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Article

Optimization of Ultrasonic-Assisted Extraction of Total Phenolics from *Citrus aurantium* L. Blossoms and Evaluation of Free Radical Scavenging, Anti-HMG-CoA Reductase Activities

Kexin Hao [1,2,†], Wenzhong Hu [1,2,*,†], Mengyang Hou [2,3,†], Duo Cao [4], Yu Wang [1,2], Qingxin Guan [1,2], Xiufu Zhang [1,2], Aosheng Wang [1,2], Jiaoxue Yu [1,2]and Binmei Guo [1,2]

[1] College of Life Science, Dalian Nationalities University, Dalian 116600, China; haokex@sina.com (K.H.); yu1922993217@163.com (Y.W.); 15140378826@163.com (Q.G.); 13734549519@163.com (X.Z.); 18742044187@163.com (A.W.); 18340873239@163.com (J.Y.); gbm2018@sina.com (B.G.)

[2] Key Laboratory of Biotechnology and Bioresources Utilization, Ministry of Education, Dalian 116600, China; mengyanghou@yahoo.com (M.H.)

[3] School of Life Science and Biotechnology, Dalian University of Technology, Dalian 116024, China;

[4] College of Life Science, Northwest University, Xi'an 710069, China; caoduo2013@163.com

* Correspondence: wenzhongh@sina.com; Tel.: +86-135-9116-2665

† These authors contributed equally to this work.

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Abstract: The objective of this study was to develop an ultrasonic-assisted procedure for the extraction of total phenolics from *Citrus aurantium* L. blossoms (CAB) and evaluate the free radical scavenging activity, anti-HMG-CoA reductase activity of total phenolics. In this work, a Box-Behnken design based on the single-factor experiments was used to explore the optimum extraction process. Under the optimum conditions (extraction solvent 70.31% ethanol, extraction temperature 61.94 °C, extraction time 51.73 min and liquid-to-solid ratio 35.63 mL/g), the extraction yield of total phenolics was 95.84 mg gallic acid equivalents (GAE)/g dry matter (DM), which was highly consistent with the theoretical value (96.12 mg GAE/g DM). The total phenolic extract showed excellent free radical scavenging properties against DPPH·, $ABTS^{+\cdot}$, ·OH and $\cdot O_2^-$, with the IC_{50} values of 197.007, 83.878, 218.643 and 158.885 μg/mL, respectively, and the extracts also showed good inhibition of HMG-CoA reductase activity, with the IC_{50} value of 117.165 μg/mL. Total phenolics from CAB could be a potential source of natural free radical scavenger and HMG-CoA reductase inhibitor.

Keywords: *Citrus aurantium* L. blossoms; total phenolics; ultrasonic-assisted extraction; Box-Behnken design; free radical scavenging activity; anti-HMG-CoA reductase activity

1. Introduction

Free radicals, atoms or groups containing unpaired electrons, are fairly active, which are essential to any physiological metabolism of organism[1]. As is known to all, organism itself has the ability to balance free radicals, but the risk of several serious diseases will increase if the organism cannot get rid of excess free radicals. Modern medical studies have demonstrated that free radicals can lead to DNA, protein and lipid oxidative damage and have a direct relationship with the cancer, cardiovascular diseases, Alzheimer's and Parkinson's disease[2-7]. Dietary supplementation of

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exogenous free radical scavengers, is associated with the reduced incidence of those frightening diseases. Despite the enormous development of chemical synthesis, many excellent natural free radical scavengers with fewer side effects from edible or medicinal plants have been successfully exploited over the past several years[8]. Plants are rich in various bioactive compounds, in which phenolics have attracted increasing attention due to their preeminent free radicals scavenging property.

In recent years, the morbidity and mortality of cardiovascular and cerebrovascular diseases have risen dramatically due to the improvement of people's living standards. And those diseases have very high correlation with hyperlipidemia, mainly manifesting as high levels of cholesterol in the blood[9]. A key regulatory enzyme, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), has a significant impact on cholesterol biosynthesis. Hence, an effective method for reducing cholesterol level is to inhibit the HMG-CoA reductase activity. The existing HMG-CoA reductase inhibitors are mainly statin drugs. Unfortunately, other potentially harmful side effects, such as myopathy and liver damage, will appear with long-time use of statin drugs[10]. Therefore, the screening of novel HMG-CoA reductase inhibitors is urgent.

Citrus aurantium L. belonging to the genus *Citrus* (Rutaceae family), is mainly cultivated in tropical and subtropical areas. *Citrus aurantium* L. is a well-known medicinal and edible plant in China and has been traditionally used for treatment of obesity, indigestion, chest congestion, bellyache, nausea and vomiting[11], and recently has attracted attention for its extensive biological activities including anticancer, antibacterial, antioxidant, antidiabetic, enhancing immunity and treatment for neurological disorders[12-16]. These activities are ascribed to active ingredients including total phenolics, triterpenoids, polysaccharides and volatile oil[17-20]. However, the development of high value-added products has not been completely realized, as a result, the huge amounts of CAB are still not sufficiently exploited and abandoned wastefully. Hence, there is an urgent need to extract bioactive compounds from CAB.

In fact, extraction technology not only has a direct influence on the extraction efficiency of phytochemical components, but also has a profound effect on the biological activities. Traditional solvent extraction methods, including cold or warm soaking, decoction and heat reflux, usually take a lot of time and energy with lower-efficiency performance[21]. Hence, in-depth exploration of green and efficient extraction methods is of great significance. Ultrasonic-assisted extraction utilizes the cavitation effect, mechanical effect and thermal effect of ultrasonic wave to extract biological active components rapidly and fully, which can also enhance the biological activities of extracts[22, 23]. As a novel technology, ultrasonic-assisted extraction has been proposed by many researchers, such as phenolic compounds from *Justicia spicigera* leaves[24], blueberry pomace[25], *Oryza sativa* L. 'Violet Nori'[26], *Brosimum alicastrum* leaves[27], *Acer Truncatum* leaves[28].

This study was committed to develop an energy-efficient technology for extraction of total phenolics from CAB by ultrasonic-assisted extraction, and the process parameters were optimized through single-factor experiment and Box-Behnken design. In addition, free radical scavenging and anti-HMG-CoA reductase activities of total phenolic extract were evaluated in vitro.

2. Results and Discussion

2.1. Single-Factor Experimental Analysis

2.1.1. Effect of ethanol concentration on the extraction yield of total phenolics

Aqueous ethanol solution is a most common extraction solvent, which has been extensively used for the extraction of natural products due to its high-efficiency, eco-friendly and recyclable characteristics. The extraction solvent with proper polarity is vital to the solubility of total phenolics[29]. And different concentrations of ethanol have different polarity, therefore, the effects of ethanol concentrations (30-80%) on the extraction yield of total phenolics from CAB were evaluated

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when controlling for other factors (extraction temperature 45 °C, extraction time 30 min and liquid-to-solid ratio 30 mL/g). As shown in Figure 1a, the extraction yield of total phenolics significantly improved from 48.95 ± 1.32 to 91.58 ± 1.12 mg GAE/g DM with the increase in ethanol concentration from 30% to 70%. However, the extraction yield declined with the further increase of ethanol concentration. Hence, 70% ethanol was considered as the optimal extraction solvent.

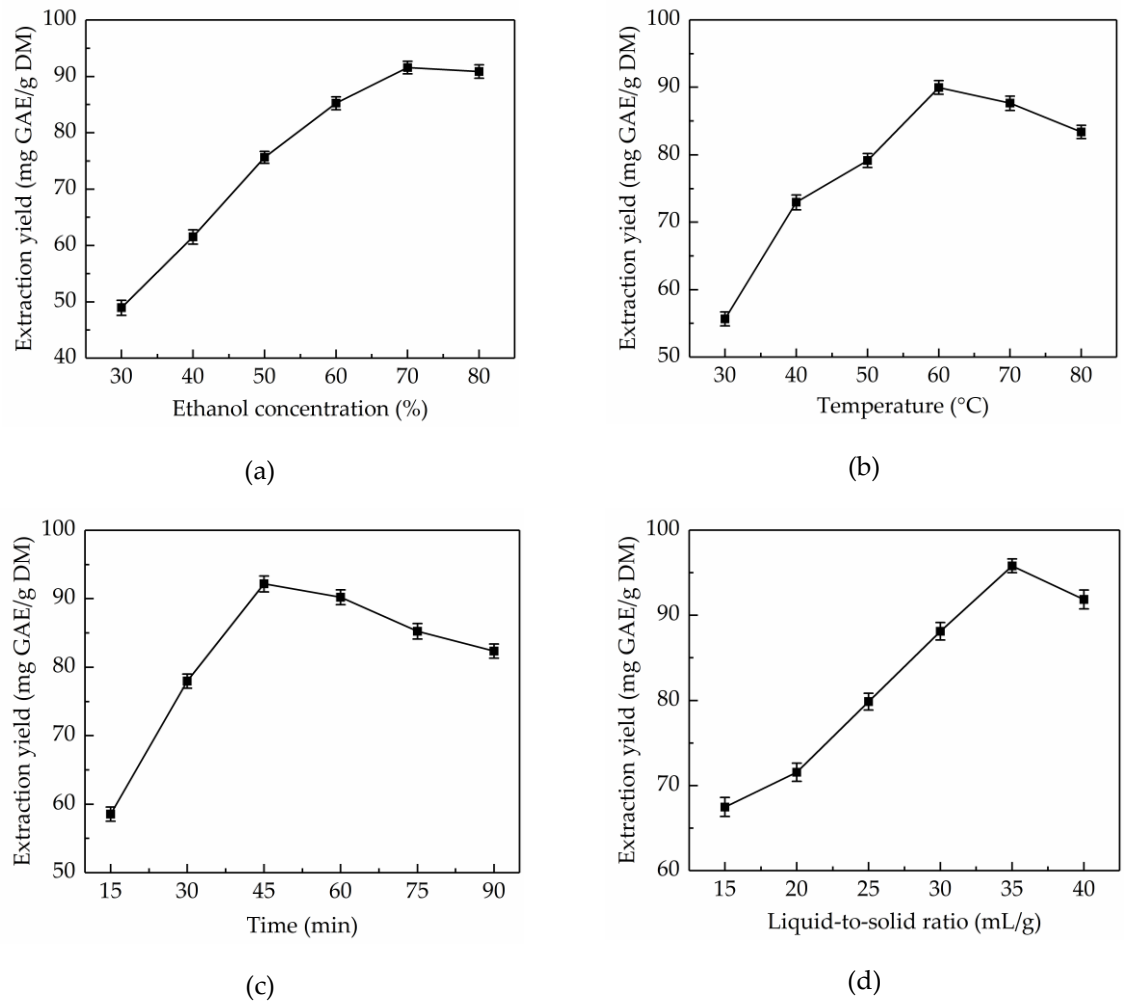

Figure 1. Effects of ethanol concentration (**a**), extraction temperature (**b**), extraction time (**c**) and liquid-to-solid ratio (**d**) on the extraction yield of total phenolics.

2.1.2. Effect of Extraction Temperature on Extraction Yield of Total Phenolics

In general, the higher extraction temperature, the higher extraction yield, which is due to the fact that molecules move faster at higher temperature, leading enhanced diffusion and permeation behaviors[30]. But if the extraction temperature is too high, the heat-sensitive components would be destroyed. Hence, to improve extraction yield, the extraction temperatures in the range of 30-80 °C were studied when controlling for other factors (ethanol concentration 70%, extraction time 30 min and liquid-to-solid ratio 30 mL/g). Figure 1b showed that the extraction yield of total phenolics from CAB obviously increased in the range of 30-60 °C, however, further increase in the extraction temperature resulted in a gradual decrease in the extraction yield. The highest extraction yield of total phenolics (89.98 ± 1.01 mg GAE/g DM) was obtained at 60 °C. Therefore, 60 °C was considered as the optimum extraction temperature.

2.1.3. Effect of Extraction Time on Extraction Yield of Total Phenolics

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Time is a significant factor influencing the extraction yield of total phenolics. A reasonable extraction time can help shorten the production cycle, reduce energy consumption and improve yield. However, too long extraction time will result in the decomposition of the target compounds[31]. To optimize the extraction time, the extraction time was set at 10-60 min, meanwhile, other variables were kept constant (ethanol concentration 70%, extraction temperature 60 °C and liquid-to-solid ratio 30 mL/g). Figure 1c showed that the extraction yield of total phenolics from CAB increased with time and reached the highest (92.16 ± 1.15 mg GAE/g DM) when the extraction time was 45 min, and after this point in time, the extraction yield started to decline. Thus, the ultrasonic time was maintained at 45 min for the next step.

2.1.4. Effect of Liquid-to-Solid Ratio on Extraction Yield of Total Phenolics

The liquid-to-solid ratio has a significant role in the mass transfer of extracts between materials and extraction solvents[32]. For obtaining the ideal extraction yield of total phenolics, selecting a suitable liquid-to-solid ratio is of the essence. The probable reason is that total phenolics from CAB cannot be fully extracted in the case of low liquid-solid ratio, conversely, when the liquid-solid ratio is too high, the content of impurity and operational cost will increase. To optimize the liquid-solid ratio, in this work, the liquid-solid ratio was set at 15-40 mL/g, meanwhile, other variables were kept constant (ethanol concentration 70%, ultrasonic temperature 60 °C and extraction time 45 min). As shown in Figure 1d, the extraction yield of total phenolics from CAB increased from 67.49 ± 1.12 to 95.81 ± 0.79 mg GAE/g DM when the liquid-to-solid ratio increased from 15 to 35 mL/g. However, further increasing liquid-to-solid ratio decreased the extraction yield of total phenolics. Thus, 35 mL/g was regarded as the optimal liquid-to-solid ratio.

2.2. Optimization of Variables by Box-Behnken Design

2.2.1. Statistical Analysis and Model Fitting

Single-factor experiments can only study the effects of changes in one factor on the response variable. The truth, however, is that the influence of variables on response variables is interdependent and mutually restricted. Hence, the influence of the interaction between the main four variables (ethanol concentration, extraction temperature, extraction time and liquid-to-solid ratio) on extraction yield of total phenolics was researched by Box-Behnken design. Detailed experimental design and results were summarized in Table 1.

Table 1. Box-Behnken design matrix and response values for the extraction yield of total phenolics.

Run	Independent variables				Y (mg GAE/g DM)	
	X_1 (%)	X_2 (°C)	X_3 (min)	X_4 (mL/g)	Experimental	Predicted
1	60	60	45	30	72.19	73.63
2	70	60	45	35	95.81	94.49
3	70	60	45	35	95.37	94.49
4	70	70	45	30	79.37	78.38
5	70	50	60	35	74.97	76.29
6	80	60	30	35	72.36	73.48
7	70	60	30	30	77.98	76.20
8	60	60	30	35	68.28	70.01

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9	80	60	45	30	77.99	79.74
10	70	60	30	40	82.47	82.40
11	70	50	45	40	72.52	73.68
12	60	50	45	35	65.69	64.30
13	70	60	45	35	93.64	94.49
14	70	60	45	35	93.47	94.49
15	70	70	45	40	85.27	85.21
16	70	60	60	30	90.21	89.55
17	60	60	60	35	81.90	80.96
18	80	70	45	35	74.01	74.67
19	70	60	45	35	94.17	94.49
20	80	50	45	35	69.86	68.77
21	80	60	60	35	84.4	82.84
22	60	60	45	40	81.27	80.07
23	70	60	60	40	88.32	89.37
24	70	70	60	35	86.22	87.01
25	70	50	30	35	69.39	69.15
26	80	60	45	40	80.19	79.31
27	70	50	45	30	74.26	74.50
28	70	70	30	35	74.61	73.84
29	60	70	45	35	73.43	73.79

Multiple regression analysis was used to determine the correlation of four variables and extraction yield of total phenolics, and a second-order polynomial equation yielded was represented as below:

$$\begin{aligned} Y = & -1309.09267 + 18.69873X_1 + 13.83737X_2 + 2.95973X_3 + 13.61713X_4 - 0.008975X_1X_2 \\ & -0.00263333X_1X_3 - 0.0344X_1X_4 + 0.01005X_2X_3 + 0.0382X_2X_4 \\ & -0.021267X_3X_4 - 0.11931X_1^2 - 0.12178X_2^2 - 0.025506X_3^2 - 0.17491X_4^2 \end{aligned} \quad (1)$$

Where, X_1, X_2, X_3 and X_4 represent the ethanol concentration, extraction temperature, extraction time and liquid-to-solid ratio, respectively. Y represents the extraction yield of total phenolics.

The parameters acquired from the analysis of variance (ANOVA) for Box-Behnken design were listed in Table 2. The model with an *F*-value of 66.3436 and *p*-value of <0.0001 meant that the model was highly significant. It also meant this model was good consistent with experimental data. The *F*-value and *p*-value of the lack of fit were 2.6222 and 0.1829, respectively, which meant that the lack of fit was not significant relative to the pure error. The coefficient (R^2=0.9852) meant this model could account for 98.52% of the response value changes and the fitting precision of this model was satisfactory. The adjusted coefficient (Adj. R^2=0.9703) was closed to the R^2, which meant that experimental values fit well with the predicted values. The value of coefficient of variation (C.V.%)

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was very low (1.91), which meant the model was repeatable[33]. In addition, the linear coefficients (X_1, X_2, X_3 and X_4), cross product coefficients (X_1X_4 and X_2X_4) and quadratic coefficient (X_1^2, X_2^2, X_3^2 and X_4^2) were significant ($p<0.05$).

Table 2. ANOVA for Box-Behnken design.

Source	Sum of Squares	df	Mean Square	*F*-value	*p*-value
Model	2196.5259	14	156.8947	66.3436	<0.0001***
X_1	21.4669	1	21.4669	9.0774	0.0093**
X_2	178.0240	1	178.0240	75.2782	<0.0001***
X_3	309.3721	1	309.3721	130.8193	<0.0001***
X_4	27.1201	1	27.1201	11.4679	0.0044**
X_1X_2	3.2220	1	3.2220	1.3624	0.2626
X_1X_3	0.6241	1	0.6241	0.2639	0.6154
X_1X_4	11.8336	1	11.8336	5.0039	0.0421*
X_2X_3	9.0902	1	9.0902	3.8438	0.0701
X_2X_4	14.5924	1	14.5924	6.1705	0.0263*
X_3X_4	10.1761	1	10.1761	4.3030	0.0570
X_1^2	923.4078	1	923.4078	390.4671	<0.0001***
X_2^2	961.9172	1	961.9172	406.7509	<0.0001***
X_3^2	213.6335	1	213.6335	90.3359	<0.0001***
X_4^2	124.0230	1	124.0230	52.4437	<0.0001***
Residual	33.1083	14	2.3649		
Lack of Fit	28.7262	10	2.8726	2.6222	0.1829
Pure Error	4.3821	4	1.0955		
Cor Total	2229.6342	28			

R^2=0.9852, Adj. R^2=0.9703, C.V.%=1.91, *p<0.05, **p<0.01, ***p<0.001.

2.2.2. Optimization of Ultrasonic-Assisted Extraction Procedure

In order to provide a better visual representation of the effects of interactions between the independent variables on the response value, three-dimensional response surface plots drawn by Box-Behnken Design were shown in Figure 2a-f. For each figure, the extraction yield of total phenolics was gained by changing two variables while the other two variables remained unchanged[34]. Figure 2a-f showed the effects of the pairwise interaction between the ethanol concentration and extraction temperature on the the extraction yield of total phenolics, it could be seen that the extraction yield of total phenolics increased rapidly with increasing ethanol concentration from 60% to about 70%, and followed by a decrease thereafter, meanwhile, the extraction yield of total phenolics increased rapidly when the extraction temperature was from 50 °C to 62 °C, and decreased afterwards. Similarly, the extraction yield of total phenolics increased greatly with the increase of extraction time from 30 min to about 52 min, whereas it decreased over 52 min. The liquid-to-solid ratio exhibited a weaker effect

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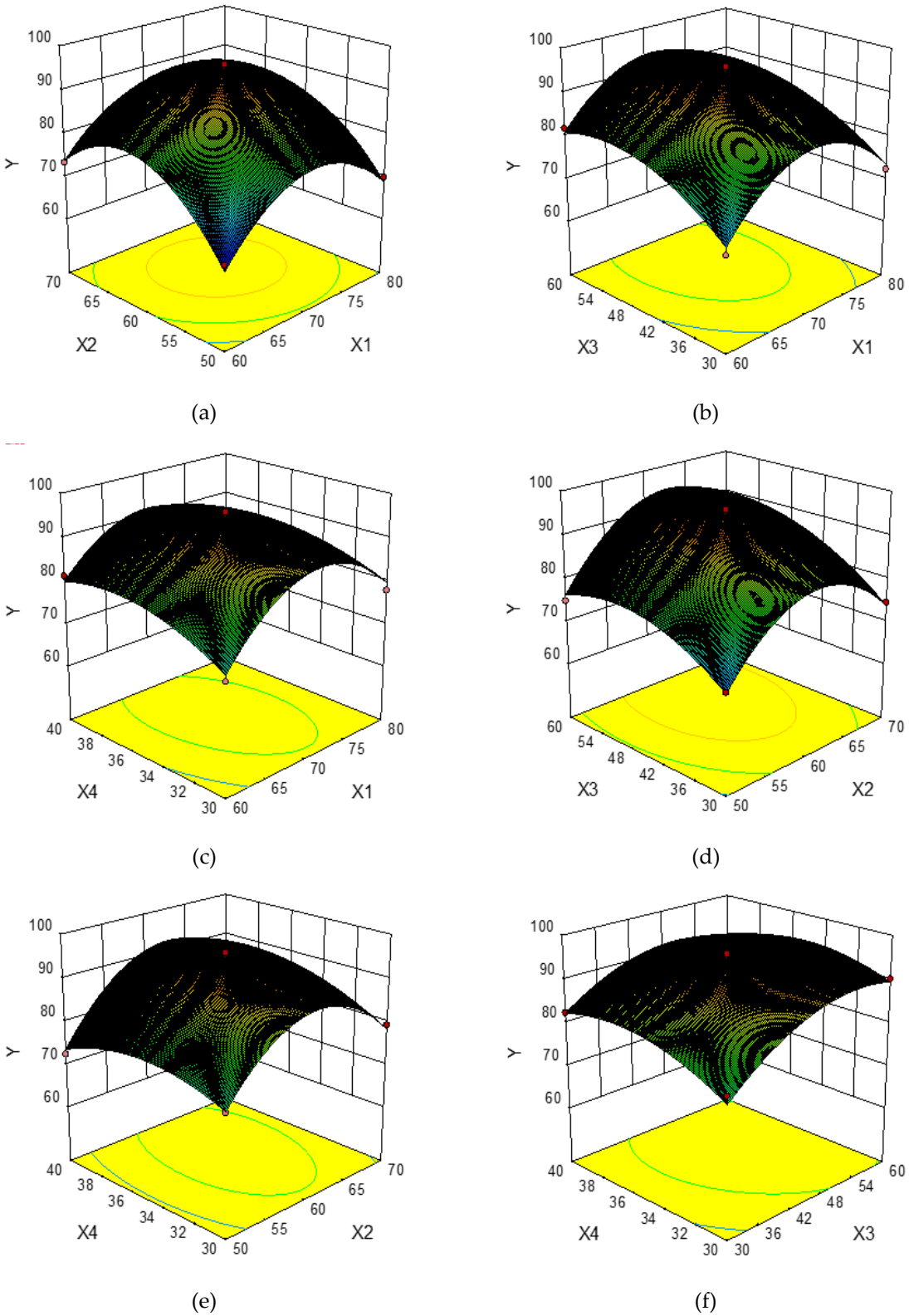
on the extraction yield of total phenolics, and the extraction yield of total phenolics increased slowly with the increase of liquid-to-solid ratio from 30 mL/g to 36 mL/g, and then declined.

Figure 2. Response surface plots showing the effects of variables on the extraction yield of total



phenolics: interaction of the ethanol concentration and extraction temperature (**a**), interaction of ethanol concentration and extraction time (**b**), interaction of ethanol concentration and liquid-to-solid

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ratio (**c**), interaction of extraction temperature and extraction time (**d**), interaction of extraction temperature and liquid-to-solid ratio (**e**), interaction of extraction time and liquid-to-solid ratio (**f**).

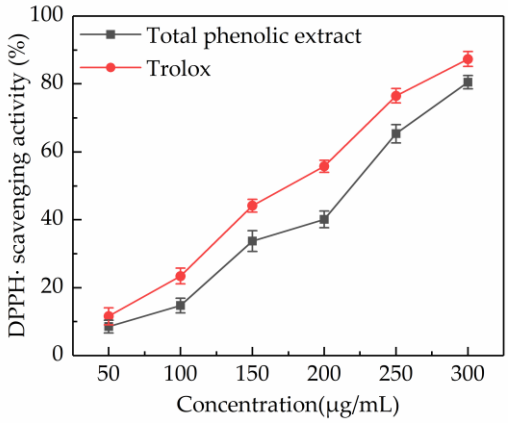
2.2.3. Verification of Predictive Model

The optimal extraction conditions (X_1 70.31%, X_2 61.94 °C, X_3 51.73 min and X_4 35.63 mL/g) were obtained from Figure 2. Under the optimal ultrasonic-assisted extraction conditions, a maximum response value (Y) of 96.12 mg GAE/g DM was yielded from the mathematical prediction of the model. Subsequently, to verify the accuracy and practicability of the model equation, verification experiments were performed. The extraction yield of total phenolics from CAB was 95.84 mg GAE/ g DM. This fact proved that the model equation was perfectly suitable for the optimization of ultrasonic-assisted extraction process.

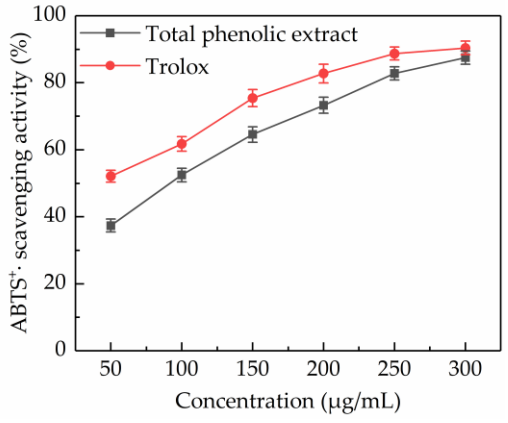
2.3. Evaluation of Free Radical Scavenging Activities

2.3.1. DPPH· Scavenging Activity

Scavenging of DPPH· is a common model used to study the free radical scavenging activity of the natural extracts. The DPPH· scavenging activity of a substance is quantitatively related to the number of electrons received by DPPH·. Figure 3a showed that the DPPH· scavenging activity of total phenolic extract from CAB increased in a concentration-dependent manner at concentrations ranging from 50 to 300 μg/mL, throughout slightly lower than trolox. At the concentration of 300 μg/mL, the DPPH· scavenging activity of total phenolic extracts from CAB and trolox were 80.54 ± 1.94% and 91.32 ± 2.21%, respectively. Besides, the IC_{50} values of total phenolics extracts from CAB and trolox were 197.007 and 157.469 μg/mL, respectively. These results showed that the total phenolic extracts from CAB under the optimal conditions had excellent DPPH· scavenging activity.



(a)



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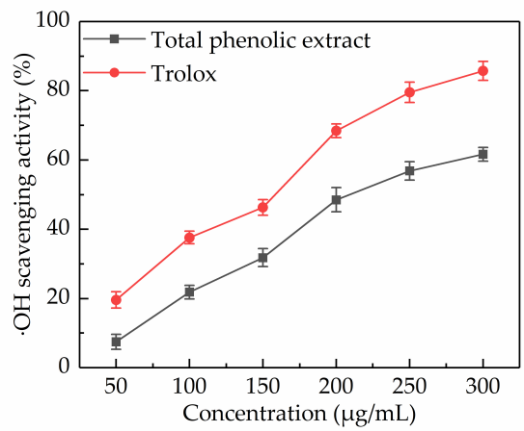

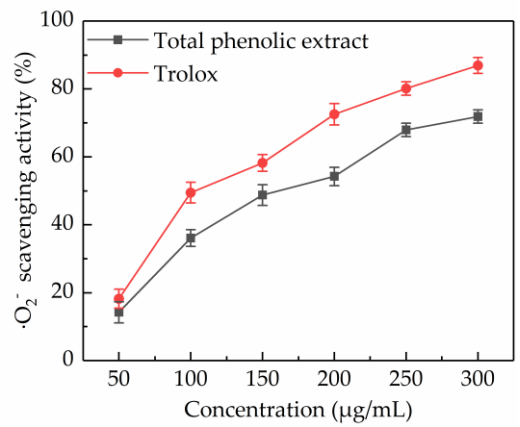

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(c) (d)

Figure 3. Free radical scavenging activities of total phenolic extract at the concentration of 50, 100, 150, 200, 250 and 300 μg/mL. DPPH· scavenging activity (**a**), $ABTS^{+}\cdot$ scavenging activity (**b**), ·OH scavenging activity (**c**) and $\cdot O_2^-$ scavenging activity (**d**).

2.3.2. $ABTS^{+}\cdot$ Scavenging Activity

$ABTS^{+}\cdot$ scavenging activity assay is another important measure to evaluate the free radical scavenging activity of the natural products. ABTS can be oxidized into green $ABTS^{+}\cdot$ under the action of oxidants, and the production of $ABTS^{+}\cdot$ will be inhibited in the presence of antioxidants. $ABTS^{+}\cdot$ scavenging activities of total phenolic extract from CAB and trolox were demonstrated in Figure 3b, it was found that their $ABTS^{+}\cdot$ scavenging activities increased with the increasing concentration. When the concentration was in the range of 50-300 μg/mL, $ABTS^{+}\cdot$ scavenging activity of total phenolic extract from CAB was slightly weaker than trolox. Additionally, the IC_{50} values were 83.878 and 53.746 μg/mL, respectively, for total phenolic extract from CAB and trolox. Hence, total phenolic extract from CAB was considered to have a high $ABTS^{+}\cdot$ scavenging activity.

2.3.3. ·OH Scavenging Activity

The ·OH has very strong oxidation ability due to its extremely strong ability to acquire electrons, which can be generated through Fenton reaction. Almost all biological molecules are exposed to oxidative damage because of the surplus ·OH. Figure 3c showed that the ·OH scavenging activity of total phenolic extract from CAB increased with an increase of concentration. A 300 μg/mL of total phenolic extract from CAB and trolox exhibited 61.65 ± 1.97% and 85.74 ± 2.72% scavenging activity respectively, and their IC_{50} values were 218.643 and 129.665 mg/mL, respectively. Those results indicated that total phenolic extract from CAB were good scavenger of ·OH.

2.3.4. $\cdot O_2^-$ Scavenging Activity

Although $\cdot O_2^-$ is a weak oxidant, it can cause damage to organism, because it was ubiquitous and can degrade to other kinds of reactive oxygen species. Hence, scavenging $\cdot O_2^-$ is also very necessary. As shown in Figure 3d, total phenolic extracts from CAB exhibited better $\cdot O_2^-$ scavenging activity than trolox at concentration from 50 to 300 μg/mL, and their $\cdot O_2^-$ scavenging activities were dose-dependent. At a concentration of 300 μg/mL, total phenolic extract from CAB and trolox respectively exhibited 74.84 ± 1.96% and 86.94 ± 2.37%. Besides, IC_{50} of total phenolic extract from CAB was 158.885 μg/mL, whereas the IC_{50} of trolox was 112.520 μg/mL. These results approved total phenolic extract from CAB can scavenge $\cdot O_2^-$ effectively.

2.4. Evaluation of anti-HMG-CoA reductase activity

The in vitro test of anti-HMG-CoA reductase activity of the total phenolic extract was carried out and the result was shown in Figure 4. It was demonstrated that the total phenolic extract significantly inhibited HMG-CoA reductase activity in a dose-dependent manner, and the inhibition drastically increased from 2.35% to 64.27 % when the concentration of total phenolics extracts was within 5-320 μg/mL, and the IC_{50} value was 117.165 μg/mL. Besides, the IC_{50} value measured for pravastatin was 68.54 nM, which was consistent with previous report[35]. These results suggested that the total phenolic extract from CAB might help in reducing the production of cholesterol.

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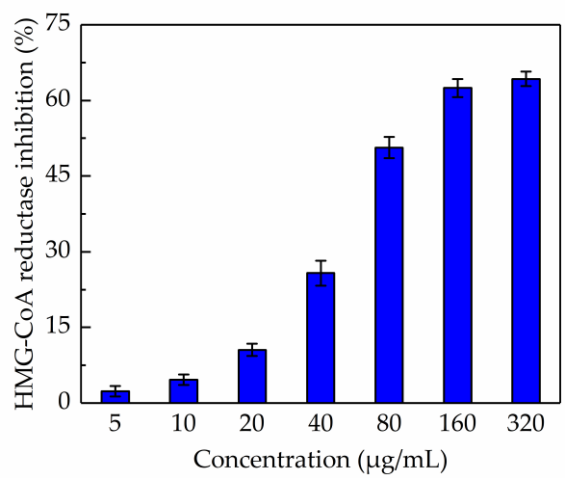

Figure 4. Inhibition of HMG-CoA reductase activity by total phenolic extract.

3. Materials and Methods

3.1. Chemicals and Reagents

Folin-Ciocalteu, trolox, gallic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS), nitroblue tetrazolium (NBT) chloride, N-methylphenazonium methyl sulfate (PMS), nicotinamide adenine dinucleotide (NADH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other analytical grade methanol, ethanol, H_2O_2, $FeSO_4$, $K_2S_2O_8$ and salicylic acid were purchased from Tianjin Kemio Chemical Co. (Tianjin, China).

3.2. Plant Material

CAB were collected in March 2018, from Jinhua city, Zhejiang province, China. A voucher specimen (DDY-20180301) was deposited at the Key Laboratory of Biotechnology and Bioresources Utilization, Ministry of Education, Dalian, China. And the raw material was dried, powdered and sifted with 80-mesh sieve, finally preserved in a desiccator.

3.3. Ultrasonic-Assisted Extraction of Total Phenolics by Single-Factor Experiments

Ultrasonic-assisted extraction of total phenolics from CAB was carried out with an ultrasonic apparatus (KQ-5200DE, Kunshan ultrasonic Co. Ltd., Suzhou, China), and ultrasound frequency and ultrasound power were set as 40 kHz and 100 W, respectively. Each 5 g of sample powder was placed into a volumetric flask (250 mL), then extracted with various ethanol concentrations (30, 40 50, 60, 70, 80%), temperatures (30, 40, 50, 60, 70, 80 °C), liquid-solid ratio (15, 20, 25, 30, 35, 40 mL/g) for various times (15, 30, 45, 60, 75, 90 min) respectively. The extracts were filtered, combined, after which extraction solvent was replenished to bring the final volume of the extract to 250 mL. The extraction yield of total phenolics from CAB for each extraction experiment was expressed as mg of gallic acid equivalent (GAE) on g of dry matter (DM).

3.4. Box-Behnken Design

The Box-Behnken is an efficient design for response surface methodology, which has been widely applied to optimize the extraction process of natural active substances. In this work, based on the single-factor experiments, a Box-Behnken design with four factors (ethanol concentration X_1, extraction temperature X_2, extraction time X_3 and liquid-solid ratio X4) and three levels (-1, 0, +1) including 29 experimental runs, was used to evaluate the combined effects on the extraction yield of

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total phenolics. A second-order polynomial model was used to describe the mathematical relationship between independent variables and response values.

$$Y = \alpha_0 + \sum_{i=1}^{4} \alpha_i X_i + \sum_{i=1}^{4} \alpha_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \alpha_{ij} X_i X_j \quad (2)$$

Where Y is the response variable, X_i and X_j are independent variables, α_0 is a constant coefficient, α_1, α_2 and α_3 represent the linear coefficient, interaction coefficient of two variables and quadratic coefficient of one variable, respectively.

Subsequently, validation experiment was performed to evaluate the feasibility and accuracy of response surface model developed.

3.5. Determination of Total Phenolic Content

The measurement of total phenolics content was conducted using Folin-Ciocalteu colorimetric method as described by Seifzadeh et al.[36]. Briefly, 0.25 mL of total phenolic extract solution was blended with 1.25 mL of the Folin-Ciocalteu reagent (diluted 10-folds by deionized water), then the mixture was neutralized by the addition of 1 mL of 7.5% Na_2CO_3. After one hour of incubation at 45 °C in the dark, the absorbance was recorded at 765 nm. Gallic acid was used as a standard. The total phenolics content in the extracts through the linear regression equation:

$$A = 0.1265\ C + 0.0228 \quad (n = 6,\ R^2 = 0.9997) \quad (3)$$

where A was the absorbance of sample, C was final total phenolics concentration.

3.6. Free Radical Scavenging Activity Assay

3.6.1. DPPH· Scavenging Activity

The DPPH· scavenging activity assay was operated as the procedure described by Shen et al. [37] with slight modifications. Briefly, 3.2 mL of freshly prepared DPPH solution (0.2 mM in ethanol) and 0.4 mL of phenolics extracts in ethanol at different concentrations (50-300 μg/mL) were mixed and shaken well in the dark at room temperature. After 60 min, the absorbance was measured at 517 nm using a spectrophotometer (UV-2600, Shimadzu, Japan). And the DPPH· scavenging activity of trolox was also analyzed for positive control. The percentage of DPPH· scavenging was calculated with the following equation:

$$\text{DPPH}\cdot \text{ scavenging activity } (\%) = (1 - \frac{A_{sample}}{A_{blank}}) \times 100 \quad (4)$$

Wher A_{sample} was the absorbance of the total phenolic extract, and the A_{balnk} was the absorbance of the sample without phenolics extract.

3.6.2. $ABTS^{+}\cdot$ Scavenging Activity Assay

The $ABTS^{+}\cdot$ scavenging activity assay was conducted as the method described by previous study with slight modifications[38]. Briefly, a redox reaction was triggered by blending the ABTS solution (7.0 mM) and $K_2S_2O_8$ solution (2.45 mM) with a ratio of 1:1. After 16 hours, the reaction solution was adjusted with ethanol to yield an absorbance between 0.68 and 0.72 at 734 nm. Then, 125 μL of each sample (50, 100, 150, 200, 250 and 300 μg/mL) was mixed with 200 μL of $ABTS^{+}\cdot$ solution. After one minute, and the absorbance was recorded at 734 nm. Trolox was used as a standard. The percentage of $ABTS^{+}\cdot$ scavenging was calculated using the following equation:

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$$\text{ABTS}^{+}\cdot \text{ scavenging activity } (\%) = (1 - \frac{A_{sample}}{A_{blank}}) \times 100 \quad (5)$$

Where A_{sample} was the absorbance of the total phenolic extract, and the A_{balnk} was the absorbance of blank (without total phenolic extract).

3.6.3. ·OH Scavenging Activity Assay

The ·OH scavenging activity of total phenolic extract was measured according to the method reported previously with some modifications[39]. The ·OH was obtained through a Fenton reaction. Explicitly, the reaction system consisted of 1.0 mL $FeSO_4$ (9.0 mM), 1.0 mL H_2O_2 (9.0 mM), and 1.0 mL of total phenolic extract at different concentrations (50, 100, 150, 200, 250 and 300 mg/mL), then 1.0 mL of salicylic acid (3.0 mM) was added to initiate the reaction. The reaction mixture was incubated at 37 °C for one hour, and the absorbance was measured at 510 nm. Trolox was used as a positive control. The ·OH scavenging activity was measured using the following equation:

$$\cdot\text{OH scavenging activity } (\%) = (1 - \frac{A_1 - A_2}{A_0}) \times 100 \quad (6)$$

Where, A_0, A_1 and A_2 are the absorbance of the blank control (without total phenolic extract), the absorbance of total phenolic extract and the absorbance without H_2O_2, respectively.

3.6.4. $\cdot O_2^-$ Scavenging Activity Assay

The $\cdot O_2^-$ scavenging activity of total phenolic extract was measured based on the modified method described previously[40]. Briefly, 3 mL of Tris-HCl buffer solution (16 mM, pH 8.0) contained 1 mL of NBT (50 μM), 1 mL of NADH (78 μM), and 1 mL of total phenolic extract at different concentrations (50, 0.100, 150, 200, 250 and 300 μg/mL). Then 1.0 mL of PMS (10 μM) was added into the system to initiate the reaction. The mixture was maintained at 25 °C for five minutes, followed by measurement of the absorbance at 560 nm. Trolox was used as a comparision. The $\cdot O_2^-$ scavenging activity was measured using the following equation:

$$\cdot O_2^- \text{ scavenging activity } (\%) = (1 - \frac{A_b}{A_s}) \times 100 \quad (7)$$

Where A_s and A_b are the absorbance of the total phenolic extract and the absorbance of blank control (without total phenolic extract), respectively.

3.7. Anti-HMG-CoA Reductase Activity Assay

HMG-CoA reductase inhibitory activity of the total phenolic extract from CAB was evaluated with the commercially available HMG-CoA reductase assay kit purchased from Sigma-Aldrich (St. Louis, MO, USA). The specific operation method was in accordance with the instruction: each well of 96-well plate contained 1 μL of different concentrations of total phenolic extract, 4 μL of NADPH (final concentration of 400 μM), 12 μL of HMG-CoA substrate (final concentration of 300 μg/mL), followed by the addition of phosphate buffer (pH 7.4) to achieve the final volume of 200 μL. Then, 2 μL of the HMG-CoA reductase was added into each well to activate the reaction system. Immediately, the 96-well plate was shaken mechanically in a microplate reader (BioTek, Synergy H1, Winooski, VT, USA) for 10 s. The consumption rate of NADPH was measured once 20 s for up to 600 s by recording the absorbance of reaction system at 340 nm. Pravastatin was used as positive control. The HMG-CoA reductase inhibition (%) was calculated using the following formula:

$$\text{HMG-CoA reductase inhibition } (\%) = \frac{\Delta A_{\text{control}} - \Delta A_{\text{test}}}{\Delta A_{\text{control}}} \times 100 \quad (8)$$

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Where A_{test} and $A_{control}$ are the absorbance of the total phenolic extract and the absorbance of control (without total phenolic extract), respectively

3.8. Statistical Methods

The data were reported as the mean ± standard deviation (SD) from three parallel experiments. The half maximal inhibitory concentration (IC_{50}) was calculated using linear regression method by the SPSS 24.0 software (SPSS Inc., USA). Design-Expert 10 (Stat-Ease Inc., Minneapolis, USA) was used for designing the experiments and statistical analysis.

4. Conclusions

In the present work, the optimum ultrasonic-assisted extraction technology to obtain total phenolics from CAB was successfully developed through single-factor experiments coupled with Box-Behnken design. The optimum parameters including ethanol concentration, ultrasonic temperature, ultrasonic time, liquid-to-solid ratio were 70.31%), 651.94 °C, 51.73 min and 35.63 mL/g, respectively. Under these conditions, the extraction yield of total phenolics from CAB was 95.84 mg GAE/g DM, which was closed with the theoretical value (96.12 mg GAE/g DM). In addition, the study on free radical scavenging activities indicated that total phenolic extract obtained under the optimum condition had excellent scavenging effects on DPPH·, $ABTS^{+}$·, ·OH and $\cdot O_2^-$, with the corresponding IC_{50} values of 197.007, 83.878, 218.643 and 158.885 μg/mL, and total phenolic extract also exhibited good potential to inhibit HMG-CoA reductase in vitro, with the IC_{50} value of .117.165 μg/mL. Therefore, these results were conducive to optimum utilization of CAB as a great source of total phenolics with good free radical scavenging, anti-HMG-CoA reductase activities.

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