INTRODUCTION

Reactive oxygen species (ROS), such as superoxide, oxygen ion, oxygen free radical, and others are produced continuously during oxidative metabolism (Fakhfakh et al., 2017). Excessive accumulation of ROS stemming from living organisms can result in oxidative-stress, which is regarded as the major pathogenesis of various diseases like immune injury, rheumatic arthritis and atherosclerosis (Halliwell et al., 1984; Mau et al., 2002). In view of this, more and more antioxidants are synthesized to scavenge free radicals and then reduce oxidative damage of the host. However, growing evidence has demonstrated that synthetic antioxidants can lead to liver damage and carcinogenesis (Yuan et al., 2008). Therefore, there is a great interest in efficient and natural antioxidant compounds which could protect the organism from free radicals and retard the onset of many chronic diseases (Luo, 2008).

Pomelo (Citrus grandis Osbeck), one of the most common citrus fruits in Rutaceae family native to China and parts of Southeast Asia, has gained massive popularity among consumers at home and abroad because of its unique flavor and potential nutritive value (Ademosun et al., 2017). The pomelo peels accounting for approximately 30% of gross weight of the fruit possess high medicinal value and health function, which are recorded in the 2010 version of the "Chinese Pharmacopoeia" (Yu et al., 2016; Methacanon et al., 2014). In recent years, the researches about pomelo peels were mainly focused on the extractive techniques of essential oil, pectin, naringin and phenolic compounds (Chen et al., 2016; Guo et al., 2017; Liu et al., 2017; Safdar et al., 2016) and their antibacterial, antidiabetic and antioxidant activities (Bocco et al., 1998; Oboh and Ademosun, 2011; Viuda-Martos et al., 2008).

Polysaccharides are polymeric carbohydrate molecules commonly existing in animals, plants, microorganisms and algae. Studies have confirmed that polysaccharides exhibit various bioactivities, including antioxidant, antitumor (Zhu et al., 2011; Zhang et al., 2017) and immunoregulatory activities (Zhao et al., 2005) via raising antioxidant enzymes activities or scavenging free radicals in the host organism (Chen R Z et al., 2015). In particular, plant polysaccharides from diverse sources have received great concerns during the past decades because of their biological activities without any damage to the host, which might be attributed to their good water solubility and leachability (Yang et al., 2008). Recently, more attentions have been paid to the extraction and
optimization of natural plant polysaccharides for higher yield and stronger bioactivities, such as sequential extraction (Xu et al., 2016; Shi et al., 2016), cellulase-assistant extraction (Yang et al., 2013) and ultrasound-microwave synergistic extraction (Liu et al., 2017). Modern researches have found that polysaccharides are one of the prominent active components in pomelo peels. However, the extraction process and *in vitro* antioxidant activities of water-soluble polysaccharides from Pomelo peels have not been reported systematically yet.

The aim of this study was to optimize the extraction conditions for maximum yield of pomelo peels polysaccharides (PPs) using the Box-Behnken design (BBD) of response surface methodology (RSM), and evaluate their *in vitro* antioxidant activity. These results will be valuable for further development of PPs as supplement or natural antioxidants.

**MATERIALS AND METHODS**

**Materials and Reagents**

The pomelos cultivated in Fujian province were purchased from a local market in Tianjin and peeled collected manually. Ascorbic acid (Vc), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and trifluoroacetic acid were obtained from Sigma-Aldrich Corp. (USA). All other chemicals and agents were of analytical grades.

**Extraction Procedure**

The fresh pomelo peels were dried to constant weight, crushed to coarse powder, and screened through 80 mesh sieves to obtain the homogeneous powder sample. Each pretreated sample (5.0 g) was extracted with distilled water under the designated liquid-solid ratio, extraction temperature, extraction time and frequency. After extraction, the supernatant was concentrated using a vacuum rotary evaporator at 60 ºC and then precipitated by the addition of 4 volumes of ethanol overnight at 4 ºC. The crude samples were centrifuged at 4500 rpm for 15 min, washed by anhydrous ethanol and acetone alternately 3 times and freeze-dried. Afterwards, the polysaccharide samples were re-dissolved in deionized water, the pectin was eliminated by pectinase and the protein was removed according to Sevag method (Alam and Gupta, 1986), and finally the mixture was concentrated, dialyzed (Mw 3500), fractionated by a freeze–thawing procedure, and lyophilized to obtain PPs for further study. The yield (%) of PPs was calculated as follows:

\[
\text{PPs yield} \% = \frac{\text{weight of PPs (g)} \times 100}{\text{weight of pretreated pomelo peels powder (g)}}
\]

**Single-factor Experiment Design**

The effects of liquid-solid ratio, extraction time, temperature and frequency on the yield of PPs were performed using single-factor experimental design, where one factor was changed while others were kept constant. Each experiment was carried out in triplicate.

**Box-Behnken Design and Statistical Analysis**

Based on the single-factor experimental results above, RSM was used to further optimize the experimental parameters. Seen from Table 1, a BBD of RSM was performed with three independent variables liquid-solid ratio (X₁), extraction temperature (X₂) and extraction time (X₃) at three levels. The whole design consisted of 17 experimental runs, each of which was carried out in triplicate in random order.

The total sugar content was measured by the phenol-sulfuric method using glucose as the standard (Dubois et al., 1956). The protein content was quantified by the Coomassie brilliant blue method using bovine serum albumin as the standard (Barbosa et al., 2009). The content of uronic acid was detected according to the carbazole-sulfuric method using glucuronic acid as the standard (Bitter and Muir, 1962).

**Antioxidant Activity In Vitro**

Superoxide anion radical scavenging activity

The scavenging effect on the superoxide anion radicals was measured as described by Liu et al. (Liu et al., 2014) with a little modification. Reaction was performed in a mixture system containing 4.5 mL Tris-HCl buffer (pH 8.2, 50 mM), 1 mL sample solution (50–800 µg/mL) and 0.4 mL pyrogallol solution (5 mM) at 25°C for 15 min. Subsequently, hydrochloric acid was added for termination reaction. The absorbance of the solution was recorded at 525 nm. The scavenging capability was calculated by the following equation: Scavenging activity (%) = (1 - Aₛ/ₐₚ) x 100, where Aₛ is the absorbance of the sample and Aₚ is the absorbance of the blank.

**DPPH Free Radical Scavenging Activity**

The DPPH free radical scavenging activity was detected according to the previous literature with some modifications (Kazuko et al., 1992). Briefly, 2.0 mL DPPH methanol solution (0.08 mM) was added into 2.0 mL polysaccharides solution (0.5, 1.0, 2.0, 3.0, 4.0, 5.0 mg/mL). The mixture was shaken thoroughly and incubated at room temperature for 30 min. The absorbance of the samples was

<table>
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<th>Coded levels (real values)</th>
<th>Yield of PPs (%)</th>
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<tr>
<td>X₁: liquid/solid ratio (mL/g)</td>
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determined at 517 nm. Ascorbic acid (Vc) was used as the positive control. The free radical scavenging activity was evaluated according to the following equation:

\[
\text{scavenging rate} \% = \left[ 1 - \frac{(A_1 - A_2)}{A_0} \right] \times 100, \text{ where } A_0 \text{ is the absorbance of the sample and DPPH, } A_1 \text{ is the absorbance of the sample only and } A_2 \text{ is the absorbance of the DPPH solution without sample.}
\]

Hydroxyl Radical (OH) Scavenging Assay
The hydroxyl radical scavenging activity was determined by the modified Fenton’s reaction as described previously (Gao et al., 2013). The reaction mixture contained 1.0 mL of FeSO₄ (9 mM), salicylic acid–ethanol (9 mM), H₂O₂ (9 mM) and different concentrations of sample solutions. The solution was mixed thoroughly, followed by incubation at 37°C for 30 min. The absorbance was determined at 510 nm. Vc was used as a positive control and distilled water served as blank control. The scavenging activity of the hydroxyl radical was calculated as follows:

\[
\text{OH scavenging activity} \% = \left[ 1 - \frac{(A_1 - A_2)}{A_0} \right] \times 100, \text{ where } A_0 \text{ is the absorbance of blank control, } A_1 \text{ is the absorbance of the sample in reactive system and } A_2 \text{ is the absorbance of the sample without OH.}
\]

Statistical Analysis
All values were presented as the mean ± standard deviation (S.D.). Statistical analyses of these data were performed with SPSS (version 19.0). The significance of difference was analyzed with one-way analysis of variance (ANOVA). A value of \( p < 0.05 \) was considered statistically significant.

RESULTS AND DISCUSSION

Single-factor Experimental Analysis
In this study, the optimization of four key parameters, including liquid to material ratio, extraction temperature, time and frequency for improving the yield of PPs were investigated. The effect of liquid/solid ratios on the yield was studied from 10 to 30 mL/g, whereas the other extraction factors were carried out as follows: extraction temperature of 80°C, extraction time of 2.5 h, and extraction frequency of 2. As shown in Fig. 1A, the extraction yield of PPs remarkably increased with the liquid/solid ratio increased from 10 to 30 mL/g, and then tended to be constant from 30 to 50 mL/g. This can be explained that larger liquid/solid ratio facilitates leaching-out rate of more targeted components into the water, which can lead to an increase of the extraction yield (Chen et al., 2015). However, a large amount of extraction solvent can cause the waste of energy and increase of workload. Therefore, the liquid/solid ratio of 30 mL/g was selected for further study.

The extraction temperature is one of the important factors that influence the extraction yield of polysaccharides. As reported, the best extraction temperature of a major polysaccharide with potential antioxidant was as high as 97.29°C (Yuan et al., 2017). Thus, the effect of extraction temperature on the polysaccharides yield was studied from 60°C to 100°C, when the liquid/solid ratio, extraction time and frequency were set at 30 mL/g, 2.5 h and 2, respectively. Fig. 1B indicated that the yield of PPs increased quickly as the extraction temperature increased from 60°C to 90°C, while above 90°C, the yield of PPs decreased slowly. It has been proved that high extraction temperature can increase the solubility of polysaccharides and accelerate mass transport of water-soluble polysaccharides (Samavati and Manoochehrizade, 2013), whereas higher temperature may cause the oxidation and thermal degradation of polysaccharides (Raza et al., 2017; Zhang and Wang, 2017). Thus, the extraction temperature of 90°C was considered to be optimal in the subsequent experiment.

Extraction time is another parameter influencing the extraction yield of the polysaccharides. Seen from Fig. 1C, the effect of different extraction time on the polysaccharides yield was investigated at 1, 1.5, 2, 2.5 and 3 h, when the liquid/solid ratio, extraction temperature and frequency were fixed at 30 mL/g, 80°C and 2, respectively. As shown, the yield of PPs reached a peak value rapidly at 2.0 h, then decreased along with continuously increase of extraction time from 2.0 h to 3.0 h. These results can be explained by the fact that excessively extended extraction time leads to thermal unstable or hydrolysis of polysaccharides, and further decrease the yield (Zhang et al., 2015; Zhang et al., 2016). Accordingly, 2.0 h was chosen as the optimum extraction time for the extraction of PPs.

To investigate the effect of extraction frequency on the PPs yield, the extraction process was tested with different extraction frequencies from 1 to 5, while other extraction conditions were fixed as follows: liquid/solid ratio of 30 mL/g, extraction temperature of 80°C and extraction time of 2.5 h. As shown in Fig. 1D, the extraction yield of PPs increased from 6.93% to 7.67% with the increase of extraction frequency. However, there was no significant increase when the extraction frequency was above 2. Taking the extraction yield and the loss into consideration, extraction frequency of 2 was selected as the fixed parameter for further experiments.

Fitting the Model
According to the results of preliminary single-factor experiments, three parameters including liquid/solid ratio (X₁), extraction temperature (X₂) and extraction time (X₃) were further investigated using BBD to obtain the optimal extraction conditions. The design matrix and corresponding experimental results were shown in Table 1, and the data analysis was carried out using Design-Expert software (Version 8.0.6). The response variable Y for extraction yield of polysaccharides can be described by the following second-order polynomial equation:

\[
Y = 8.64 + 0.37X_1 - 0.14X_1^2 + 0.46X_2 + 0.090X_2^2 + 0.11X_3 - 0.23X_1X_2 - 0.43X_1X_3 - 0.097X_2X_3 - 1.14X_3^2
\]

The analysis of variance (ANOVA) and adequacy of quadratic model were summarized to evaluate the “goodness of fit”, which is displayed in Table 2. F-test and \( p \)-value are used to check the significance of each coefficient and assess the interaction strength of each factor (Wei et al., 2009). According to the results, the high F-value of the model was 59.60 and small p-values were less than 0.0001, which suggested that the regression model was fitted more significantly. Furthermore, the “lack of fit” of this model was not significant with the \( p \)-value of 0.0545. The values of determination coefficient (\( R^2 = 0.9871 \)), adjusted determination coefficient (\( R_{adj}^2 = 0.9706 \)) and low coefficient of variance
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Analysis of Response Surface

Three-dimensional (3D) response surface plots and their corresponding contour plots about the reciprocal interactions between two independent extraction parameters were created, as shown in Fig. 2. The circular contour plot indicates that the mutual influences of corresponding variables on extraction yield are negligible, while elliptical or saddle response contour plots indicate that the interactions between corresponding variables are significant (Liu et al., 2015). Given this, it was observed that the mutual interactions between extraction temperature and extraction time were significant, which was in agreement with the results shown in Table 2 ($p < 0.05$). As shown in Fig. 2 (A and D) and (B and E), when the extraction temperature and time were designated at level 0, the maximum yield was noticed with the increase of liquid-solid ratio. When liquid-solid ratio was fixed at level 0, the PPs yield increased firstly with increase of the extraction temperature and time, and then decreased. It could be explained that long extraction time and high temperature accelerated the solubility and diffusion of the plant polysaccharides into water, leading to a highest polysaccharides yield. However, excessive extraction temperature and time could also cause the oxidization and degradation of polysaccharides, which might be responsible for the decrease of extraction yield. Seen from Fig. 2 (C and F), a significant decrease of the polysaccharides yield was observed with the increase of extraction temperature and extraction time. These two variables had negative reciprocal action on response of extraction yield (Eq. (1)). Therefore, the decrease of extraction yield could be explained by the hydrolyzation and thermal

(C.V.% = 1.72) verified the significance of regression models and the high accuracy of experimental data. Meanwhile, Table 2 showed that the linear coefficients ($X_1$, $X_2$, $X_3$), one cross product coefficient ($X_2X_3$) and two quadratic coefficients ($X_1^2$ and $X_2^2$) had a significant effect on the yield ($p < 0.05$), while the other interaction coefficients ($X_1X_2$, $X_1X_3$ and $X_2^2$) were not significant with high $p$-values ($p > 0.05$).
FIGURE 2 | 3D response surface plots (A, C, and E) and 2D response contour plots (B, D, and F) showing the effects of variables (X₁: liquid-solid ratio; X₂: extraction temperature; X₃: extraction time) on the extraction yield of polysaccharides.
conditions, the maximum extraction yield of PPs was 8.73%, which was consistent with the predicted value of 8.78%. These findings indicated that the adequacy of model equation was confirmed and the optimizing extraction process was suitable for PPs.

Physicochemical Characteristics of Polysaccharides

The content of total sugar, uronic acid and protein in PPs was 72.01%, 20.37% and 3.56%, respectively, based on the chemical analysis.

Antioxidant Activity In Vitro

Reactive oxygen species (ROS) are generated after the inflammatory phase, the superoxide and hydroxyl radicals can deleteriously damage DNA, proteins and lipids and finally result in cell death, reduction of cell proliferation and mutation, and inactivation of innate antioxidant system (Komeri et al., 2017; Nimse and Pal, 2015). DPPH is a stable free-radical compound which can accept an electron or a hydrogen atom from the antioxidant scavenger molecule, to be converted to a more stable DPPH molecule, and it is widely used to evaluate the free radical scavenging ability of antioxidants (Carmona-Jimenez et al., 2014). It was reported that water-soluble polysaccharides extracted from potatoes and tangerine peels exhibited potent antioxidant capacity (Chen et al., 2016; Jeddou et al., 2016). Hence, the antioxidant activity of polysaccharides from pomelo peels was investigated. As shown in Fig. 3, PPs showed higher scavenging effects on DPPH with scavenging rate of 88.60% at the concentration of 5 mg/mL, while relatively lower scavenging effects on OH (75.60%) and O₂ (78.60%), which were correlated positively with increasing concentrations. While the Vc showed scavenging rates on DPPH, HO and O₂ of 95.60%, 94.6% and 95.8%, respectively. The results implied that PPs were likely to contain substances that were hydrogen donors and could react with free radicals, suggesting that PPs could serve as free radical inhibitors or scavengers.

CONCLUSIONS

In this study, bioactive PPs were obtained using conventional hot water extraction and ethanol precipitation from pomelo peels. The maximum yield of polysaccharides was 8.73% under the optimum conditions of liquid-solid ratio (34 mL/g), extraction temperature (87°C), extraction time (2.0 h) and extraction frequency (2). Chemical analysis indicated that PPs contained total sugar of 72.01%, uronic acid of 20.37% and protein of 3.56%, respectively. In addition, the antioxidant assays in vitro revealed that PPs exhibited higher scavenging activity on DPPH, whereas relatively lower activities on hydroxyl and superoxide radicals. Our results demonstrated that the polysaccharides from pomelo peels had potential to be explored as supplement or natural antioxidants. It is valuable to develop the natural plant polysaccharides as potential antioxidant agents, and further work is needed to elucidate the mechanism.
CONFLICTS OF INTEREST
All authors declare no conflicts of interest.

ACKNOWLEDGMENT
The authors would like to acknowledge the financial support provided by the Natural Science Foundation of Agriculture Department (No. 201303082-3), National Natural Science Foundation of China (No. 31271975) and Innovation fund for the Excellent Doctoral Dissertation (2018) of Tianjin University of Science and Technology (No. 201801).

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