Preparation, Preliminary Characterization and Antiangiogenic Activities of Polysaccharides from Pomegranate Peels

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Summary: In the present study, the crude polysaccharides from pomegranate peels **(**CPP**)** were prepared by compound enzyme-extraction technology, and the response surface methodology was used to optimize the extraction parameters. Box–Behnken design (BBD) was applied to estimate the effects of extraction temperature, extraction pH, and dosage of compound enzyme on the yield of CPP. A mathematical model with high fitness was obtained. Extraction temperature, extraction pH, and dosage of compound enzyme exhibited independent and interactive effects on CPP yield. CPP were purified by macroporous resin HP-20 and Sephadex G-100 column chromatography to afford purified fraction of CPP-2. The relative molecular weight of CPP-2 was 93.5 kDa. In CPP-2, and FT-IR spectra showed that the main components among CPP-2 may be pectic polysaccharides.The effect of CPP-2 on angiogenesis was measured *in vivo* by using the chick embryo chorioallantoic membrane (CAM) assay. The results demonstrated that CPP-2 suppressed angiogenesis in chicken embryos.

Keywords*:* Pomegranate peel, Polysaccharide, Preparation, Characterization, Antian-giogenic activity.

Introduction

Pomegranate (*Punica grantum* L.) is an arbor belonging to the family of punicaceae, which originated from the surrounding areas of Iran and Balkan Peninsula [1]. Pomegranate has gained popularity in recent years due to its multi-functionality and nutritional value in human diet. The fruit is grown globally in many different geographical regions, satisfying the nutritional and medicinal needs of populations of various countries [2]. Pomegranate peel is byproduct of pomegranate processing plants, which mainly contains tannin, flavone, organic acid, pectin, alkaloids, and saccharides [3, 4], which is almost discarded except a small quantity used as traditional chinese medicines at present. Over the last decade, some scientific publications indicate that the pomegranate peel possesses a number of biological activities such as anti-inflammatory [5], removing heavy metal, antioxidant, antimetalloproteinases [6], antibacterial, antiviral, antitumor, antimutagenic, hepatoprotective and immunoregulatory activities [7-9]. Pomegranate peels are rich in polyphenols and polysaccharides [10]. Almost all the researches are focused on the polyphenols extracted from the pomegranate peels, while a little attention was devoted to the polysaccharides [7, 11]. Whereas it is difficult to make significant progress to elucidate the underlying mechanism by which pomegranate takes effect only by polyphenols. Indeed, it is necessary to extract the polysaccharides from the pomegranate peel and study the biological activities of the polysaccharides [12, 13].

Therefore, the main objective of the present study was to develop a response surface methodology to optimize the comprehensive compound enzyme extraction technology parameters, and evaluate the characterization and antiangiogenic activity *in vitro* of polysaccharides from pomegranate peel. Thus, these results could help us to take action for future study the polymers.

Experimental

Reagents and Materials

Ripened pomegranates were obtained from Huaiyuan, Anhui Province of China. The peels were separated manually from the fruit, sun-dried and powdered, and then kept at room temperature for further study. Chick embryos were obtained from Xingdian Poultry Plant (Nanjing, China). All other chemicals used were ultra-pure or analytic grade.

Extraction of polysaccharides

The crude CPP was prepared according to our previous method with minor modifications [14]. Briefly, the fresh peels collected were cut into pieces, dried by oven at 60 ℃ and pulverized into powder. A

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60-mesh sift was used to screen the powder, and then extracted with compound enzyme. The extraction process was performed at different extraction temperature, extraction pH and dosage of enzyme. The optimal extraction conditions were calculated by response surface methodology. The extract was filtered through a Whatman No. 1 filter paper. Then, more anhydrate ethanol was added to the filtrate to a final concentration of 80% (v/v). It was kept overnight at 4℃ to precipitate CPP. All the samples were lyophylized. Phenol/sulfuric acid method was used to determine the polysaccharide content with glucose as the standard. The polysaccharide yield of extraction was calculated according to the formula below:

Extraction yield (%) = $W_1/W_0 \times 100$

where $W_1$is the weight of CPP and W_0 is the dried weight of Pomegranate peels.

Box–Behnken design for the extraction of polysaccharides

The software Design Expert (Trial Version 7.1.3, Stat-Ease Inc., Minneapolis, Minnesota, USA) was employed for experimental design, data analysis and model building. A Box–Behnken design with three variables was used to determine the response pattern and then to establish a model. Three variables used in this study were extraction temperature (45–65℃, A), extraction pH (4.5–5.5, B) and dosage of enzyme (1.0%–1.5%, C), with three levels for each variable, while the dependent variables was the yield of CPP. The polysaccharide extract was filtrated and concentrated. Four-fold volume of absolute ethanol was added directly to precipitate CPP. The symbols and levels are shown in Table 1. Five replicates at the center of the design were used to allow for estimation of a pure error sum of squares. Experiments were randomized to maximize the effects of unexplained variability in the observed responses due to extraneous factors. A full quadratic equation or the diminished form of this equation, shown as follows, was used for this model.

$$Y = \beta_0 + \sum_{j=1}^{k}\beta_j X_j + \sum_{j=1}^{k}\beta_{jj} X_j^2 + \sum\sum_{i<j}\beta_{ij} X_i X_j \quad (1)$$

where Y is the estimated response, β_0, β_j, β_{jj} and β_{ij} are the regression coefficients for intercept, linearity, square and interaction, respectively, while X_i and X_j are the independent variables coded ($i \neq j$).

Purification of crude polysaccharides

The crude CPP was separated and sequentially purified through a column of macroporous resin HP-20 and Sephadex G-100. Crude CPP solution was applied to macroporous resin HP-20 and eluted with deioned water. Eluate (10 ml/tube) was collected automatically and the carbohydrates were determined by the phenol–sulfuric acid method. As results, one fraction of polysaccharides was obtained, concentrated, dialyzed and further purified through Sephadex G-100 column (2.6×60 cm) to afford CPP-2. Finally, purified polysaccharides was lyophilized for further study.

Analysis of polysaccharides characterization

UV spectrometric analysis

The *CPP* and *CPP-2* were scanned at 190-800 nm by TU1901 ultraviolet spectrophotometer. The spectrum was collected and analyzed.

Relative molecular weight of CPP-2

The relative molecular weight of CPP-2 was determined by HPLC according to our previous method [15].

FT-IR spectrometric analysis

FT-IR of CPP-2 was carried out by the potassium bromide (KBr) pellet method on Fourier transform-infrared spectrometer type MB154S (Bomen, Canada) in the range of 500–4000 cm^{-1}.

Chicken chorioallantoic membrane assay

Ex vivo anti-angiogenic activity of CPP-2 was measured by CAM assay as described elsewhere with minor modifications [16]. A group of 7-day-old fertilized eggs was incubated at 37.5 ℃ with 55% relatively humidity. On day 8, eggs were rinsed with 70% ethanol and a 1 cm^2 window was carefully created with a puncher on the broad side of the egg. A volume of CPP (20 µl containing 25, 50 or 100 µg/egg) or PBS (20 µl) or 5-Fluorouracil (20 µl, containing 2 µg/egg) were applied on a sterile cotton ball and then placed into CAM, after which a permeable sticky tape was immediately appended to the window. After incubation for 3 days (until day 11), the eggshell was pushed aside around the window, and the blood vessels were photographed under a stereomicroscope (Stemi 2000-C, Germany).

Table-1: Box–Behnken design matrix and the response values for the yield of CPP.

Standard order	A Temperature（°C）	B pH	C Dosage of enzyme（%）	Polysaccharide yield (%)	
				Experimental	Predicted
1	55(0)	5.0 (0)	1.25 (0)	14.85	14.79
2	65(1)	5.0 (0)	1.0(-1)	14.38	14.40
3	55(0)	4.5 (-1)	1.0 (-1)	13.37	13.41
4	45(-1)	5.5 (1)	1.25 (0)	14.57	14.62
5	55(0)	5.5 (1)	1.5 (1)	13.51	13.47
6	55(0)	5.5 (1)	1.0 (-1)	14.55	14.47
7	55(0)	5.0 (0)	1.25 (0)	14.75	14.79
8	65(1)	4.5 (-1)	1.25 (0)	14.31	14.26
9	45(-1)	4.5 (-1)	1.25 (0)	13.27	13.20
10	65(1)	5.0 (0)	1.5 (1)	13.56	13.52
11	55(0)	5.0 (0)	1.25 (0)	14.83	14.79
12	55(0)	4.5 (-1)	1.5 (1)	14.18	14.26
13	55(0)	5.0 (0)	1.25 (0)	14.79	14.79
14	45(-1)	5.0 (0)	1.0 (-1)	13.79	13.82
15	65(1)	5.5 (1)	1.25 (0)	13.05	13.12
16	45(-1)	5.0 (0)	1.5 (1)	14.55	14.54
17	55(0)	5.0 (0)	1.25(0)	14.73	14.79

Statistical analysis

Data was statistically analyzed using SPSS 16.0 software package by one-way analysis of variance (ANOVA). Significant differences between two means were observed by Student-Newman-Keuls test. Difference was considered to be statistically significant if $P < 0.05$.

Results and discussion

Single factor test

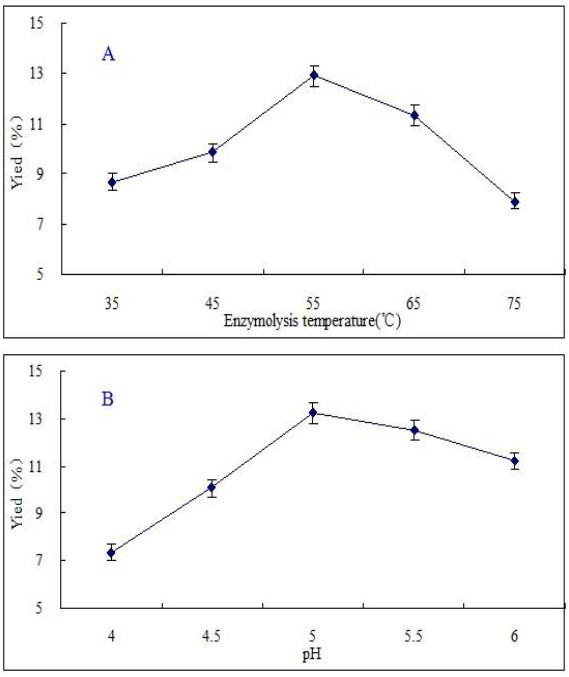
Effect of enzymolysis temperature on the yield of CPP

The influence of temperature on the enzyme activity is twofold, enzyme activity gradually increases with the increasing of the temperature, but the activity decreases after exceeding the optimal temperature. Fig. 1A illustrated the effect of different enzymolysis temperature of 35℃, 45℃, 55℃, 65℃ and 75℃ on the CPP yield, while the other extraction factors were fixed at pH 5 and compound enzyme dosage 1.25%, respectively. The yield of CPP increased with the elevation of temperature from 35℃ to 55℃. However, the CPP yield decreased when enzymolysis temperature varied from 55℃ to 75℃, which due to higher temperature could lower the activity of enzyme. This phenomenon could be explained as higher temperature could increase mass transfer of CPP from material cell to extraction solutions and strengthen the interaction between cytoderm and enzyme, thus the yield improved [17]. On all of above, in the following single factor tests and response surface experiments, the enzymolysis temperature was selected to be fixed at 55℃.

Effect of enzymolysis pH value on the yield of CPP

The pH value affects the enzyme activity owing to various enzymes had their own optimum pH, the reason probably lies on the active sites or functional groups region of enzyme conformation could be altered or controlled by adjusting the pH value [18].As shown in Fig. 1B, the effects of different pH (4–6) on the CPP yield were tested, respectively. The enzymolysis temperature 55℃ and compound enzyme dosage 1.25%. The yield of CPP increased with the elevation of pH values at first, however, a higher pH value (>5) could lower the yield of CPP, which should be due to the highest enzyme activity depends on the optimum pH value of the enzyme. Based on the results, pH 5 was considered to be the optimal value and it will be fixed in the other single factor tests and the following response surface experiments.

Effect of enzyme dosage on the yield of CPP



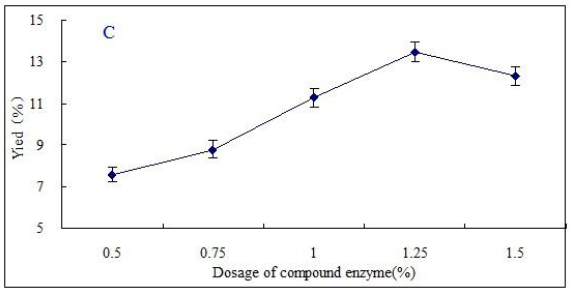


Fig. 1: Effects of extraction temperature (A), extraction pH (B) and dosage of enzyme (C) on the yield of CPP.

Enzyme reaction rate is proportional to the enzyme concentration under certain condition, small amount of enzyme may not fully interact with materials, otherwise, large amount of enzyme may cause the degradation of extraction products or higher processing cost [19]. Fig. 1C showed the effect of enzyme dosage on the CPP yield. The dosage of compound enzyme was set at 0.5%, 0.75%, 1.0%, 1.25%and 1.5% (the quality percent of the raw material). When the enzymolysis temperature 55℃and pH 5, the CPP yield reached the maximum value when the compound enzyme dosage was 1.25%. This indicated that the dosage of compound enzyme 1.25% was sufficient to obtain a high CPP yield. Therefore, the enzyme dosage range from 1.0% to 1.5%was considered to be optimal in the present experiment.

Model fitting and optimization for extraction of CPP

Model fitting

By using the software of Design Expert version 7.1.3, a polynomial model describing the correlation between the extraction yield of CPP and the three variables was obtained as follows:

$$Y=14.79-0.11A+0.069B-0.036C-0.64AB-0.40AC-0.46BC-0.41A^2-0.58B^2-0.31C^2$$

where Y represents the yield of crude CPP, A, B and C represent extraction temperature, pH, and dosage of enzyme, respectively.

The results of ANOVA, lack-of-fit and the adequacy of the model are summarized in Table-2. The model *F*-value of 1111.06 implied that the model was highly significant. There was only a 0.01% chance that a model *F*-value could occur due to noise. The determination coefficient R^2 of the model was 0.9930, indicating that 99.30% of the variability in the response could be explained by the model. In addition, the p-value of $p = 0.1030$ for lack-of-fit implied the lack-of-fit was not significant relative to the pure error, indicating the model equation was adequate to predict the extraction yield of CPP within the range of experimental variables.

The significance of the regression coefficients was tested by *F*-test, and the corresponding *P*-values for the model terms are also listed in Table-2. The *p*-value is used as a tool to check the significance of each coefficient, which in turn may indicate the pattern of the interaction between the variables. Smaller the *p*-value is, more significant the corresponding coefficient is. Accordingly, A, B, AB, AC, BC, A^2, B^2, and C^2 were significant ($p < 0.05$), while C was not significant ($p > 0.05$).

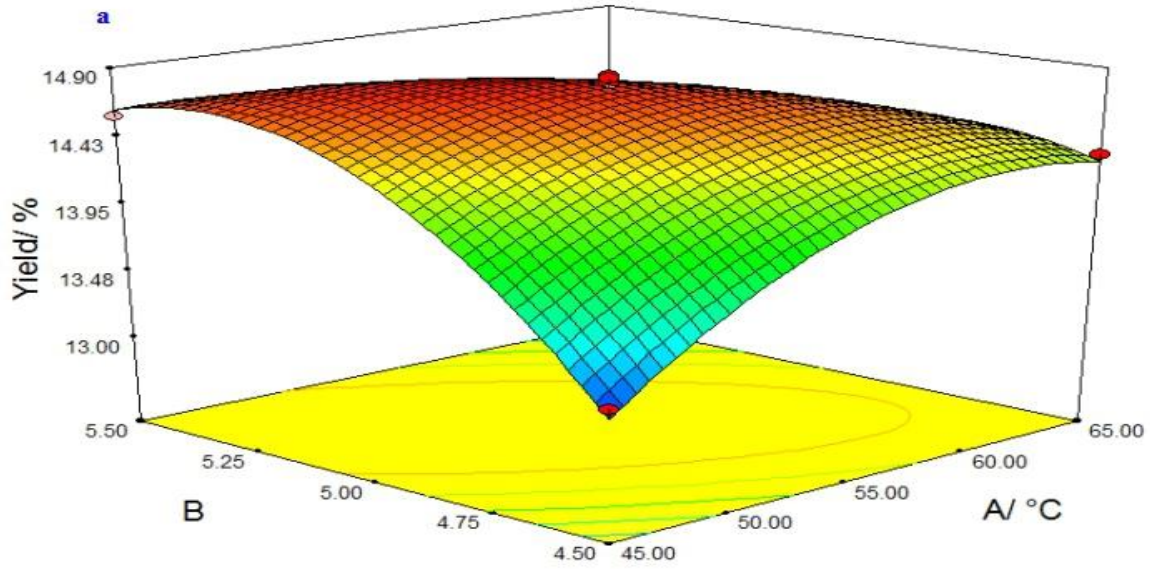
Optimization for extraction of CPP

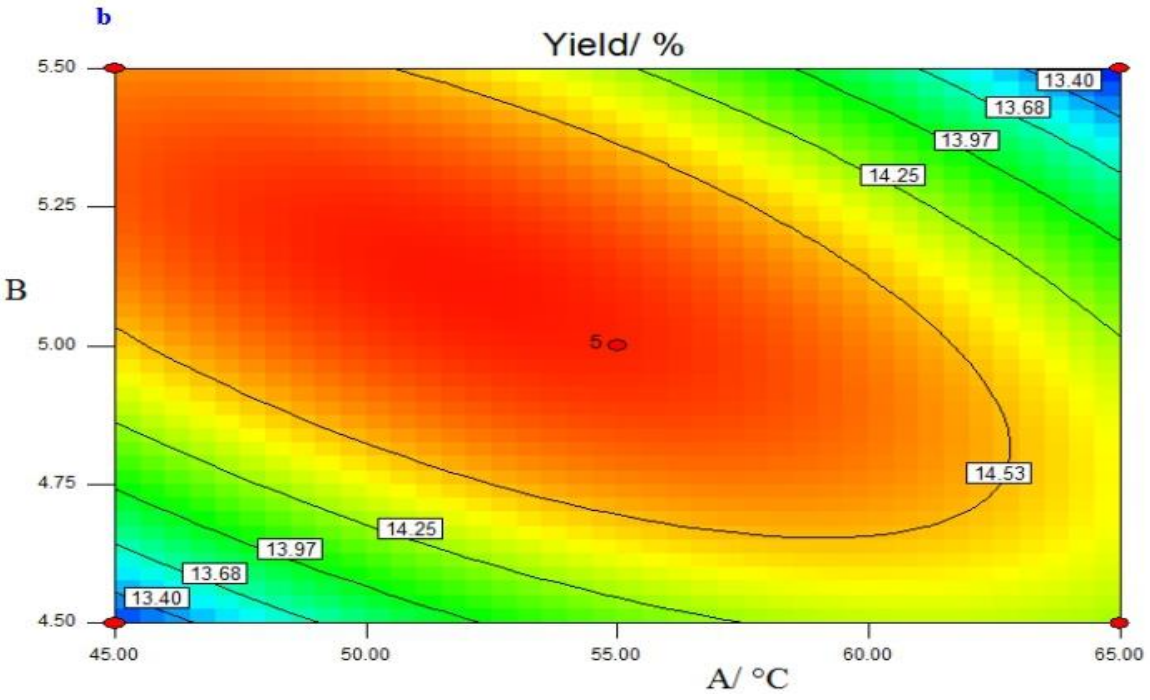
The fitted response surface plots and contour plots for the model were generated by the Stat-Ease Design-Expert software in order to better understand the interactions of the variables. The shape of the contour plots indicates whether the mutual interactions between variables are significant or not. A circular contour plot indicates that the interaction between related variables is negligible, while an elliptical contour plot indicates that the interaction between related variables is significant [20]. The response surface plots and contour plots as shown in Fig. 2 were generated using Design-Expert, which depicted the interactions between two variables by keeping the other variables at their zero levels for crude CPP production. It is evident that these three-dimensional plots and their corresponding contour plots provided a visual interpretation of the interaction between two variables and facilitated the location of optimum experimental conditions. By employing the software Design-Expert, the solved optimum values of the tested variables for the extraction of CPP were extraction temperature 55℃, extraction pH 5.0 and dosage of enzyme 1.25%. Using the optimal conditions, the maximum predicted extraction yield of CPP was 14.79%, which corresponded fairly well to that of real extraction (14.79±5.09%). The result suggested that the regression model was accurate and adequate for the prediction of CPP extraction.

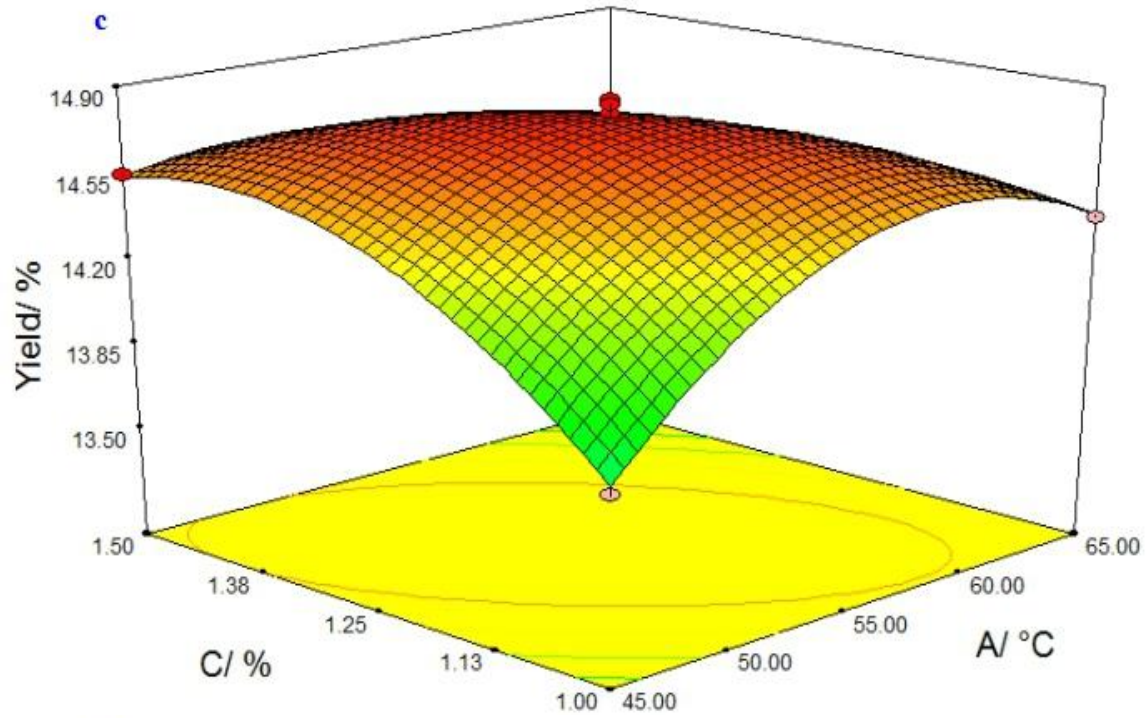
Table-2: Analysis of variance for the response surface quadratic model for CPP yield[a].

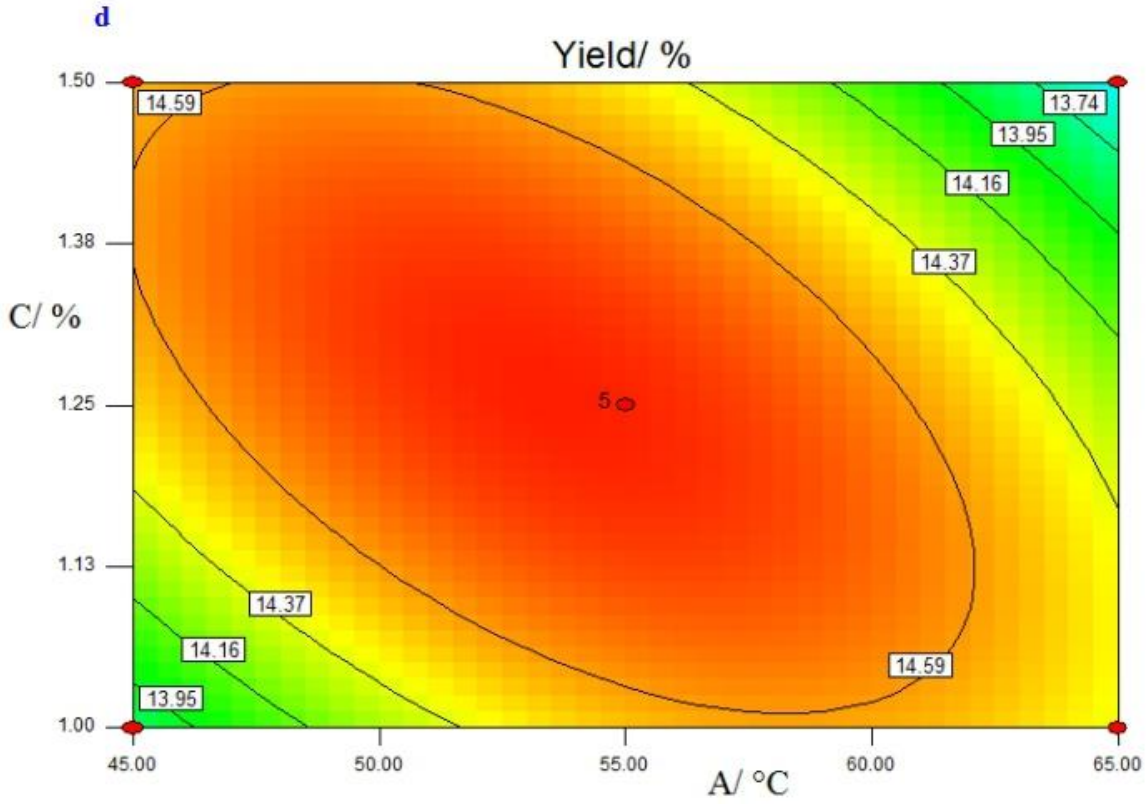
Source	Sum of squares	Degrees of freedom	Mean square	F-value	P-value
Model	**6.06**	**9**	**0.67**	**111.06**	**< 0.0001**
A	**0.097**	**1**	**0.097**	**15.97**	**0.0052**
B	**0.038**	**1**	**0.038**	**6.24**	**0.0411**
C	**0.011**	**1**	**0.011**	**1.73**	**0.2293**
AB	**1.64**	**1**	**1.64**	**270.33**	**< 0.0001**
AC	**0.62**	**1**	**0.62**	**102.97**	**< 0.0001**
BC	**0.86**	**1**	**0.86**	**141.18**	**< 0.0001**
A^2	**0.71**	**1**	**0.71**	**117.50**	**< 0.0001**
B^2	**1.41**	**1**	**1.41**	**232.70**	**< 0.0001**
C^2	**0.40**	**1**	**0.40**	**66.23**	**< 0.0001**
Residual	**0.042**	**7**	**6.061E-003**		
Lack of fit	**0.032**	**3**	**0.011**	**4.11**	**0.1030**
Pure error	**0.010**	**4**	**2.600E-003**		
Total	**6.10**	**16**			

[a] A, extraction temperature; B pH; C, Dosage of enzyme.
R^2 = 0.9930, adjusted R^2 = 0.9841






c
14.90
14.55
14.20
13.85
13.50
Yield/ %
1.50
1.38
1.25
1.13
1.00
C/ %
45.00
50.00
55.00
60.00
65.00
A/ °C


d
Yield/ %
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13.74
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A/ °C

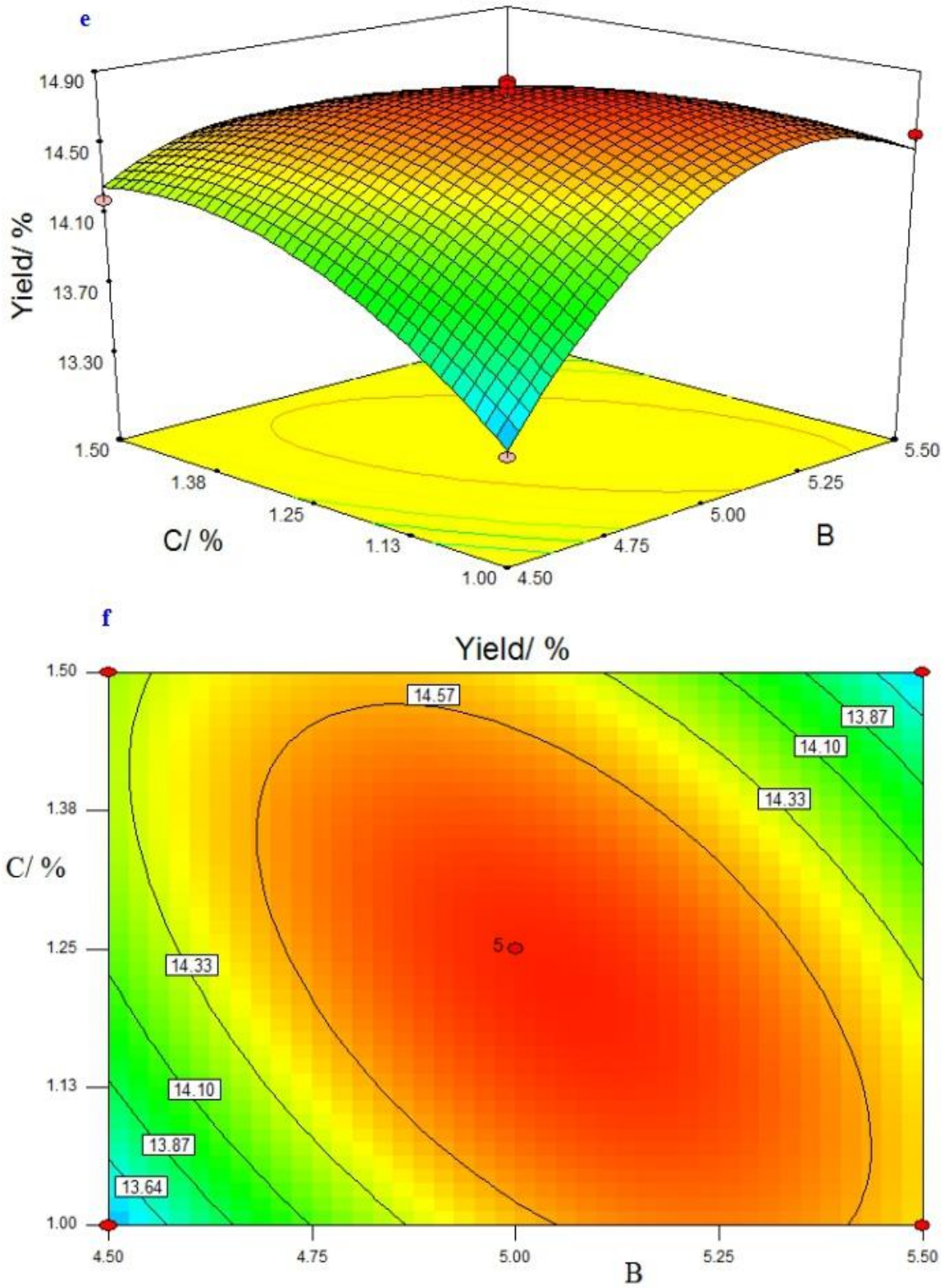

Fig. 2: Response surface plots (a, c and e) and contour plots (b, d and f) showing the effects of extraction temperature, extraction pH, dosage of enzyme and their mutual effects on CPP.

Purification and characterization of CPP

Chromatograms CPP and CPP-2

The crude CPP solution was firstly separated through macroporous resin HP-20 and Sephadex G-100 respectively, and the column was eluted with deioned water. As results, the fraction generated single elution peak (Fig. 3), named as CPP-2. To determine the relative molecular weights of CPP-2, size-exclusive HPLC was employed and standard glucans were used as references. As results, the relative molecular weight of CPP-2 was 93.5kDa.

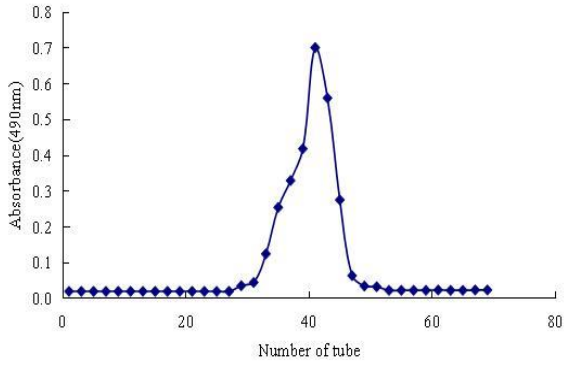

Fig.3: Elution curve of CPP by HP-20 and Sephadex G-100 column chromatography.

UV spectrometric of CPP and CPP-2

Fig.4 shows that the CPP and CPP-2 has an obvious absorption peak at 190 nm at the wavelength of 190-600 nm.

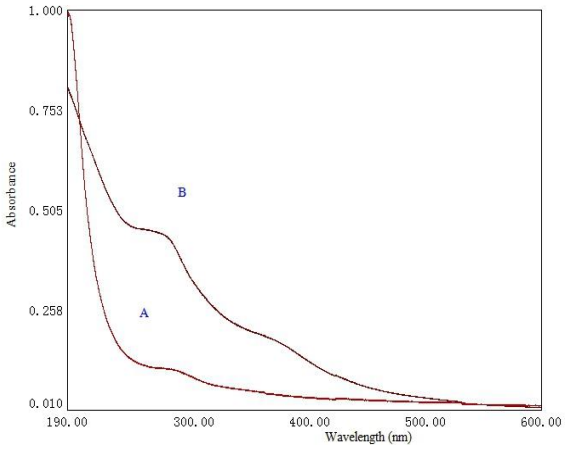

Fig. 4: UV spectrometric of CPP (A) and CPP-2(B)

FT-IR spectrum of CPP-2

The FT-IR spectra of CPP-2 was showed in Fig. 5. Two characteristic absorptions of polysaccharides, a strong and wide absorption band of about 3100–3700 cm^{-1} for O–H stretching vibrations and a strong absorption peak of about 2800–3000 cm^{-1} for C–H stretching vibrations, were observed. Two characteristic absorptions, a peak of about 1600–1700 cm^{-1} (C=O asymmetric stretching vibrations) and a band of about 1300–1400 cm^{-1} (C=O symmetric stretching vibrations), indicated that there were carboxyl groups in CPP-2. In addition, absorption peak of 1450.97 and 1411.84 cm^{-1} were also for C=O bending vibration. It was shown that the main components among *CPP-2* may be pectic polysaccharides [21].

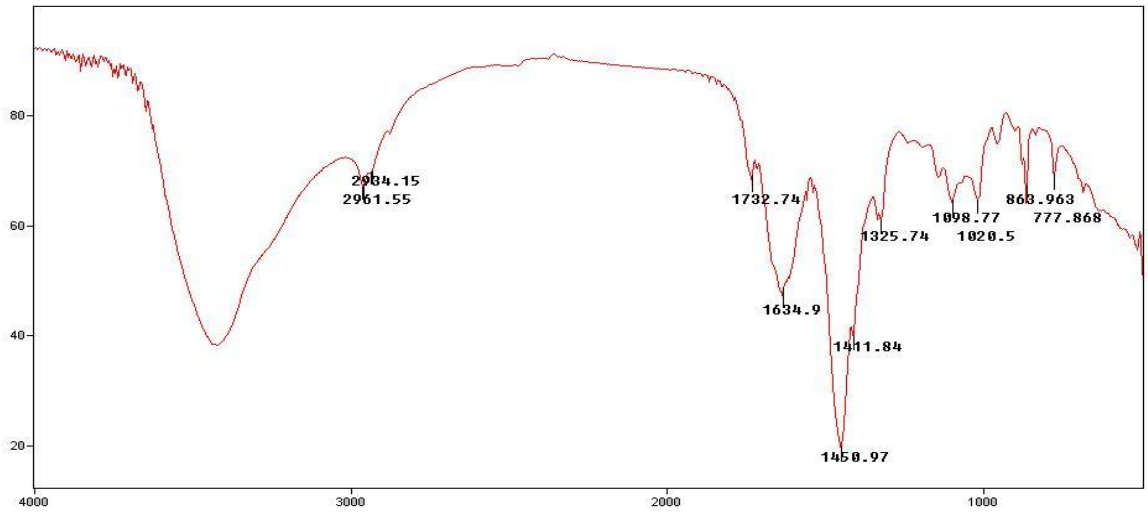

Fig. 5: FT-IR spectrum of CPP-2.

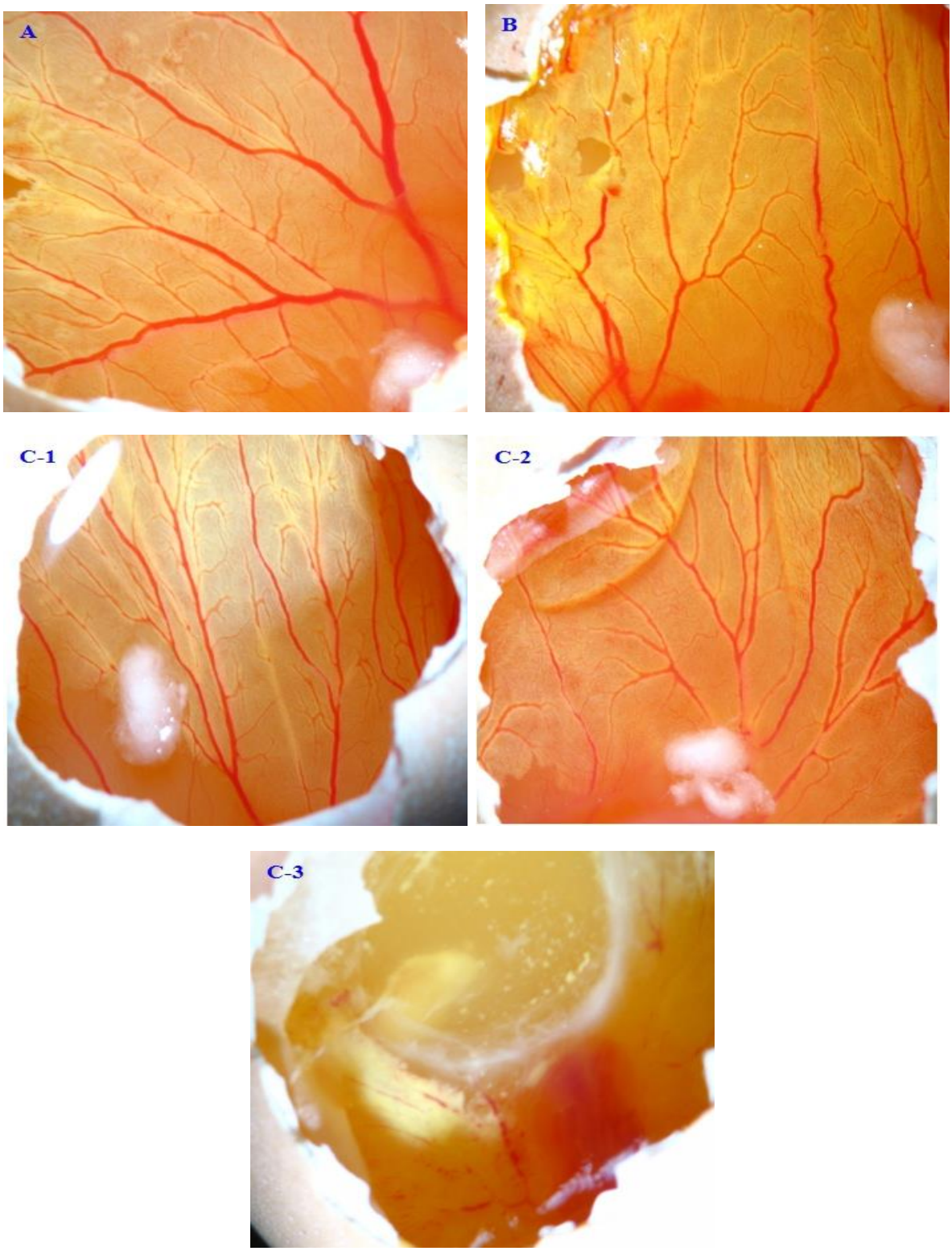

Fig. 6: Effects of CPP-2 on angiogenesis of chicken eggs (n=3) by chorioallantoic membrane assay. A: PBS (20 µl, control); B: 5-Fluorouracil (20 µl, 2µg/egg, positive control); C: CPP-2 (25 µg/egg, C-1; 50 µg/egg, C-2; 100 µg/egg, C-3).

Antiangiogenic activity in vitro *of CPP-2*

The CAM of the chicken embryo is a unique model for evaluating neovascularization [22]. Using this model, we examined the potential anti-angiogenic activities of CPP-2 *in vivo*. In the control eggs, blood vessels formed densely branching vascular networks (Fig. 6A). Treatment with CPP-2 significantly inhibited the number of newly formed blood vessels in a dose-dependent manner (Fig. 6C). As compare with the control group (Fig. 6A), CPP-2 added at 25 µg/egg for 72 h only moderately inhibited the neovascularization (Fig. 6, C-1), whereas CPP-2 added at 50 and 100 µg/egg significantly inhibited neovascularization (Fig. 6, C-2, C-3). As compared with the positive control group (Fig. 6B), Low concentration CPP-2 (Fig. 6, C-1) showed similar effect on antiangiogenic activity, and high concentration CPP-2 **(**Fig. 6, C-2, C-3**)** showed better effects on antiangiogenic activity.

Angiogenesis, the process of the sprouting of new capillaries from pre-existing blood vessels, is essential for sustained growth of solid tumors and metastasis [23]. The results suggested that CPP-2 suppresses angiogenesis in chicken embryos. The antiangiogenic mechanism of CPP-2 may be that CPP-2 blocked a signaling cascade at or immediately following ligand-receptor interaction [24].

Conclusions

In this study, BBD was employed to optimize the parameters for the extraction of CPP. As a result, the maximum yield of CPP (14.79±5.09%) was obtained under the following conditions: extraction temperature 55℃, extraction pH 5.0, and dosage of enzyme 1.25 %. In the present study, the antiangiogenic activities of CPP-2 from pomegranate peel were demonstrated by using the chick embryo chorioallantoic membrane (CAM) assay. In a CAM model, CPP-2 could suppress angiogenesis in chicken embryos. Further works on the structure and biological activity in vivo of CPP-2 is in progress.

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