) and Nicotinamide Adenine Dinucleotide (NADH), potassium ferricyanide ($K_3[Fe(CN)_6]$), ferrous sulfate ($FeSO_4$), ferric chloride ($FeCl_3$) and hydrogen peroxide (H_2O_2) were provided by Sigma Aldrich Chemical Co., Ltd (St. Louis, MO, USA). Other reagents and solvents with analytical grade such as aluminum nitrate ($Al(NO_3)_3$), sodium nitrite ($NaNO_2$), methanol and ethanol, etc. were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Reagent kits used for the *in vivo* antioxidant evaluation including Malonaldehyde (MDA), Total Superoxide Dismutase (T-SOD), Glutathione (GSH) and Protein Carbonyls (PCO) were provided by Jiancheng Biotechnology Co., Ltd. (Nanjing, Jiangsu, China).

TFCB Extraction

Cellulase-Assisted Extraction (CAE)

One gram of corn bract powders and 10 mL of Disodium Hydrogen Phosphate (DHP)-citric acid buffer solution (pH = 5) were mixed together, then added certain cellulase (0.1% ~ 0.6%, respected to solid material) and incubated at 40°C for certain time (0.5 h ~ 3 h). After enzyme inactivation (boiling water bath, 5 min), added absolute ethanol and distilled water to adjust to certain liquid-to-solid ratio (20: 1 mL/g ~ 45: 1 mL/g) and certain ethanol concentration (40% ~ 90%, v/v), followed by extraction at 80°C for 2 h. After cooling, the mixture was filtered and the total flavonoids in filtrate were determined by using an UV-visible spectrophotometer (722N, Jingke Scientific Instrument Co., Ltd., Shanghai, China). The TFCB extraction yield was expressed as follows:

$$Yield\ (\%) = \frac{C \times V}{m} \times 100 \quad (1)$$

where, C was the concentration of flavonoids (mg/mL), V was the volume of filtrate (mL) and m was the weight of corn bract powders (mg).

Heat Reflux Extraction (HRE)

One gram of corn bract powders and 10 mL of DHP-citric acid buffer solution (pH = 5) were mixed together and incubated at 40°C for 2 h, then added about 25 mL of absolute ethanol to adjust the ethanol

concentration to 71% (v/v), followed by extraction at 80□ for 2 h. After cooling, the mixture was filtered and the total flavonoids in filtrate were determined and the extraction yield was calculated.

TFCB Content Determination

TFCB content was determined according to the previously reported method (Khorasani *et al*., 2015) with some modifications. As much as 1 mL of diluted extract was mixed with 0.3 mL of 5% $NaNO_2$ solution, 0.3 mL of 10% $Al(NO_3)_3$ solution and 4 mL of 30% (v/v) methanol, then incubated and shaken for 6 min, followed by the addition of 2 mL of 1 mol/L NaOH solution. Then, the mixture was diluted to 10 mL with 30% (v/v) methanol and the absorbance was measured at 510 nm, which was put into the regression equation to calculate TFCB content. The regression equation using rutin as standard substance was expressed as follows:

$$A = 36.557C - 0.0002\left(R^2 = 0.9998\right) \quad (2)$$

where, A was the absorbance, C was the concentration of flavonoids (mg/mL) and the linear range was from 0.004 to 0.02 mg/mL.

Experimental Design of Response Surface Optimization

In order to assess the effect of each factor on TFCB extraction yield, single-factor experiments were conducted to analyze the influences of the four independent variables including amount of cellulase (0.1% ~ 0.6%, w/w), incubation time (0.5 h ~ 3 h), liquid-to-solid ratio (20: 1 mL/g ~ 45: 1 mL/g) and ethanol concentration (40% ~ 90%, v/v). Then, the CAE optimization of TFCB was further performed by using RSM (Zhang *et al*., 2013). A four-variable and three-level BBD comprising 29 runs was applied at the center point (Table 1).

Regression analysis was used for the experimental data and fitted to the following second-order polynomial equation:

$$Y = \beta_O + \sum_{i=1}^{4}\beta_i X_i + \sum_{i=1}^{4}\beta_{ii}X_i^2 + \sum_{i=1}^{3}\sum_{j=i+1}^{4}\beta_{ij}X_iY_j \quad (3)$$

where, Y represents the response function. β_0 is an intercept. β_i, β_{ii} and β_{ij} are coefficients of the linear, quadratic and interactive terms, respectively. X_i and X_j represent the coded independent variables.

The experimental design, results analysis and responses prediction were carried out by using Design-Expert software 8.0.6.1 (Stat-Ease, Minneapolis, MN, USA).

Table 1: The code and level of factors selected for the trials

	Level		
Independent variable	-1	0	1
Amount of cellulase (w/w, %, X_1)	0.3	0.4	0.5
Incubation time (h, X_2)	1.5	2.0	2.5
Liquid-to-solid ratio (mL/g, X_3)	30: 1	35: 1	40: 1
Ethanol concentration (v/v, %, X_4)	60	70	80

In vitro Antioxidant Activity of TFCB

DPPH Radical-Scavenging Capacity

The DPPH radical-scavenging assay of TFCB was conducted according to the previously reported method (Liu *et al*., 2009) with some modifications. As much as 2 mL of TFCB solution with various concentrations (10 μg/mL ~ 60 μg/mL) was mixed with 2 mL of 0.1 mmol/L DPPH solution (dissolved in ethanol) and incubated at room temperature for 30 min, followed by measuring the absorbance at 517 nm (A_s). The reaction system without DPPH was employed as normal control (A_c), while, system without TFCB was used as blank solution (A_0). VC solution at different concentrations (4 μg/mL ~ 20 μg/mL) was used as positive control. The capacity to scavenge DPPH radical was calculated by using the following equation:

$$DPPH\ radical-scavenging\ rate(\%) = \left(A_s - A_c\right)\times 100 / A_0 \quad (4)$$

Hydroxyl Radical-Scavenging Capacity

Hydroxyl radical-scavenging capacity of TFCB was investigated based on the method reported by Ke and Chen (2016) with some modifications. As much as 2 mL of TFCB solution with different concentrations (100 μg/mL ~ 600 μg/mL) was mixed with 1 mL of 0.75 mmol/L 1, 10-phenanthroline (dissolved in phosphate buffer saline (PBS), pH 7.4), then added 1 mL of 0.75 mmol/L $FeSO_4$ as well as equal volume of 0.12% (v/v) H_2O_2 solution, followed by reacting at 37°C for 60 min. The absorbance (A_s) was measured at 536 nm. The reaction system without H_2O_2 was used as normal control (A_c) and the system without TFCB was used as blank control (A_0). VC solution in the concentration ranging from 25 μg/mL to 150 μg/mL was used as positive control. The capacity to scavenge hydroxyl radical was calculated as the following formula:

$$Hydroxyl\ radical-scavenging\ rate(\%) = \left(A_s - A_0\right)\times 100 / \left(A_c - A_0\right) \quad (5)$$

Superoxide Anion Radical-Scavenging Capacity

Superoxide anion radical-scavenging capacity of TFCB was evaluated by using the method reported by

El-Beshbishy *et al*. (2009) with some modifications. As much as 1 mL of TFCB solution with varying concentrations (10 μg/mL ~ 60 μg/mL) was mixed with 3 mL of 16 mmol/L Tris-HCl buffer (pH 8.0) containing 0.5 mL of 0.47 mmol/L NADH and 0.5 mL of 0.3 mmol/L NBT, followed by adding 0.5 mL of 0.06 mmol/L PMS to start the reaction. After incubation at room temperature for 5 min, the absorbance was measured at 560 nm (A_s). The mixture in absence of TFCB was used as blank control (A_0). VC solution with different concentrations (5 μg/mL ~ 30 μg/mL) was used as positive control. The capacity to scavenge superoxide anion radical was calculated by the following equation:

$$\begin{aligned} &Superoxide\ anion-scavenging\ rate(\%) \\ &=(A_0-A_s)\times 100/A_0 \end{aligned} \quad (6)$$

Reducing Power

Reducing power of TFCB to ferric iron was determined based on the method reported by Vaquero *et al*. (2010) with some modifications. As much as 1 mL of TFCB solution with different concentrations (3 μg/mL ~ 18 μg/mL) was mixed with 2.5 mL of 0.2 mol/L phosphate buffer (pH 6.6) and 2.5 mL of 1% (w/v) $K_3[Fe(CN)_6]$ solution, followed by incubating at 50°C for 20 min. Then 2.5 mL of 10% (v/v) trichloroacetic acid was added, after shaking, the mixture was centrifuged at 3000 rpm for 10 min. As much as 5 mL of the supernatant was mixed with 4 mL of distilled water and 1 mL of 0.1% (w/v) $FeCl_3$ solution for 10 min, then the absorbance at 700 nm was read. VC solution in the concentration range 5 μg/mL to 30 μg/mL was used as positive control.

In vitro Antibacterial Activity of TFCB

Bacteria and Culture Condition

The test bacteria including three Gram positive ones (*Staphylococcus aureus* ATCC 49775, *Bacillus pumilus* ATCC 14884 and *Bacillus subtilis* ATCC 21332) and two Gram negative ones (*Escherichia coli* ATCC 33456 and *Salmonella typhimurium* ATCC 14028) were provided by ATCC Global Bioresource Center (Manassas, VA, USA) and stored at -80°C in 30% (v/v) glycerol media.

Prior to experiment, all the strains were subcultured in Luria-Bertani (LB) broth (Sangon Biotech Co., Ltd., Shanghai, China) for 24 h at 37°C. The activated bacteria were diluted with sterile water to obtain the suspension of bacterial strain at concentration of 1.0×10^8 cfu/mL.

Minimum Inhibitory Concentration (MIC) Determination

The minimum inhibitory concentration (MIC) of TFCB was determined by serial dilution microplate method (Bisi-Johnson *et al*., 2017). The tested concentrations of TFCB were from 0.5 μg/mL to 50 μg/mL. MIC values were defined as the lowest concentration of TFCB inhibiting bacterial growth after being incubated at 37°C for 18 h.

Animals and in vivo Antioxidant Activity of TFCB

Experimental Animals

Male ICR mice with SPF grade (aged 4 weeks, weighed 18 g ~ 22 g) were obtained from JOINN Laboratories (Suzhou) (Approval No. SCXK (Su) 2013-0003, Jiangsu, Suzhou, China). Mice were allowed free access to food and water and reared in polypropylene cages. The feeding conditions were as follows: temperature: 18°C ~ 22°C; relative humidity: 50% ~ 60%; light/dark regime: 12 h. Animal experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and approved by the Animal Care and Welfare Committee of the First Affiliated Hospital of Soochow University (Approval No. 2018-092).

In vivo Antioxidant Activity of TFCB

Sixty mice were randomly divided into six groups (n = 10 in each group) as follows: Normal Control (NC), Positive Control (PC), Model Control (MC) as well as three TFCB-treated groups, which were administered with TFCB in different doses of 50, 100 and 200 mg/kg once a day for 30 consecutive days, respectively. Mice in PC were dosed with VC in a dose of 200 mg/kg, while, mice in NC and MC groups were treated with distilled water. On the last day, all the animals except for ones in NC were orally treated with 50% (v/v) ethanol solution in a dose of 12 mL/kg to induce oxidative stress, followed by being anesthetized with pentobarbital sodium after 6 h. Then blood samples were harvested from orbit to obtain serum for the quantification of MDA and T-SOD. After that, animals were euthanized by using carbon dioxide, the livers were immediately dissected, washed and homogenized for the determination of GSH and PCO. Levels of MDA, T-SOD, GSH and PCO were determined based the methods provided in the kits instructions (Jiancheng Biotechnology Co., Ltd, Nanjing, Jiangsu, China) (Peng *et al*., 2015).

Statistical Analysis

Data was expressed as means ± or + SD (standard deviation). Statistical analysis was conducted by using SPSS19.0 software (SPSS Inc., Chicago, USA). The *t*-test and one-way Analysis of Variance (ANOVA) were used to evaluate the significance of distances between two means or multiple means, respectively.

Results and Discussion

Optimization of CAE Parameters for TFCB Extraction

Effects of Amount of Cellulase, Incubation Time, Liquid-to-Solid Ratio and Ethanol Concentration on the Extraction Yield of TFCB

As shown in Fig. 2a, in the range of 0.1% ~ 0.4% (w/w) cellulase, TFCB yield increased with the increase of cellulase amount, which reached the highest level at cellulase amount of 0.4%. Then, with the increase of cellulase, TFCB yield decreased. These results indicated that cellulase could enhance the TFCB release by decomposing the components of cell wall, but when enzyme is fully saturated with substrate, the TFCB release could be reduced due to the fact that excess cellulase might make the solution viscous, which is not conducive to the enzymatic hydrolysis (Huang *et al*., 2016).

To examine the effects of incubation time for enzymatic reaction on TFCB yield, a range of 0.5 h ~ 3 h incubation time was selected (Fig. 2b). Results showed that TFCB yield achieved maximum value at incubation time of 2 h, while, after 2 h, prolonged incubation time caused the decrease of TFCB yield, suggesting that cellulase could completely decompose cell wall of corn bract and release the maximum amount of total flavonoids within a 2 h-incubation period.

As shown in Fig. 2c, TFCB yield was remarkably raised with the increase of solvent amount spanning from 20: 1 mL/g ~ 25: 1 mL/g, then was slightly raised with the solvent ranging from 25: 1 mL/g ~ 35: 1 mL/g and reached the highest level at the liquid-to-solid ratio of 35: 1 mL/g, after which it was reduced. The decreased TFCB yield may be due to the fact that larger volume of solvent could result in excessive swelling of corn bract powders and absorbing TFCB (Xiao *et al*., 2008).

The effects of ethanol concentration on TFCB yield were investigated from 40% to 90% (v/v). It was seen in Fig. 2d that ethanol concentration showed great impacts on TFCB yield and the ethanol concentration of 70% led to a highest extraction yield. Too low or too high ethanol concentrations are not conducive to the optimum extraction of flavonoids, which depend on the polarity of ethanol solution as well as the types of flavonoids present in corn bract (Huang *et al*., 2016).

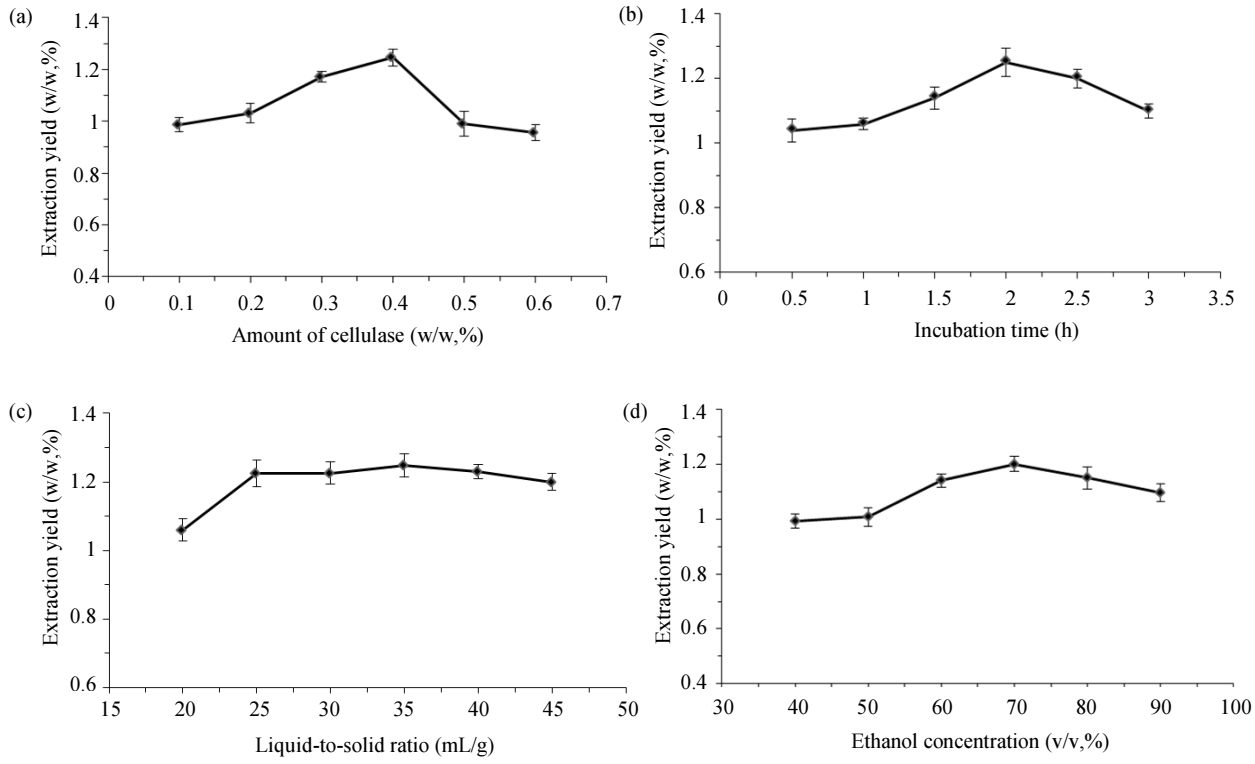

Fig. 2: Effects of (a) amount of cellulase (b) incubation time (c) liquid-to-solid ratio and (d) ethanol concentration on the extraction yield of TFCB. Data was expressed as the means ± SD (n = 3)

Table 2: Box-Behnken design and observed responses

	Independent variable				
Run	X_1	X_2	X_3	X_4	Y
1	0	1	1	0	1.098
2	1	0	-1	0	0.996
3	-1	0	0	1	1.120
4	1	1	0	0	1.012
5	0	0	1	-1	1.064
6	0	0	0	0	1.273
7	1	0	0	1	1.132
8	0	0	0	0	1.286
9	0	1	0	1	1.124
10	0	0	-1	1	1.021
11	0	-1	-1	0	1.021
12	-1	0	0	-1	0.986
13	0	-1	0	1	0.998
14	0	-1	1	0	1.051
15	0	1	0	-1	1.028
16	-1	0	1	0	1.008
17	0	-1	0	-1	1.025
18	0	0	0	0	1.298
19	1	-1	0	0	0.998
20	0	0	0	0	1.330
21	-1	-1	0	0	1.023
22	-1	1	0	0	0.976
23	1	0	1	0	1.099
24	1	0	0	-1	1.019
25	-1	0	-1	0	1.098
26	0	1	-1	0	1.106
27	0	0	-1	-1	0.989
28	0	0	0	0	1.332
29	0	0	1	1	1.019

Model Fitting and Statistical Analysis

According to the principles of BBD, the values of independent variables including amount of cellulase (w/w, %), incubation time (h), liquid-to-solid ratio (mL/g) and ethanol concentration (v/v, %) were selected based on the results of single-factor experiments and applied to the RSM test using TFCB extraction yield (w/w, %) as the response variable (Table 2). 29 different experimental combinations and response values were described in Table 2 and the TFCB extraction yield ranged from 0.976% ~ 1.332% (w/w), which could be described by a response surface quadratic model via multiple regression analysis. The response variable Y (TFCB extraction yield) could be related by the following second-order polynomial equation:

$$Y = 1.3 + 0.00375X_1 + 0.019X_2 + 0.009X_3 + 0.025X_4$$
$$+0.015X_1X_2 + 0.048X_1X_3 - 0.00525X_1X_4$$
$$-0.0095X_2X_3 + 0.031X_2X_4 - 0.019X_3X_4$$
$$-0.14X_1^2 - 0.14X_2^2 - 0.12X_3^2 - 0.13X_4^2$$

where, Y means TFCB extraction yield (w/w, %) and X_1, X_2, X_3 and X_4 represent amount of cellulase (w/w, %), incubation time (h), liquid-to-solid ratio (mL/g), ethanol concentration (v/v, %), respectively.

Table 3 exhibited one-way Analysis of Variance (ANOVA) for the fitted equation. The F-value was calculated to be 10.85 and the P-value was less than 0.0001, implying that the model obtained was highly significant. The determination coefficient value of model (R^2) was 0.9156, indicating that approximately 91.56% of the response variability could be explained by the model. In addition, the F-value for lack of fit was 3.76 and the P-value was 0.1070, greater than 0.05, implying a statistical non-significance thereby confirming the validity of the model. In this model, the linear parameters X_1, X_2, X_3 and X_4 were not significant (p>0.05). The quadratic terms X_1^2, X_2^2, X_3^2 and X_4^2 were highly significant (p<0.01). The interaction parameters X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4 and X_3X_4 were not significant (p>0.05), which indicated that interactions of any two of the four variables were not significant. Response surfaces were plotted by using Design-Expert software (version 8.0.6.1) to visualize interactions between the variables. The relationships between TFCB extraction yield and any two independent variables (the other variables were set to "0" level) were shown in Fig. 3.

Verification of Predictive Model

The optimal conditions of CAE for TFCB obtained by using RSM were as follows: Amount of cellulase, 0.40% (w/w); incubation time, 2.04 h; liquid-to-solid ratio, 35.15: 1 mL/g; ethanol concentration, 71.05% (v/v); the predicted extraction yield of TFCB was 1.306% (w/w). Considering the convenience of practical operation, the optimal conditions were modified as follows: amount of cellulase, 0.4% (w/w); incubation time, 2 h; liquid-to-solid ratio, 35: 1 mL/g; ethanol concentration, 71% (v/v), under these conditions, the actual extraction yield was 1.284±0.01% (w/w) (n = 3), which were also higher than any single factor experiments.

The Influence of Cellulase on TFCB Release

In this study, TFCB extraction by CAE was compared with that of Heat Reflux Extraction (HRE). As shown in Table 4, the yield of TFCB obtained by CAE was significantly (p<0.01) higher than that obtained by HRE, suggesting that cellulase plays an important role on the release of TFCB from cell.

In vitro Antioxidant Activity of TFCB

Figure 4a ~ 4c showed the *in vitro* antioxidant activities of TFCB against 1, 1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl and superoxide anion radicals, Figure 4d exhibited the reducing power to ferric ion and the half inhibitory concentration (IC_{50}) values were depicted in Table 5. The DPPH radical-scavenging capacities of TFCB were elevated with the increase of concentration

ranging from 10 μg/mL to 60 μg/mL, the highest scavenging rate was 80.54±1.06%, the IC_{50} value was 34.02±0.52 μg/mL, which was significantly (p<0.01) higher than that of vitamin C (VC) with a IC_{50} value of 5.82±0.07 μg/mL (Fig. 4a). As shown in Fig. 4b, in the concentration range 100 μg/mL to 600 μg/mL, TFCB exerted scavenging capacities against hydroxyl radical, which increased with the increase of concentration, the highest scavenging rate was 81.52±2.42%, but inferior to VC, the IC_{50} value was still significantly (p<0.01) higher than that of VC (200.55±6.43 μg/mL *vs*. 47.36±1.20 μg/mL). As for the superoxide anion radical (Fig. 4c), in the concentration range 10 μg/mL to 60 μg/mL, the free radical scavenging capacities of TFCB were raised with the elevation of concentration, the highest scavenging rate was 92.38±2.29% in the concentration of 60 μg/mL, the IC_{50} value was 17.18±0.97 μg/mL, which was a little higher than that of VC (15.78±0.058 μg/mL), but no significant differences (p>0.05) were found. The reducing powers of TFCB to ferric ion were shown in Fig. 4d, in the concentration range 3 μg/mL to 18 μg/mL, the absorbance (A) of TFCB at 700 nm was gradually raised with the elevation of concentration, when $A_{700\ nm}$ was 0.2, the concentration of TFCB was 10.39±0.21 μg/mL, while, the concentration of VC was 7.87±0.017 μg/mL, significant differences (p<0.01) were observed. These results indicated that TFCB exerts certain scavenging capacities against DPPH, hydroxyl and superoxide anion radicals, as well as reducing powers to ferric ion in different concentration ranges. According to the IC_{50} values presented in Table 5, in addition to superoxide anion radical, TFCB showed weaker scavenging or reducing capacities against DPPH, hydroxyl and ferric ion compared with VC.

Table 3: Box-Behnken design and observed responses

Source[a]	Sum of squares	DF[b]	Mean square	*F*-value	*P*-value	Significance[c]
Model	0.31	14	0.022	10.85	< 0.0001	**
X_1	1.688×10^{-4}	1	1.688×10^{-4}	0.082	0.7791	n.s.
X_2	4.332×10^{-3}	1	4.332×10^{-3}	2.10	0.1694	n.s.
X_3	9.720×10^{-4}	1	9.720×10^{-4}	0.47	0.5037	n.s.
X_4	7.651×10^{-3}	1	7.651×10^{-3}	3.71	0.0747	n.s.
X_1X_2	9.303×10^{-4}	1	9.303×10^{-4}	0.45	0.5129	n.s.
X_1X_3	9.312×10^{-3}	1	9.312×10^{-3}	4.51	0.0519	n.s.
X_1X_4	1.103×10^{-4}	1	1.103×10^{-4}	0.053	0.8205	n.s.
X_2X_3	3.610×10^{-4}	1	3.610×10^{-4}	0.17	0.6821	n.s.
X_2X_4	3.782×10^{-3}	1	3.782×10^{-3}	1.83	0.1972	n.s.
X_3X_4	1.482×10^{-3}	1	1.482×10^{-3}	0.72	0.4109	n.s.
X_1^2	0.12	1	0.12	57.84	< 0.0001	**
X_2^2	0.12	1	0.12	58.59	< 0.0001	**
X_3^2	0.098	1	0.098	47.38	< 0.0001	**
X_4^2	0.11	1	0.11	51.83	< 0.0001	**
Residual	0.029	14	2.063×10^{-3}			
Lack of fit	0.026	10	2.611×10^{-3}	3.76	0.1070	n.s.
Pure error	2.781×10^{-3}	4	6.952×10^{-4}			
Cor total	0.34	28				
R^2	0.9156			Adjusted R^2	0.8312	

[a] X_1: amount of cellulase (w/w, %); X_2: Incubation time (h); X_3: Liquid-to-solid ratio (mL/g); X_4: Ethanol concentration (v/v, %); [b] Degree of freedom; [c] *p<0.05 significant; **p<0.01 highly significant; n.s. means not significant

Table 4: The influence of cellulase on TFCB release.

Method	X_1	X_2	X_3	X_4	Time (h)	Temperature (°C)	Yield (w/w, %)[a]
HRE	−	2	35: 1	71	2	80	0.958±0.04
CAE	0.4	2	35: 1	71	2	80	1.284±0.01 **

X_1: Amount of cellulase (w/w, %); X_2: Incubation time (h); X_3: Liquid-to-solid ratio (mL/g); X_4: ethanol concentration (v/v, %); [a]TFCB extraction yields were expressed as means ± SD; **p<0.01 highly significant versus HRE

Table 5: The IC_{50} values and reducing power of TFCB and VC.

	IC_{50} (μg/mL)			
Sample	DPPH radical	Hydroxyl radical	Superoxide anion	Reducing power (μg/mL)[a]
TFCB	34.02±0.52**	200.55±6.43**	17.18±0.97	10.39±0.21**
VC	5.82±0.07	47.36±1.20	15.78±0.058	7.87±0.017

[a] The corresponding concentrations of TFCB and VC, when $A_{700\ nm}$ = 0.2; data was expressed as the mean ± SD (n = 3); symbol indicates statistically significant differences, **p<0.01 highly significant versus VC.

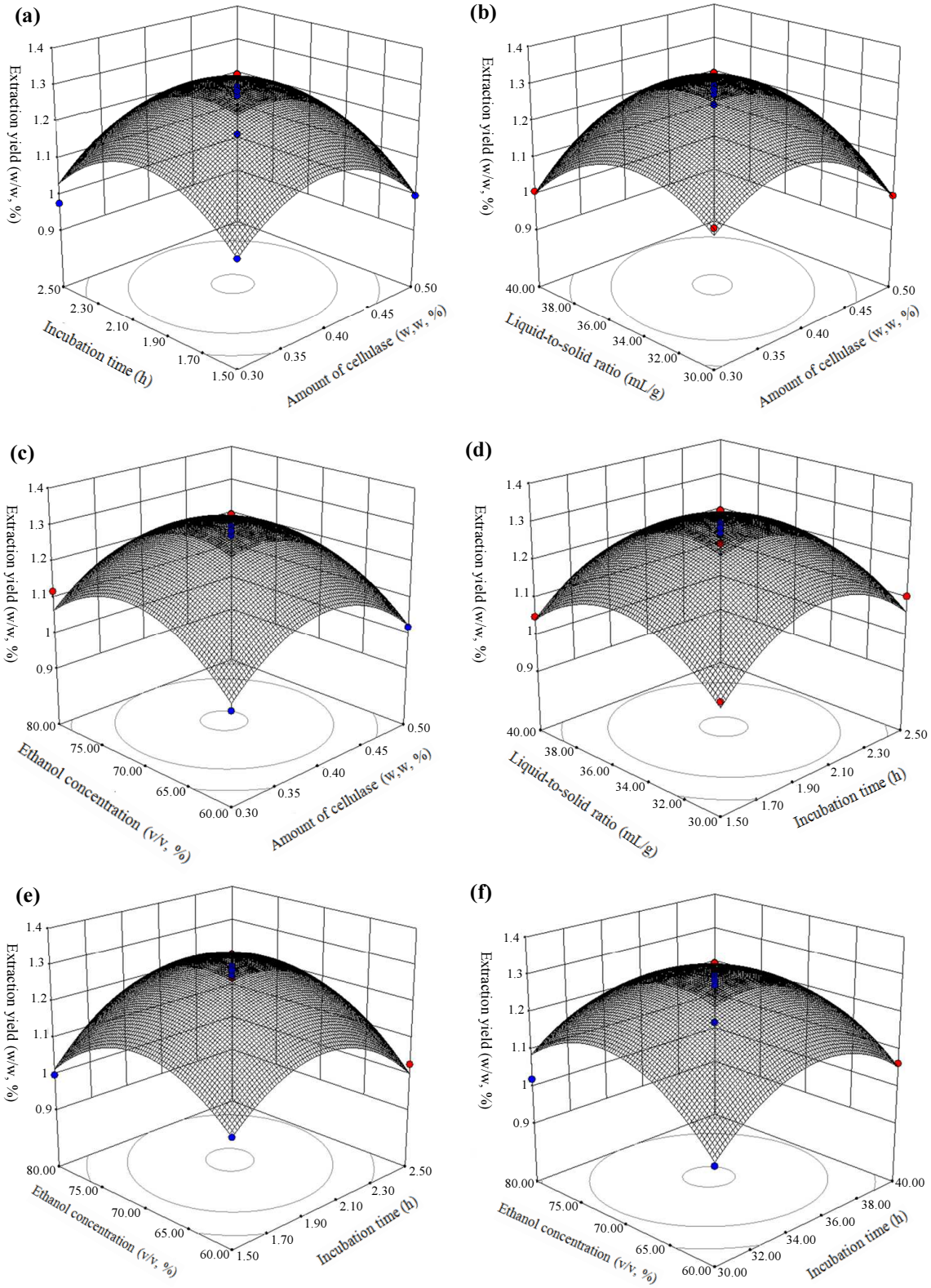

Fig. 3: Response surface plots (3-D) showing the effects of variables on TFCB extraction yield: (a) at varying amount of cellulase (X_1) and incubation time (X_2); (b) at varying amount of cellulase (X_1) and liquid-to-solid ratio (X_3); (c) at varying amount of cellulase (X_1) and ethanol concentration (X_4); (d) at varying incubation time (X_2) and liquid-to-solid ratio (X_3); (e) at varying incubation time (X_2) and ethanol concentration (X_4); (f) at varying liquid-to-solid ratio (X_3) and ethanol concentration (X_4)

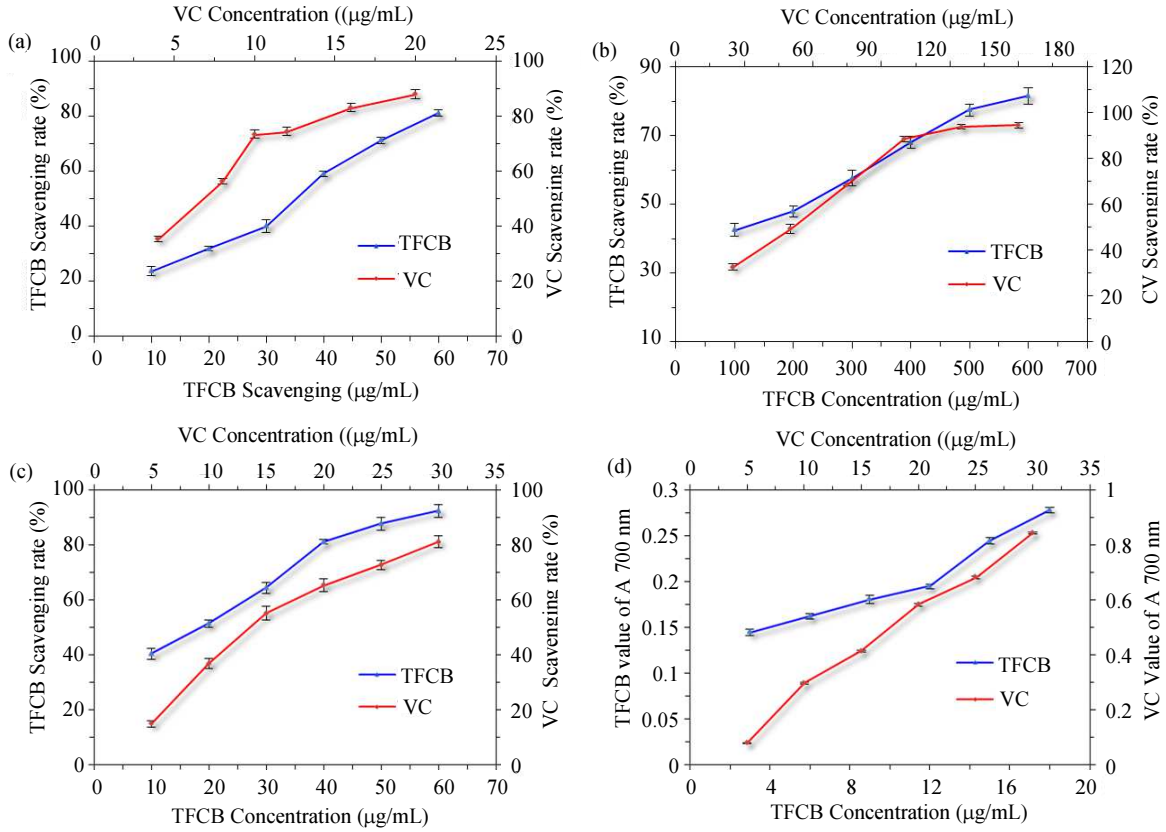

Fig. 4: The *in vitro* antioxidant evaluation of TFCB using VC as positive control. (a) DPPH radical-scavenging capacity; (b) Hydroxyl radical-scavenging capacity; (c) superoxide anion radical-scavenging capacity; (d) reducing power. Data was expressed as the means ± SD (n = 3)

In vitro Antibacterial Activity of TFCB

The Minimum Inhibitory Concentration (MIC) of TFCB against five bacterial species were summarized in Table 6. In the concentration range 0.50 μg/mL to 50.0 μg/mL, the MIC values of TFCB against *S. aureus*, *B. pumilus*, *B. subtilis* and *S. typhimurium* were 18.0, 6.48, 10.8 and 50.0 μg/mL, respectively. These results indicated that TFCB may exhibit more inhibitory effects on Gram-positive bacteria than Gram-negative ones, which deserved to be further investigated in the near future.

In vivo Antioxidant Activity of TFCB

In order to confirm the *in vivo* antioxidant activity of TFCB, an ethanol-induced oxidative stress mouse model was adopted using VC as positive control and four antioxidant-related biomarkers including Malondialdehyde (MDA), Total Superoxide Dismutase (T-SOD), Glutathione (GSH) and Protein Carbonyls (PCO) were selected.

Effects of TFCB on MDA

MDA is one of the most common final metabolic products of lipid peroxidation, its quantity is usually in proportion to the oxidative stress-related damages (Esterbauer *et al*., 1991). As shown in Fig. 5a, MDA content decreased in a dose-dependent manner ($p<0.05$), when dose was 200 mg/kg, MDA content reached the lowest level of 7.22±1.92 mmol/L, 1.8-fold lower than that in MC and 1.4-fold lower than that in PC. Statistical analysis revealed that significant differences ($p<0.01$) between NC and MC were found; there were significant differences ($p<0.01$) between MC and all TFCB-treated groups; when compared with PC, significant differences ($p<0.01$) were observed in 200 mg/kg of TFCB-treated group.

Effects of TFCB on T-SOD

SOD is a kind of antioxidase that possesses scavenging capacities against superoxide by changing the high reactive superoxide to the low hydrogen peroxide via dismutation reaction. Three isozymes of SOD, namely SOD-1, SOD-2 and SOD-3 have been reported, they show different biological localizations and enzyme characteristics, but catalyze the same reaction (Hu *et al*., 2007). As shown in Fig. 5b, when compared with NC, T-SOD activities in MC were reduced significantly ($p<0.01$). There were statistically

significant differences (p<0.05, p<0.01) between MC and all TFCB-treated groups. Oral administration of TFCB can enhance T-SOD activities in a dose-dependent manner (p<0.05), significant differences (p<0.05) were found in 200 mg/kg of TFCB-treated group compared with PC.

Table 6: The Minimum inhibitory concentration of TFCB

TFCB (μg/mL)	*S. aureus*	*B. pumilus*	*B. subtilis*	*E. coli*	*S. typhimurium*
0.50	-	-	-	-	-
0.84	-	-	-	-	-
1.40	-	-	-	-	-
2.33	-	-	-	-	-
3.89	-	-	-	-	-
6.48	-	+	-	-	-
10.8	-	++	+	-	-
18.0	+	+++	++	-	-
30.0	++	++++	++	-	-
50.0	+++	+++++	+++	-	+

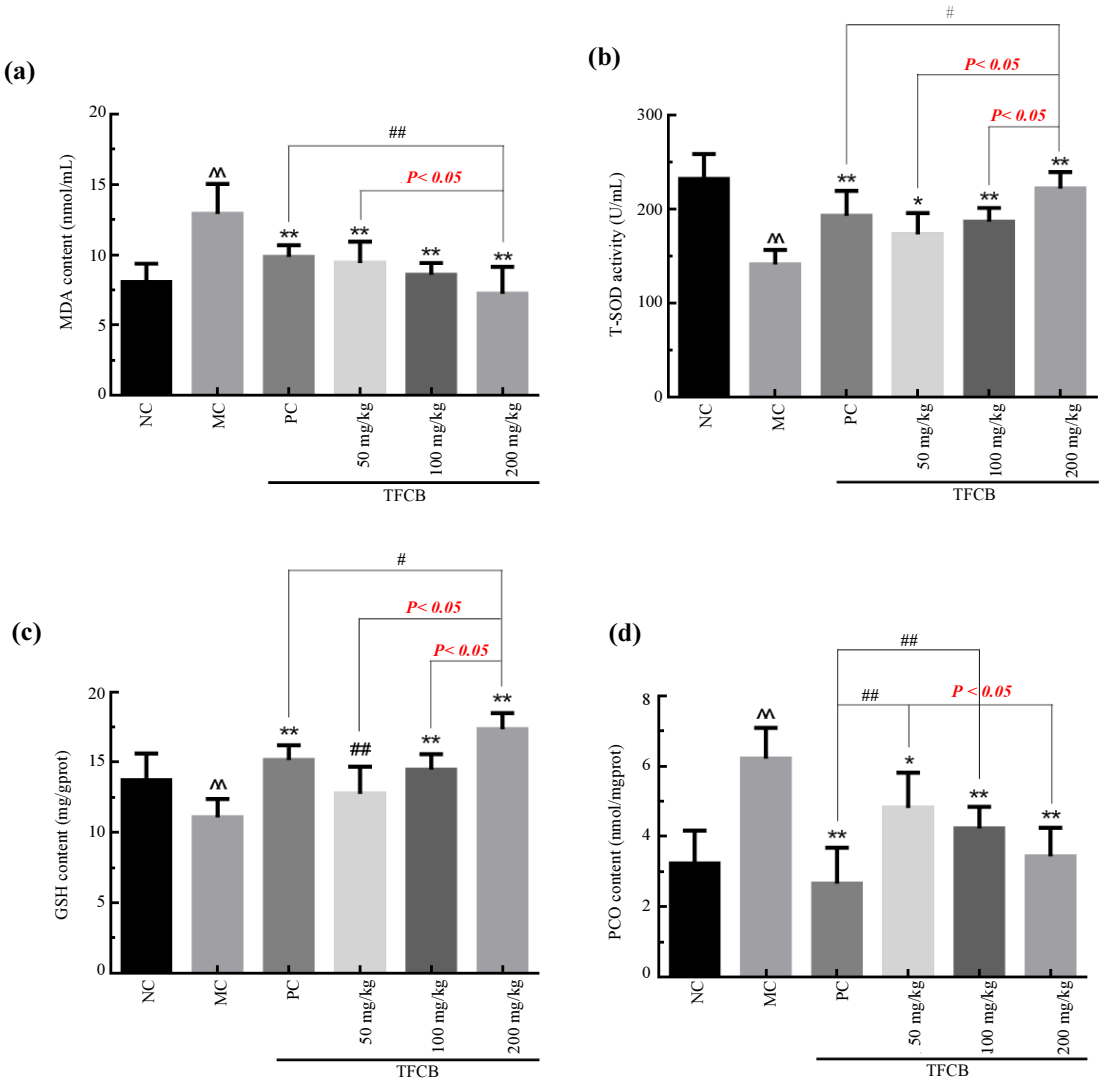

Fig. 5: Effects of TFCB on (a) MDA, (b) T-SOD, (c) GSH and (d) PCO. Data denoted were means + SD (n = 10). Different symbols indicate statistically significant differences, $^{\wedge\wedge}p$<0.01 as compared with NC group; $^{*}p$<0.05, $^{**}p$<0.01 as compared with MC group; $^{\#}p$<0.05, $^{\#\#}p$<0.01 as compared with PC group. NC: Normal Control; MC: Model Control; PC: Positive Control (VC in a dose of 200 mg/kg)

Effects of TFCB on GSH

GSH is one of the most important antioxidants that widely participate in the detoxification of xenobiotic substances, it is a tri-peptide condensed by glutamic acid, cysteine and glycine, thus owing to the presence of thiol group, GSH can directly react with free radicals (Bray and Taylor, 1993). As shown in Fig. 5c, significant differences (p<0.01) in GSH content were noted between NC and MC. When compared with MC, there were significant differences (p<0.01) in PC and two TFCB-treated groups (100 and 200 mg/kg). GSH content was raised in a dose-dependent manner (p<0.05), when compared with PC, GSH content in 50 mg/kg of TFCB-treated group was significantly lower (p<0.01), however, when dose was elevated to 200 mg/kg, GSH content was significantly higher (p<0.05) than that in PC.

Effects of TFCB on PCO

PCO is an irreversible form of protein oxidation, which is more stable than MDA and usually forms earlier than other oxidative stress-related biochemical indicators (Weber *et al*., 2015). As shown in Fig. 5d, PCO content in NC was significantly lower (p<0.01) than that in MC. Significant differences (p<0.05, p<0.01) were found in PC and all TFCB-treated groups compared with MC. PCO content decreased in a dose-dependent manner (p<0.05), but inferior to PC, PCO contents in 50 and 100 mg/kg of TFCB-treated groups were significantly higher (p<0.01) than that in PC and PCO content in 200 mg/kg of TFCB-treated group was 3.44±0.82 nmol/mgprot, which was still higher than that of PC (2.67±1.02 nmol/mgprot).

These results revealed that oral administration of TFCB can depress the oxidative stress induced by ethanol in mice and exerts more effects on MDA, T-SOD and GSH than on PCO. The underlying mechanisms may involve in reducing MDA and PCO formation as well as increasing T-SOD activities and GSH biosynthesis. The *in vivo* antioxidant activity of TFCB was raised in a dose-dependent manner (p<0.05), when dose was elevated to 200 mg/kg, significant differences (p<0.05, p<0.01) in MDA, T-SOD and GSH were observed compared with PC (VC in a dose of 200 mg/kg), which contradicted with the results of the in vitro evaluation of antioxidant activity, where TFCB showed weaker free radical-scavenging capacities and reducing power to ferric ion than those of VC in most cases (Fig. 4). These results were consistent with the general findings that several *in vivo* metabolites of flavonoids, especially flavonoid glycosides may equally contribute their antioxidant activities (Arora *et al*., 1998; Miyake *et al*., 2000; Lemmens *et al*., 2015). However, the exact mechanisms of the *in vivo* antioxidant activity of TFCB are still unclear.

Conclusion

In this study, RSM was applied to optimize the extraction conditions of CAE for TFCB by using BBD to obtain the optimal conditions as follows: Amount of cellulase, 0.4% (w/w); incubation time, 2 h; liquid-to-solid ratio, 35: 1 mL/g; ethanol concentration, 71% (v/v). Under these conditions, the TFCB yield was 1.284±0.01% (w/w), which was 25.39% higher than that of heat reflux extraction. *In vitro*, TFCB showed scavenging capacities on DPPH, hydroxyl and superoxide anion radicals as well as reducing power to ferric iron in different concentration ranges; exhibited inhibitory effects against the Gram positive bacteria (*S. aureus*, *B. pumilus* and *B. subtilis*), but little impact on the Gram negative ones (*E. coli* and *S. typhimurium*). *In vivo*, TFCB exerted antioxidant activities via reducing MDA and PCO formation as well as increasing T-SOD activity and GSH biosynthesis in ethanol-induced oxidative stress mice model. The stronger *in vivo* antioxidant activity was contradicted with the results obtained from the *in vitro* antioxidant evaluation compared with VC, suggesting that TFCB metabolites could also contribute to the antioxidant activities, which deserved to be further studied. In addition, the chemical compositions of TFCB will be equally needed further study to explore in the near future.

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Author's Contributions

Yang Zhang and Zhaowei Yan: Conceived and designed the experiments.

Jing Lu: Performed the experiments of *in vitro* antioxidant and antibacterial evaluations.

Leijie Ben, Lixue Zheng, Pengfei Xu and Yingang Jia: Optimized the extraction process.

Zhenyu Cheng: Processed the data.

Zhaowei Yanb Carried out the antioxidant activities of TFCB in mice.

Yang Zhang: Wrote manuscript.

Zhaowei Yan: Revised the manuscript.

Ethics

Authors declared no ethical issues that may arise after the publication of this manuscript.

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