Optimization of polysaccharide process from *Fructus corni* with box-behnken design and antioxidant capacity

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Abstract: In the present study, the ultrasound-assisted extraction (UAE) process of polysaccharide from *Fructus corni* (FCP) was opitimized using the Box-Behnken design (BBD). The main parameters including ultrasound time (min), temperature (^{o}C) and solvent to raw material ratio (mL/g) were chosen as the process variables for the optimization of UAE process. The results of analysis of variance indicated that the equation obtained from the experiments could represent the data and the predicted responses satisfactorily. The optimum conditons obtained by BBD were ultrasonic time (51min), temperature (69^{o}C) and solvent to solid ratio (20mL/g) with actual yield (12.68±0.16%), which was good agreement with value predicted by the model. The antioxidant properties of FCP were assessed *in vitro* based on scavenging effect of the DPPH radical, hydroxyl radical and super oxide radical tests. The FCP possessed strong antioxidant abilities on DPPH and super oxide radical at the high concentration. The results on hydroxyl radical demonstrated that FCP exhibited high scavenging effect when the concentration was over 3000μg/mL. The findings suggested that the FCP had antioxidant capacities and could be developed as a source of natural antioxidants and functional food material.

Keywords: *Fructus corni*, polysaccharide process, box-behnken design, antioxidant capacity.

INTRODUCTION

Fructus corni (FC), which is derived from the dried fruit of *Cornus officinalis* Sieb. Et Zucc., is a commonly used Chinese herbal. It is used as both medicine and food. In China the material has long been employed to keep kidney essence, invigorate the liver and kidney, reduce urination, and check perspiration and hemorrhage (China Pharmacopoeia Committee, 2015). In previous studies, the phytochemicals in FC have been extensively investigated (Kroes *et al*., 1992; Ding, *et al*., 2008; Cao *et al*., 2012; Jiang *et al*., 2016). The polysaccharide from FC (FCP) has been found to have plenty of pharmacological and physiological properties, as reviewed in our previous published paper (Wu *et al*., 2013).

At present, various methods have been used for the extraction of FCP, including hot water extraction (Li *et al*., 2003; Zhang *et al*., 2007), UAE (Wang *et al*., 2016), microwave-assisted extraction (Cheong *et al* 2016; Hu *et al*., 2011) and enzymatic extraction (Chang 2011; Cheng *et al*., 2010). Among these extraction methods, UAE as is an economical and highly efficient procedure and has been reported in many studies (Ying *et al*., 2014; Chen *et al*., 2015; Dong *et al*., 2016). The mechanism of ultrasonic treatment is attributed to the micro fractures and disruption of cell wall, which causes the more release of target ingredients and improves the mass transfer (Vinatoru 2001; Tsochatzidis *et al*., 2001). Further, there is no chemical involvement in UAE, which will minimize possible structural changes and degradation of active components (Wang *et al*., 2006).

Box-Behnken design (BBD) is an effective statistical method used to determine the optimal levels of two or more treatment variables. The main virtue of this technique is that it can reduce number of test trials required to estimate multiple variables and their interactions, which needs less laborious and time-consuming than other techniques to optimize a process. It is extensively carried out to optimize the polysaccharides extraction process variables from various botanical materials (Maran *et al*., 2015; Raza *et al*., 2017; Zhang *et al*., 2017). However, according to our knowledge and literature survey, there are no studies available on the use of BBD on UAE process optimisation for the FCP. In this study, UAE for the polysaccharides extract from FC was investigated and the extraction process variables were optimized by BBD. Moreover, we assessed the antioxidant effect of FCP with several established *in vitro* systems. The results obtained from the present study will be useful to further utilize and develop the material.

MATERIALS AND METHODS

1,1-diphenyl-2-picrylhydrazyl (DPPH) and D-Glucose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The other chemical reagents used in the present study were of analytical grade and obtained from Nanjing Chemical Co. (Jiangsu Province, China).

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FC was collected from the Funiu Mountains, Henan Province, China and identified by the Prof. Yanfang Wu from Xinxiang Medical University. The sample was first dried at 60^{o}C in an oven and then smashed to make it pass through a 40-mesh sieve. The powder was sealed and stored at 4^{o}C before analysis.

Ultrasound-assisted extraction process

The powdered FC was defatted using petroleum ether (60-90^{o}C) in a Soxhlet extractor for 5hours and treated by 80% ethanol twice, which can remove coloured materials, monosaccharides, oligosaccharides, and other small molecular compounds. The organic solvent was distilled off and the defatted dry sample was collected, as reported previously (Wang *et al*., 2015). The defatted FC sample was extracted using distilled water based on the design of experiments (table 2). The ultrasonic process was carried out in a temperature controlled ultrasonic cleaner (KH300SP, 25kHz, 300W, Kunshan Ultrasonic Instrument Co. Jiangsu, China). Furthermore, the temperature was also measured using a thermometer. After ultrasonic extraction, the sample was centrifuged at 1,650g for 15min and the supernatant was collected. And then it was concentrated to one-fifth of starting volume and the condensed solution was prepared a final mixture of FCP in 80% (v/v) by adding anhydrous ethanol. The mixture sample was stored overnight at 4^{o}C. After centrifugation at 4,000rpm for 20min, the precipitate was collected and washed using absolute ethanol and dried by vacuum to obtain the FCP.

Quantity analysis of polysaccharides

The quantity of polysaccharide was measured using the minor modified sulphuric-acid phenol approach reported by Yan *et al*. (2011). Briefly, 1.0mL FCP solution was added 1.0mL distilled phenol (5%) solution and the solution was mixed with a rotor. And then 5mL concentrated sulphuric acid was added. Finally, the mixed was kept for 10min and incubated for 15min in a boiling water bath. The mixed solution was cooled to room temperature before the absorbance was measured against blank at 490nm. The FCP yield (%) was expressed as D-glucose equivalent and analyzed as follows:

$$Yield(\%) = \frac{C \times \alpha \times v}{M \times 1000} \times 100\%$$

where C (mg/mL) is the concentration of D-glucose and obtained using the standard curve; α and v are the dilution ratio and total volume of extraction solution (mL), respectively. And M represents the dried weight of PCR (g).

Box-Behnken design

The optimization of the UAE for FC was performed with the BBD and the BBD was mainly employed form of response surface methodology at present. According to the preliminary test, a three-parameter, three-level was implemented in the present investigation. Ultrasound time (min, X_1), temperature (^{o}C, X_2) and ratio of solvent to solid (mL/g, X_3) were the independent parameters selected to be optimized for the UAE process of FCP. It is generally thought that high ultrasonic power can improve the release of target compounds (Wang *et al*., 2013). Therefore, the optimal ultrasound variable is the highest power of 300W and was not further investigated. The table 1 represents the coded and uncoded (actual) levels of the independent factors. The variables were coded using the formula 1:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (1)$$

where x_i is the independent factor coded value, X_i and X_0 is the actual value and is the independent parameter actual value on the center point, respectively. The ΔX_i is the step change value. The complete design performed in random order is given in table 2. It can be observed from table 2 that there are 17 combinations including five replicates at the center point. The results obtained from BBD was analyzed by multiple regressions to fit the following quadratic polynomial model.

$$Y = \gamma_0 + \sum_{i=1}^{3} \alpha_i X_i + \sum_{i=1}^{3} \alpha_{ii} X_i^2 + \sum_{i \neq j=1}^{3} \alpha_{ij} X_i X_j \quad (2)$$

where Y is the predicted value, γ_0 is a constant, α_i, α_{ii} and α_{ij} are the linear, quadratic and interactive coefficients of the model, respectively. And accordingly X_i and X_j denote the levels of the independent parameters, respectively. The Design-Expert 8.0 (Trial Version, State-Ease Inc., Minneapolis, MN, USA) software was used to invetigate the regression analyses, statistical significance and response surfaces. P-values of less than 0.05 represents statistically significant.

Antioxidant activity

Scavenging capacity on DPPH radical

The DPPH radical, which is a stable and commonly accepted technique, is used to estimate the free radical scavenging capacity of different materials *in vitro* (Zhang *et al*., 2015, Hafsa *et al*., 2016). The antioxidant ability of FCP was studied by evaluating the capacity of scavenging DPPH radical based on the procedure published by Wang *et al*. (2012), with some modifications. Briefly, the stock solution of FCP was first prepared with water and then working solutions of different concentrations were obtained by diluting stock solution in water. Finally, a 2 mL aliquot of sample and 1mL of 0.1mmol/L ethanolic solution of DPPH were mixed. The absorbance at 517nm was reported against a blank after the mixed solution was left to keep at 30^{o}C for 30min. Ascorbic acid was used as positive reference. The DPPH free radical scavenging capability was calculated in following formula:

DPPH radical scavenging capability

$$(\%) = \frac{[A_0 - (A_i - A_c)]}{A_0} \times 100 \quad (3)$$

Here, A_0, A_i and A_c are the absorbance value of pure DPPH solution, sample and DPPH mixture solution, and pure sample solution, respectively.

Scavenging capacity on hydroxyl radical

The scavenging capacity of FCP on the hydroxyl radical was measured using the approach reported by Smirnoff (Smirnoff, *et al*., 1989) with some minor changes. Briefly, 1mL of FCP solution was added using 1mL of 9mmol/L ferrous sulfate and 1ml of 9mmol/L 95% ethanolic salicylic acid solution. And then 1mL of 8.8mmol/L H_2O_2 was added the mixed solution and generated the reaction. The mixed solution was left to keep for 30min at 37°C. The absorbance value was measured at 510nm against a reagent blank. Ascorbic acid was employed as a standard for comparison. The scavenging capability on hydroxyl free radical was calculated using the following formula:

Hydroxyl radical scavenging capability

$$(\%) = \frac{[A_0 - (A_i - A_c)]}{A_0} \times 100 \quad (3)$$

where A_i, A_0 and A_c represent the absorbance of mixture, mixture without H_2O_2 and mixture without sample, respectively.

Super oxide radical scavenging capacity

The scavenging capacity on super oxide radical of FCP was assessed according to the reference (Marklund, *et al*., 1974). Briefly, 1mL of FCP solution, 2ml of 50mmol/L Tris-HCl buffer (pH8.2) and 0.85mL of water were mixed and then 0.15mL of 3mmol/L pyrogallol solution was added to initiate the reaction. The standard control was the ascorbic acid. The absorbance of the mixed solution at 325nm was recorded within 5min of preparation and the scavenging capacity on super oxide radical was evaluated using following formula:

Super oxide radical scavenging capability

$$(\%) = \frac{A_0 - A_i}{A_0} \times 100 \quad (5)$$

where A_0 and A_i are the absorbance values without sample and with sample, respectively.

RESULTS

The actual and predicted yields of polysaccharides

The yields of FCP (*Y*) from all the experiments and the predicted values were presented in table 2.

Analysis of variance for the fitted model

Multiple linear regressions were carried out on the results of table 2 with the quadratic polynomial model (Eq.(2)). Table 3 shows ANOVA for the fitting model.

Response surface and contour plots

In order to help the visualization of the statistically significant parameters obtained from the statistical analysis, the contour plots and response surface for FCP extraction yield as functions of extraction parameters are given in fig. 1a-c. As seen from plots, they demonstrate influences of two variables on the response at a time and the other one parameter is kept constant at level zero.

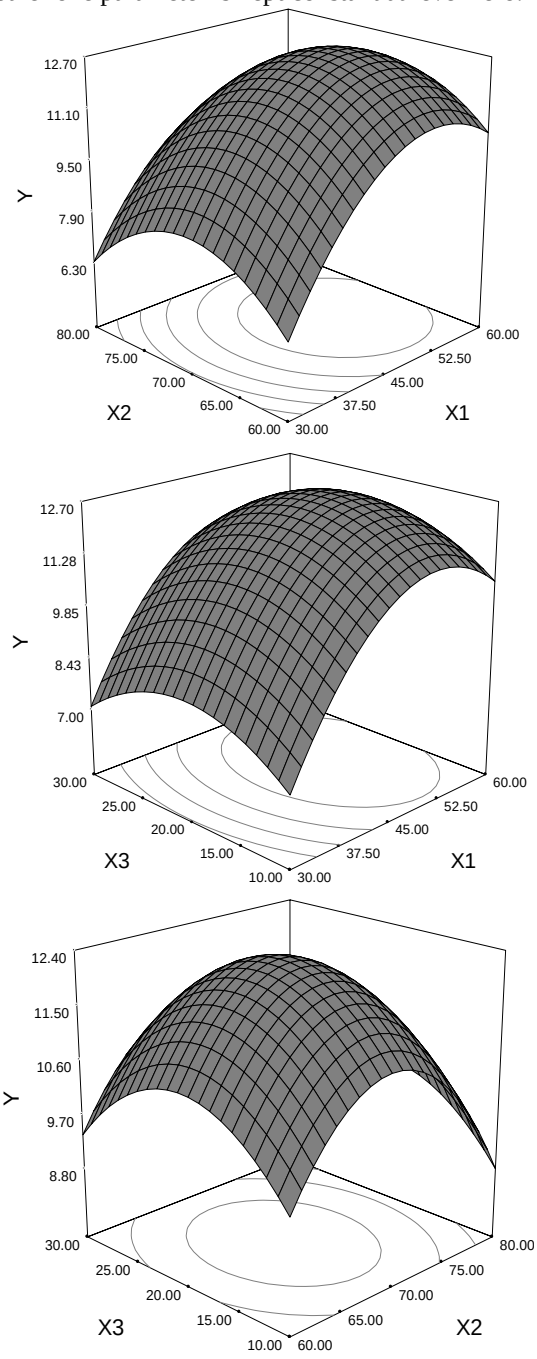

Fig. 1a-c: Response surface (3-D) and contour plots showing the effect of the extraction time, extraction temperature and ratio of water to raw material on the response *Y*

Predicted and experimental values at optimal conditions

In order to confirm the suitability of the model equation, a certification test was performed using the optimal

parameters. Table 4 presents the optimum conditions based on experimental data analysis. The experimental value was 12.68±0.16% for the yield of FCP. It is obvious that the results were in good agreement with value predicted by the model. The results of our experiments verify that the response model sufficiently reflected the predicted optimization.

In vitro antioxidant effect of FCP

In vitro antioxidant abilities of FCP were determined using DPPH radical, hydroxyl radical and super oxide radical tests. The results were depicted figs. 2, 3 and 4.

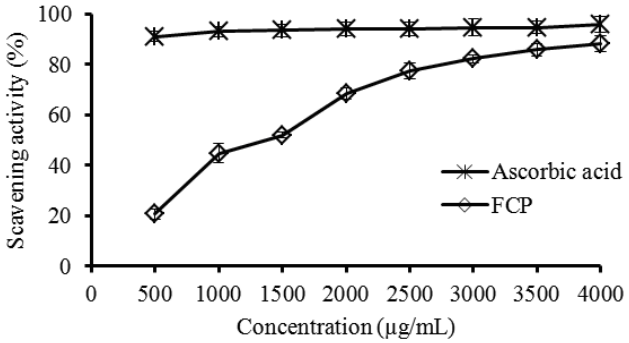

Fig. 2: Scavenging effect of FCP on DPPH radical.

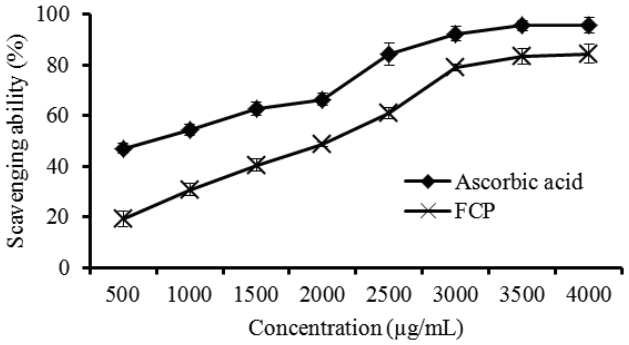

Fig. 3: Scavenging effect of FCP on hydroxyl radical

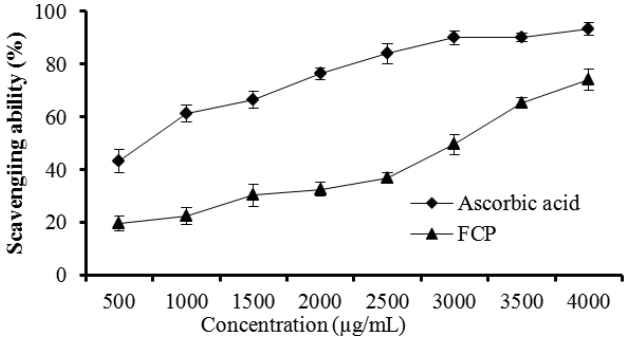

Fig. 4: Scavenging effect of FCP on superoxide radical

DISCUSSION

Fitting the response surface models

In general, the selected model, which has no significant lack-of-fit, indicated it is proper for the description of the response surface. As can be seen from table 3, the lack-of-fit is insignificant for the model at the 95% confidence level. It is obvious that the model can fit the data satisfactorily. The R^2, adj-$R^2$and coefficient of variation (CV) were used to evaluate the model adequacy (Karazhiyan *et al*., 2011). The R^2, adj-R^2 and CV were 0.993, 0.983 and 2.84, respectively. This showed that there were a high degree of precision of the experimental value and a good relationship between the experimental and predicted values. It is generally believed that the R^2 value exceeds 0.9 the regression model is thought to have a high relationship (Haaland, 1989). Furthermore, the 0.983 of adj-R^2 was also high enough to demonstrate the significance to this model. On the other hand, a high CV shows that variation in the mean value is high, which does not satisfactorily develop an adequate response model (Chandrika and Fereidoon, 2005). In this case the small value of CV (2.84) gave well reproducibility.

Analysis of response surface

Based on independent parameters ultrasound time and temperature, the Design-Expert software creates the three-dimensional (3-D) plot and the contour plot (fig. 1a). The results indicate that FCP yield would be a maximum when the ultrasound time and temperature were 50.90 min and 68.97^{o}C, respectively. As seen from fig. 1a, when ultrasound time is from 30.00 to 50.90 min the FCP yield always increases and then decreases with further ultrasound time. This phenomenon can be explained in terms of ultrasound degradation. It is reported that ultrasound can induce acoustic cavitation and breaking of cells (Mason *et al*. 1996). The breaking of the bubbles caused by cavitation would enhance the penetration of the extraction liquid into the plant cells to extract target compounds there. However, the plant cells would be completely disrupted by the mechanical effect and target compounds would no longer be dissolved with increasing ultrasound time. As the plant cells ruptured, varieties of ingredients including insoluble substances and cytosol suspended in the solvent, which causes the lower permeation of the extraction liquid (Zhao *et al*., 2007).

Objective constituents would also re-adsorb to the shattered plant particles because of their relatively big surface areas lowering yield of recovered ingredients (Dong *et al*., 2010). The temperature is a significant factor for FCP extraction. However, the extraction yield of FCP with temperature using UAE is affected by the combination of the cavitation effect and thermal effect according to Sun *et al*. (2011). With the temperature range from 60 to 68.97^{o}C, the yield of FCP increased. This was because that the increasing temperature accelerated the softening and swelling of plant sample, which resulted in solubility and diffusivity of target compounds (Braga *et al*., 2006). On the other hand, increasing temperature could generate low cavitation intensity (Entezari *et al*., 1996). Hence, the 68.97^{o}C was chosen as the optimal temperature in considering the combination of the thermal and cavitation effects. For ultrasound time and ratio of

Table 1: Variables and their levels for Box-Behnken design.

Symbols	Independent variables	Factor level		
		-1	0	+1
X_1	Extraction time (min)	30	45	60
X_2	Extraction temperature (^oC)	60	70	80
X_3	Ratio of solvent to raw material (mL/g)	10	20	30

Table 2: Box-Behnken design (coded) and results for extraction yield of FCP and the predicted.

Run	X_1	X_2	X_3	*Y* (%)	*Predicted value* (%)
1	0	+1	-1	9.06	8.80
2	-1	+1	0	6.33	6.35
3	+1	-1	0	10.43	10.42
4	0	0	0	12.32	12.30
5	0	-1	-1	9.68	9.48
6	0	0	0	12.16	12.30
7	+1	0	+1	10.72	10.48
8	-1	-1	0	6.58	6.54
9	-1	0	+1	7.31	7.09
10	0	0	0	12.13	12.30
11	0	-1	+1	9.12	9.38
12	+1	0	-1	10.36	10.58
13	-1	0	-1	6.82	7.07
14	0	0	0	12.51	12.30
15	+1	+1	0	9.33	9.37
16	0	+1	+1	8.61	8.82
17	0	0	0	12.38	12.30

Table 3: Analysis of variance for the fitted model.

Source	Coefficient	Sum of square	Degree of freedom	Mean square	*F*-value	*p*-value
Residual		0.54	7	0.077		
Lack of fit		0.44	3	0.15	5.90	0.0597
Pure error		0.099	4	0.025		
Total		73.02	16			
R^2	0.993					
Adj-R^2	0.983					
CV	2.84					

liquid to solid, the 3-D plot and the contour plot were presented in fig. From fig. 1b, it can be seen that the FCP yield had a maximum value when the ultrasound time and solvent-solid ratio were 50.90 min and 19.85mL/g, respectively. The 3-D plot and the contour plot of the interaction of ultrasound temperature and solvent to solid ratio are given in fig. 1c. It is apparent that the yield of FCP reached maximum when the ultrasound temperature was 68.97^{o}C and solvent-solid ratio was 19.85mL/g. Analysis of the 3D plots and their respective contour plots, indicates that the predicted optimal levels of the investigated parameters for providing a maximum yield of FCP are in following: ultrasound time 50.90 min, temperature, 68.97^{o}C and solvent-material ratio, 19.85mL/g. The optimal variables were rounded as follows: ultrasound time 51min, temperature, 69^{o}C and solvent- material ratio, 20mL/g, taking into account practical production.

Effect of scavenging DPPH radical

DPPH, which has electron-donating ability, is usually used as a substrate to assess capacity of antioxidants (Chen, *et al*., 2008). In the work, the antioxidants reduced the stable DPPH radical to the yellow-coloured diphenylpicrylhydrazine (Blois, 1958). Fig. 2 indicates that inhibiting abilities of FCP increases in a concentration dependent manner. As can be seen, at the high dose (4mg/mL) FCP indicated high DPPH radical

Table 4: Predicted and experimental values of the responses obtained at optimal conditions.

Process variables			Extraction yield of FCP (%)	
Extraction time (min)	Extraction temperature (^oC)	Ratio of solvent to material (mL/g)	Experimental value	Predicted value
51	69	20	12.68±0.16*	12.66

scavenging ability. Vitamin C has very substantial scavenging activity on the DPPH radical from 0.5mg/mL to 4.0mg/mL. It is noticeable that PCF has high antioxidant ability only at the higher doses.

Effect of scavenging hydroxyl radical

It is widely believed that the hydroxyl radical in excess is harmful to human health, this is because the hydroxyl radical can damage various biomolecules such as carbohydrates, amino acids, lipids and nucleic acids in cells. Thus, it is very significant for scavenging hydroxyl radicals to protect living systems. In this study, the Fenton reaction and salicylate were mixed and reacted to generate a colored material (2,3-dihydroxybenzoate). Using this color change of the reaction system, the hydroxyl radical ability could be evaluated (Teismann *et al*., 2000). The fig. 3 represents the results of the hydroxyl radical scavenging activity of FCP and those of vitamin. As is illustrated, both substances showed noticeable scavenging capacity in a concentration dependent manner. The FCP was observed that the hydroxyl radicals possessed scavenging ability at concentrations between 0.5 and 4.0 mg/mL. However, this activity was lower than that of vitamin C. Free radical scavenging ability increased according to the concentration in the range of 0.5-3.0mg/mL. But when it was over the concentration of 3.0 mg/mL, the increase of scavenging ability was unconspicuous and FCP and vitamin C exhibited high scavenging effect on hydroxyl radical. Hence, FCP could be used to antioxidant to scavenge hydroxyl free radical.

Effect of scavenging super oxide radical

In generally, the super oxide anion is thought as an original free radical of all reactive species generated *in vivo* and can generate other cell-damaging reactive oxygen species such as hydrogen peroxide, hydroxyl radical, or singlet oxygen in living systems (Lee *et al*., 2004). The total super oxide radical scavenging effects of both FCP and vitamin C at varying concentrations were measured and the findings are illustrated in fig. 4. As seen from the fig. 4, the samples exhibited evidently scavenging ability in a concentration dependent manner. The ability to scavenge super oxide radical was found to be low compared to the same concentration of vitamin C. However, at the highest dose (4.0mg/mL), the super oxide radical scavenging ability of FCP achieved 74.1 %, which was close to that of vitamin C at 2.0mg/mL.

CONCLUSION

UAE was employed to extract polysaccharides from fructus corni and BBD was successfully applied for optimization process. The optimum conditions were ultrasonic time (51min), temperature (69^oC) and solvent to solid ratio (20mL/g). Under these parameters, the actual yield of FCP was 12.68±0.16%. The results of antioxidant assays showed that FCP possessed high DPPH, hydroxyl and super oxide radical scavenging capacities at the high concentration.

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