

Optimization of the flash extraction of flavonoids from the leaves of *Salix babylonica* using the response surface method and an evaluation of the leaves' high antioxidant activity

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Abstract

Many biological activities of *Salix babylonica* leaves are attributed to the plants' high total flavonoid content. Flash extraction has the advantages of high efficiency and maximum retention of the active ingredient. In this study, flash extraction was used to extract the total flavonoids, and a Box–Behnken design was used to optimize the extraction conditions for the first time. The effects of four independent variables, including ethanol concentration, extraction voltage, time, and ratio of liquid to material on flavonoid yield, was determined, and the optimal conditions for flavonoid extraction were evaluated using response surface methodology. Statistical analyses showed that the linear and quadratic terms of these four variables had significant effects. The fitted second-order model revealed that the optimal conditions consisted of an ethanol concentration of 67.91%, extraction time of 87 s, extraction voltage of 116 V and ratio of liquid to material of 42.79. Under the optimum conditions, the experimental value of $66.40 \pm 0.80\%$ nearly coincided with that predicted by the model. In the ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) assays, the extracts showed significant antioxidant and scavenging capacity for free radicals, respectively. This study helps to better exploit the resources of *Salix babylonica* leaves and provides new insights for effective extraction of flavonoids.

Introduction

Currently, there are increasing applications of flavonoids in food and medicine for their extensive biological functions in terms of antibacterial, antiviral and antioxidant activities [1-5]. As a biological response modifier, most of these flavonoids are derived from traditional Chinese medicine, including *Salix babylonica*. This plant is economically notable and is distributed

throughout Asia, Europe, and the Americas. The leaf of *Salix babylonica* has been studied in China for antifungal and anti-obesity utilization due to its high content of flavonoids [6, 7]. Currently, there are limited studies on the extraction process and antioxidant activity of the total flavonoids from *Salix babylonica* leaves [8], and the primary focus is on the enrichment and purification of the total flavonoids. In addition, most of the extraction methods continue using the outmoded methods of reflux extraction, ultrasonic extraction and microwave extraction [9, 10]. In these cases, extraction required a long time and was labor- and material-intensive, and the most important factor was that the active ingredients of the extract were easily degraded. To use *Salix babylonica* leaf resources more optimally, there is an urgent need to establish the optimum procedure for the effective extraction of flavonoids.

The flash extraction method is a new method in recent years [11, 12]. This method uses the principle of high-speed shear force and molecular filtration to break up the stems, leaves, roots and other materials of plants into tiny particles such that the concentration of the extraction solvent reaches equilibrium in a short time [13, 14]. In addition, the solute transfer process is completed in tens of seconds or even seconds, and the active ingredients in the plant are only destroyed to a small extent. Compared with the traditional hot water extraction, enzyme extraction and other physical field-assisted extraction methods, such as microwave, ultrasonic, and ultrahigh pressure extraction, the flash extraction method uses small amounts of solvent, a short extraction time and is highly efficient [15-18]. In recent years, there have been many reports of the use of this technology to extract phytochemicals. It was used to extract water-soluble components, such as polyphenols [19], glycosides [20], and polysaccharides [21], as well as water-insoluble components, such as tannins [22], peony [23], and volatile oil [24]. Therefore, the flash extraction method with its unique

advantages of speed, energy, and the savings in solvents is bound to exert greater potential for herbal extractions.

In humans, oxidation is involved in the generation of energy, but the body may be affected by a variety of factors, such as illness, aging, and free oxygen radicals produced by oxidation, that are uncontrollable and harmful to the human body. Antioxidants, such as flavonoids, can eliminate these free radicals, reduce the risk of death from related diseases, and slow aging [25, 26]. Therefore, the preparation and application of antioxidants to protect the body from free radical damage is popular [27]. In this study, flash extraction and response surface methodology (RSM) were combined to seek an effective extraction procedure. In addition, the antioxidant capacity of the extracts was investigated to verify the effectiveness of flash extraction. The results indicated that the flash extraction method could maximize the protection of the active ingredients of flavonoids with high biological function. These findings will provide a theoretical basis to develop and utilize *Salix babylonica* leaf resources.

Materials and methods

Materials

The leaves of *Salix babylonica* were collected in Jilin Province (China). Rutin and oligomeric proanthocyanidins (OPC) standards were purchased from the National Institutes for Food and Drug Control (Beijing, China). Aluminum nitrate, sodium hydroxide, ethanol, sodium nitrite, hexahydrate ferric chloride and ferrous sulfate were analytically pure and obtained from the Tianjin Reagent Company (Tianjin, China). 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) were purchased from J&K Scientific Co. (Beijing,

China).

Extraction of total flavonoids

The leaves of *Salix babylonica* were crushed using a grinder and passed through a 20-mesh screen. With the use of a flash extractor, a number of samples were accurately weighed and extracted under different extraction times, extraction numbers, ratio of liquid to raw material, ethanol concentration and extraction voltage. The extract was separated from the insoluble residue using centrifugation (3000 rpm, 5 min), and the extract was filtered using a 0.45 µm microporous membrane. The filtrate was concentrated and dissolved to 100 mL to determine the concentration of the flavonoids.

Determination of the flavonoid contents

The colorimetric method was used to determine the content of the flavonoids in the extract [28, 29]. One milliliter of flavonoid extract, 1 mL of 5% (w/w) sodium nitrite and 5 mL of 80% (v/v) ethanol were accurately measured and mixed for 6 min. One milliliter of 10% (w/w) aluminum trichloride were added and incubated for 6 min, and 10 mL of 1 mol/L sodium hydroxide was added. The absorbance of the solution was measured using a UV-2900 spectrophotometer at 510 nm after 15 min. A blank control was used. The standard curve ($y = 0.0164 + 0.0097x$, where y is absorbance value of sample, and x is sample concentration) ranged from 16.32–65.28 µg/mL ($R^2=0.9993$).

Experimental design

The extraction rate of the flavonoids is influenced by many factors, such as the extraction voltage, extraction time, the ratio of liquid to raw material, ethanol concentration and extraction

number [30, 31]. Based on the single factor test results, factors that have a significant impact on the flavonoids were selected to test using response surface methodology. The four significant impact factors were designated X_1 , X_2 , X_3 , and X_4 , and each factor was set at three levels, coded +1, 0, -1 for high, medium and low values, respectively (Table 1). Four variables were encoded based on the following equation:

$$X_i = \frac{x_i - x_0}{\Delta x}, \quad i = 1 - 4 \quad (1)$$

where X_i represented the encoded value of the variable; x_i represented the actual value of the variable; x_0 represented the actual value of the independent variable at the center point, and Δx represented the change value of the variable, respectively.

Table 1. Variables and design levels of response surface methodology.

Independent variables	Coded symbols	Levels		
		-1	0	1
Extraction voltage (V)	X_1	110	120	130
Ethanol concentration (%)	X_2	50	60	70
Extraction time (s)	X_3	80	90	100
Ratio of liquid to material	X_4	30	40	50

To use the Box-Behnken design and obtain the best extraction conditions, a second order polynomial model was used to illustrate the relation between the response values and independent variables. The model is as follows:

$$Y = \beta_0 + \sum_{i=0}^4 \beta_i X_i + \sum_{j=0}^4 \beta_{ii} X_i^2 + \sum_{i=0}^4 \sum_{j=0}^4 \beta_{ij} X_i X_j \quad (2)$$

Where Y represented the response function; β_0 represented the constant, and β_i , β_{ii} and β_{ij}

represented the coefficients of the linear, quadratic and interactive terms, respectively. X_i , X_i^2 and X_iX_j were the encoded independent variables, interaction and quadratic terms, respectively.

To design the experiments and analyze the experimental data, design-expert software (version 8.0) was used. Based on the analysis of variance, the regression coefficients of individual linear, quadratic, and interaction terms were determined. The regression coefficients were used to provide statistical calculations to generate dimensional and contour maps from the regression model. The lack of fit and the coefficient of determination (R^2) were generated using the Design-Expert software. They represented the accuracy of the model. Fischer's F-test at a probability (P) of 0.001, 0.01 or 0.05 examined the significance of the model, as well as the encoded independent variables, interaction and quadratic terms.

Antioxidant activity assay of flavonoids

A ferric reducing antioxidant power (FRAP) experiment was used to measure the total antioxidant activity of the extracts and standards (FeSO_4 solution). A 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) experiment was used to measure the free radical scavenging activity of the extracts and standards using OPC.

FRAP assay

FRAP reagents included 50 mmol/L acetate buffer, which contained 20.4 g $\text{C}_2\text{H}_3\text{NaO}_2$ and 80 mL $\text{C}_2\text{H}_4\text{O}_2$ per liter, 10 mmol/L TPTZ (2,4,6-tripyridyl-s-triazine) solution in which 40 mmol/L HCl solution was used as the solvent, and 20 mmol/L $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$. The working FRAP reagent was obtained by mixing 100 mL acetate buffer, 10 mL TPTZ solution, and 10 mL $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution

[32]. One hundred microliters of different amounts of FeSO₄ solutions or extracts were added to 6 mL FRAP reagent and incubated in a 37° C water bath for 30 min. The wavelength of 593 nm was used to monitor the absorbance of the samples.

DPPH assay

Sixty micromolar DPPH was dissolved in 3 mL ethanol, and 0.5 mL of different amounts of extracts or OPC were added. Blank experiments, which contained 0.5 mL of 99% ethanol instead of the extract, were also conducted. The absorbance values were monitored at 517 nm [33]. The inhibition rate (IR) of the DPPH free radical was calculated using the following equation [34]:

$$IR = (A_0 - A_s) / A_0 \times 100\% \quad (3)$$

where A_0 was the absorbance of the blank experiments, and A_s was the absorbance in the presence of the samples. When the IR is 50%, the corresponding concentration is called the inhibition concentration 50 (IC₅₀). Therefore, the value of the IC₅₀ was obtained by fitting the sample concentration and inhibition rate [35].

Statistical analysis

Statistical analysis of the single-factor experimental data was performed using SPSS 11.5 software (SPSS Inc., Chicago, IL, USA). Dunnett's one-tailed t-tests permitted the examination of the statistical significance of the means in the different levels of parameters. Stat-Ease Design-Expert 8.0.0 (Trial version, Stat-Ease Inc., Minneapolis, MN, USA) was used for the experimental design and regression analysis of the experimental data. Student's t-test permitted the determination of the statistical significance of the regression coefficient, and Fischer's F-test determined the second-order model equation at a probability (P) of 0.001, 0.01 or 0.05. The

adequacy of the model was determined by evaluating the lack of fit, the coefficient of determination (R^2), and the F-test value obtained from the analysis of variance (ANOVA) that was generated. All the experiments were conducted in triplicate, and the average values $\pm$ SD (standard deviation) were calculated.

Results

Single factor test results

Effect of extraction voltage in single factor analysis

The extraction voltage was one of the important factors affecting the yield of total flavonoids [36]. The effects of different voltages (60, 80, 100, 120, and 140 V) on the yield of total flavonoids were investigated, while the other extraction conditions were as follows: ethanol concentration of 70%, ratio of ethanol to raw material of 30:1, extraction time of 60 s, and extraction number of 3. The results showed that the yield increased greatly when the voltage increased from 60 to 140 V, and the highest yield was obtained at 120 V (Fig 1A). With the increase in voltage, the yield of flavonoids increased. This phenomenon might be attributable to the increase of the rotational speed of the head of the flash extractor, the increase in the temperature of the extraction solvent at the same time, and the flavonoids undergoing side reactions, such as oxidation or decomposition, resulting in a decrease in the content of flavonoids [36]. In this experiment, the optimal extraction voltage was considered to be 120 V.

Fig 1. Column chart showing the effect of different factors on the yield of total flavonoids. The effect of (A) different voltage, (B) ethanol concentration, (C) ratio of ethanol to raw material, (D) time and (E) extraction number on the yield of flavonoids was determined. (t-test, * $p < 0.05$, ** $p <$

0.01, *** $p < 0.001$)

203

204 **Effect of ethanol concentration in single factor analysis**

205 Different concentrations of ethanol (40, 50, 60, 70, and 80%) were utilized to study the effect
206 of the ethanol concentration on the extraction rate, while the other experimental parameters were as
207 follows: the extraction voltage was 120 V, the ratio of liquid to material was 30, the extraction time
208 was 60 s, and the number of extraction was 3.

209

210 As shown in Fig 1B, the extraction yield of the flavonoids was greatly influenced by the
211 concentration of ethanol [37, 38]. This yield increased with the augmentation of the ethanol
212 concentration until it reached a maximum production of 37.53 ± 1.12 mg/g at 60% of ethanol. It
213 also indicated that the maximum extraction yield was obtained with 60% ethanol. Therefore, 60%
214 ethanol was used as the center point for the additional RSM experiments.

215

216 **Effect of the ratio of liquid to material in single factor analysis**

217 The effect of the ratio of material to liquid on the yield of flavonoids is also important [39, 40].
218 Different ratios of liquid to material (20, 30, 40, 50 and 60) were designed to investigate their effect
219 on the extraction rate. Simultaneously, the other conditions were as follows: extraction voltage of
220 120 V, ethanol concentration of 60%, extraction time of 60 s, and number of extraction of 3. As
221 displayed in Fig 1C, the yield increased strongly as the ratio increased from 20 to 60. When the
222 ratio of the liquid to the material continued increasing, the output was maintained at a stable level.
223 Therefore, the ratio of liquid to material of 40 was used in this study.

224

Effect of extraction time in single factor analysis

Using a fixed extraction voltage (120 V), ethanol concentration (60%), ratio of liquid to material (40) and extraction number (3), the effects of different extraction times (30, 60, 90, 120, and 150 s) on the extraction rate of the flavonoids were determined. The yield of the flavonoids increased significantly with the increase in extraction time from 30 s to 150 s (Fig 1D). However, at more than 90 s, the flavonoid production decreased slightly. This finding may be attributable to the prolonged extraction time; a large amount of heat is generated due to the rapid rotation of the head of the flash extractor; the temperature of the extraction solvent is increased, and the flavonoids undergo side reactions, such as oxidation or decomposition, resulting in a decrease in the content of the flavonoids [41]. Therefore, 90 s was used as the center of the extraction time for RSM.

Effect of the extraction number in single factor analysis

The number of the extraction was also important to extract the active compound [42, 43]. The number of the extraction was related to the extraction efficiency, cost, and flavonoid yield. Respectively, different extraction numbers (1, 2, 3, 4 and 5) were used to extract the flavonoid under the optimal parameters obtained above. As shown in Fig 1E, with the increase in the number of the extraction from 1 to 2, the yield of flavonoids increased significantly. However, when the number of extractions was more than 2, the increase in flavonoid production was not significant. Considering the efficiency of extraction, the number of extraction was set at 2 in this study.

Model fitting and statistical analysis of the extraction process optimization

Response surface methodology optimization is more advantageous than the traditional single

parameter optimization, because it saves time, space and raw material. To optimize the four individual parameters of the Box-Behnken design, the 27 experiments designed were completed. Using multiple regression analyses, the following second order polynomial equations are used to correlate the response variables and test variables:

$$Y = -1486.75792 + 9.06700 \cdot X_1 + 0.74617 \cdot X_2 + 19.82517 \cdot X_3 + 6.21783 \cdot X_4 + 0.021275 \cdot X_1 \cdot X_2 - 0.050850 \cdot X_1 \cdot X_3 + 5.57500 \text{E-}003 \cdot X_1 \cdot X_4 + 3.90000 \text{E-}003 \cdot X_2 \cdot X_3 + 0.041075 \cdot X_2 \cdot X_4 - 6.82500 \text{E-}003 \cdot X_3 \cdot X_4 - 0.027050 \cdot X_1^2 - 0.039200 \cdot X_2^2 - 0.079338 \cdot X_3^2 - 0.10586 \cdot X_4^2$$

where X_1 , X_2 , X_3 and X_4 were the encoded values on extraction voltage, ethanol concentration, extraction time and the ratio of liquid to material, respectively.

To determine whether the model was significant, the significance of each coefficient was determined using the F -test and p -value in Table 2. Smaller p -values indicated higher significances for the corresponding coefficient [44]. In this study, the ANOVA of the quadratic regression model demonstrated that the model was highly significant, as was evident from the F -test with a very low probability value ($p < 0.0001$). With the determination coefficient ($R^2 = 0.9755$) close to 1, this finding indicated that the actual values had a strong correlation with the predicted values [45]. With Adj. R^2 values of 0.9469, it could be concluded that more than 90% of the total flavonoid content could be predicted. Only 5% could not be predicted by the model.

Table 2. ANOVA table for the response surface quadratic model.

Source	Sum of squares	Degree of freedom	Mean square	F-Value	p -Value (Prob > F)	Significant
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Model	2163.56	14	154.54	34.10	<0.0001	***
X_1	302.40	1	302.40	66.73	<0.0001	***
X_2	416.54	1	416.54	91.91	<0.0001	***
X_3	427.09	1	427.09	94.24	<0.0001	***
X_4	86.24	1	86.24	19.03	0.0009	***
X_1X_2	18.11	1	18.11	4.00	0.0688	
X_1X_3	103.43	1	103.43	22.82	0.0005	***
X_1X_4	1.24	1	1.24	0.27	0.6100	
X_2X_3	0.61	1	0.61	0.13	0.7204	
X_2X_4	67.49	1	67.49	14.89	0.0023	**
X_3X_4	1.86	1	1.86	0.41	0.5334	
X_1^2	39.02	1	39.02	8.61	0.0125	*
X_2^2	81.95	1	81.95	18.08	0.0011	**
X_3^2	335.70	1	335.70	74.08	<0.0001	***
X_4^2	597.70	1	597.70	131.89	<0.0001	***
Residual	54.38	12	4.53			
Lack of fit	27.50	10	2.75	0.20	0.9760	Not significant
Pure error	26.89	2	13.44			
Cor total	2217.94	26				
R^2	0.9755					
Adj. R^2	0.9469					
Pred. R^2	0.9013					

Adequate precision	18.194					
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(t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

The failure of the model was represented by the lack-of-fit shown in Table 2. The lack of fit of the F -value was 0.2000, and the P -value was 0.9670, which illustrated that the pure error was not significantly relative. In summary, the model was appropriate. Simultaneously, the adequate precision of 18.194 indicated that the model discrimination was adequate. Therefore, this study model could be used to navigate the design response surface space.

The P -values of each model term presented in Table 2 indicated that the total flavonoid yield was significantly affected by four independent variables (X_1 , X_2 , X_3 , X_4) and all of the quadratic terms (X_1^2 , X_2^2 , X_3^2 , X_4^2). In addition, the ethanol concentration and extraction time were the most significant factors on the total flavonoid yield followed by the extraction voltage and the ratio of liquid to material.

Response surface analysis of extraction process optimization

The interaction effects of the factors in response surface experiments were displayed using three-dimensional response surface plots (Fig 2) and two-dimensional contour plots (Fig 3). The former described the sensitivity of the variable change on the response value, and the latter illustrated the important coefficients between the different variables [46, 47]. The impact of the two factors on the response could be displayed at one time by these plots. In these figures, the other two factors remained at level zero.

289

290 The influences of the extraction voltage and ethanol concentration on the flavonoid yield are
 291 shown in Figs 2A and 3A. When the ethanol concentration was less than 68%, the flavonoid yield
 292 increased, while when it was higher than 68%, the flavonoid yield decreased, and when the voltage
 293 was low, the yield changed more significantly. When the ethanol concentration increased from 50%
 294 to 60%, the extraction voltage had little effect. However, when the ethanol concentration increased
 295 from 65% to 70%, the voltage had a greater effect on the flavonoid yield.

296

297 **Fig 2. Response surface method analysis (3D) of the different interaction factors.**

298 **Fig 3. Contour plots (2D) of the different interaction factors.**

299

300 Figs 2B and 3B showed the reciprocal interactions of the extraction voltage and extraction time
 301 on the yield of flavonoids. The mutual interactions were significant. With the increase of extraction
 302 voltage, the flavonoid yield clearly decreased, especially when the extraction time was longer. After
 303 a period of time, the flavonoid yield increased. However, at more than this range, the output did not
 304 increase noticeably. Simultaneously, when the extraction voltage was raised to 116 V, the extraction
 305 time had a significant effect on the flavonoid yield.

306

307 The effect of extraction voltage and ratio of liquid to material on the flavonoid yield is shown
 308 in Figs 2C and 3C. With the extraction voltage dropping, when the ratio of liquid to material was
 309 relatively low, there was little effect on the flavonoid yield. However, when the ratio of liquid to
 310 material was relatively high, the decrease in voltage had a greater effect on the flavonoid yield.
 311 When the ratio of liquid to material increased from 30 to 42.79, the flavonoid yield increased

significantly to 42.79 and began to decline. Therefore, in order to access the maximum increase, the ratio of liquid to material was more effective at 42.79.

The influences of ethanol concentration and extraction time on the yield of flavonoids are visible from Figs 2D and 3D. This result showed that the extraction time exhibited a significant effect on the flavonoid yield, while the ethanol concentration had a weaker effect. With the extraction time increasing to 87 s, the flavonoid extraction rate increased and then decreased. When the ethanol concentration increased from 50% to 67.79%, the extraction of the flavonoids increased significantly, while at more than 67.79% it decreased. Therefore, to achieve maximum increase, the ethanol concentration used was 67.79%.

The effects of the mutual interactions between the ethanol concentration and the ratio of liquid to material were significant (Figs 2E and 3E). With the ethanol concentration increasing from 50% to 67.79%, the flavonoid production increased linearly, but at more than 67.79%, it decreased. This finding was probably attributable to the scope of the solvent; the polarity was appropriate and more suitable to extract flavonoids [48]. When the ratio of liquid to material increased from 30 to 42.79, the flavonoid yield increased at first and then decreased.

In Figs 2F and 3F, the impact of the interaction of the ratio of liquid to material and extraction time was displayed. An appropriate extraction time was essential for the extraction of flavonoids. When the extraction time increased from 80 to 87 s, the flavonoid yield increased, but when the extraction time was longer than 87 s, the yield decreased. The ratio of liquid to material was lower, and the yield also stayed lower. When the ratio of the liquid to material increased from 30 to 42.79,

the flavonoid yield increased significantly, and after 42.79, the change was very small.

Validation of the model

As shown in Table 3, the optimum extraction conditions were obtained, and the flavonoid yield predicted by the Design Expert Software was 66.54 mg/g. To validate the adequacy of the model equations, a verification experiment was conducted under the optimal conditions (within the experimental range): extraction voltage 116 V, ethanol concentration 68%, extraction time 87 s, the ratio of liquid to material 43 and extraction number 2, respectively. A mean value of 66.40 ± 0.80 mg/g (N=3) was obtained from the practical experiments. The average was close to the predicted value of the RSM model. Therefore, this model could be used to extract flavonoids, and the yield of flavonoids was higher than that of other extraction conditions.

Table 3. Experimental and predicted values of the model under optimal conditions.

Optimum condition				Extraction yield	
				(mg/g)	
Extraction voltage (V)	Concentration of ethanol (%)	Extraction time (s)	Ratio of liquid to material	Experimental	Predicted
116	67.91	87.00	42.79	66.40 ± 0.80	66.54

FRAP assay

As shown in Fig 4, a linear correlation between the concentration of the FeSO_4 solution or extracts and the absorbance was displayed. In addition, the concentration of extracts was expressed as milligrams of *Salix babylonica* leaves per liter. In addition, the total antioxidant activity of the 1

mg extracts approximately equaled the total antioxidant activity of 0.5–0.75 mmol FeSO₄.

Fig 4. Total antioxidant activity of (A) FeSO₄ solutions and (B) extracts.

DPPH assay

DPPH is a stable and well-characterized solid radical source, and it is a traditional and the most popular free radical used to assay free radical scavenging activity [49]. OPC, which has a strong ability to scavenge a free radical, was used as the standard to evaluate the free radical scavenging activity of the extracts. As shown in Fig 5, the inhibition rate (IR) and IC₅₀ were used to characterize the free radical scavenging activity of the extracts. With the concentration increasing, the value of the IR increased. The IC₅₀ value of the extracts from the *Salix babylonica* leaves was 0.8937 mg/L, while the IC₅₀ value of the OPC was 0.0196 mg/L. Therefore, the free radical scavenging activity of the 1 mg extracts was approximately equivalent to that of 0.021 mg OPC.

Fig 5. Scavenging activity of (A) OPC and (B) extracts on DPPH free radicals.

Discussion

Belonging to *Salicaceae* and *Salix*, *Salix babylonica* is a fast-growing deciduous tree, which is widely cultivated in Asia, Europe and America. In foreign countries, the leaves of these trees are an important source of feed for ruminants, particularly in areas that experience harsh environmental conditions. Plant extracts from *Salix babylonica* leaves contain saponins and other secondary natural metabolites, which can improve nutrient digestibility and feed utilization [50-53]. In China, its leaves, branches, and floes are all included in the Chinese Dictionary of Traditional Chinese

376 Medicine as a traditional Chinese medicine. The Compendium of Materia Medica recorded the
 377 following: *Salix babylonica* is the lower grade of this product. This plant is cold in nature and tastes
 378 bitter. The plant has the functions of hurricane, diuretic, analgesic, and swelling. According to
 379 reports, *Salix babylonica* is rich in flavonoids [54, 55], which have diverse physiological activities,
 380 such as anti-inflammatory, anti-tumor, antioxidant, anti-dementia and anti-obesity [56-64]. It must
 381 have widespread utilization in the medicine and food industry in the future.

382

383 *Salix babylonica* is rich in resources and cultivated throughout China, but its medicinal value
 384 has not been properly developed and utilized to date. The optimal extraction procedures of the
 385 flavonoids from *Salix babylonica* leaves with new techniques had not been studied frequently, and
 386 most of the extraction methods are limited to conventional extraction methods with low efficiency
 387 and obvious shortcomings. Xiaoyan Yi *et al.* used the reflux extraction method, and the yield of
 388 flavonoids was 5.15% [10]. Keyue Liu *et al.* adopted the microwave extraction method, and the
 389 flavone yield was 5.67% [65]. The flash extraction method, also known as the tissue fragmentation
 390 extraction method, is an emerging extraction method [66-68]. The flash extraction method is
 391 applicable to the extraction of the roots, stems, leaves, flowers, fruits, seeds (except for fine seeds)
 392 and whole plants and can be entered into the extraction system only by appropriate treatment. In
 393 addition to ether and other volatile solvents, water, methanol, ethanol, acetone, and microemulsion
 394 [69] can be directly selected as the solvent according to the desired effective site or chemical
 395 composition. The extraction process flow is simple, rapid, energy saving, and environmentally
 396 friendly. It can be implemented at room temperature, avoiding heat interference, effectively
 397 protecting the heat-sensitive components [22, 24] and has obvious advantages compared with
 398 traditional extraction methods. In traditional Chinese Medicine research, the flash extraction

method will inevitably exert greater potential. In this study, the flesh extraction method was used to extract flavonoids from *Salix babylonica*. The extraction rate of the flavonoids was 60.4 mg/g (6.04%), which is significantly higher than those of the other extraction methods.

In the human body, free radicals and oxidants are produced from either normal cell metabolism or external sources. Excessive free radicals and oxidants that accumulate in the human body could generate a phenomenon called oxidative stress and result in oxidative damage to cause the deterioration of the normal function of proteins, nucleic acids and many other biomasses [70]. The antioxidant activity of the flavonoid extract of *Salix babylonica* leaf was examined by Yaoyao Su *et al.* who used the ultrasound and microwave methods to extract α -glucosidase inhibitors from weeping willow leaves and found that the inhibition rate of α -glucosidase was 77.85% [9]. Ruiping Ji *et al.* used indirect iodine quantitative methods to determine the peroxidation of animal and plant oils and fats and showed that the highest antioxidant value of *Salix babylonica* leaf was 0.204% [8]. In addition, Jie Xie *et al.* used a homogenate extraction technique to extract bamboo leaf flavonoids. The yield of total flavonoids extracted from the bamboo leaves was 8.50 mg/g, which was 20.2% higher than that of the ethanol reflux extraction. The IC_{50} of the total flavonoids obtained by flash extraction was 67.35 μ g/mL, which scavenged free radicals. The effect is better than ethanol refluxing [71]. However, there was no report on the activity of the total flavonoids scavenging free radicals in *Salix babylonica* leaves. For the first time, this study describes the application of the flash extraction and response surface optimization methods. On the basis of improving the yield of flavonoids, the free radical scavenging activity was maintained at a high level with an IC_{50} of 0.8937 mg/L.

In this study, response surface methodology was used to establish the optimal conditions for the extraction of total flavonoids from *Salix babylonica* leaves using flash extraction. The yield of the flavonoids was maximized and increased significantly compared with those of the conventional extraction methods. In addition, antioxidant assays showed that the extracts with the established procedures in this experiment had stronger antioxidant ability, and 1 mg extracts equaled approximately 0.5–0.75 mmol FeSO₄ or 0.021 mg OPC, respectively. These results will help to better exploit the resources of *Salix babylonica* leaves and provide new insights for the effective extraction of flavonoids with greater bioactivity.

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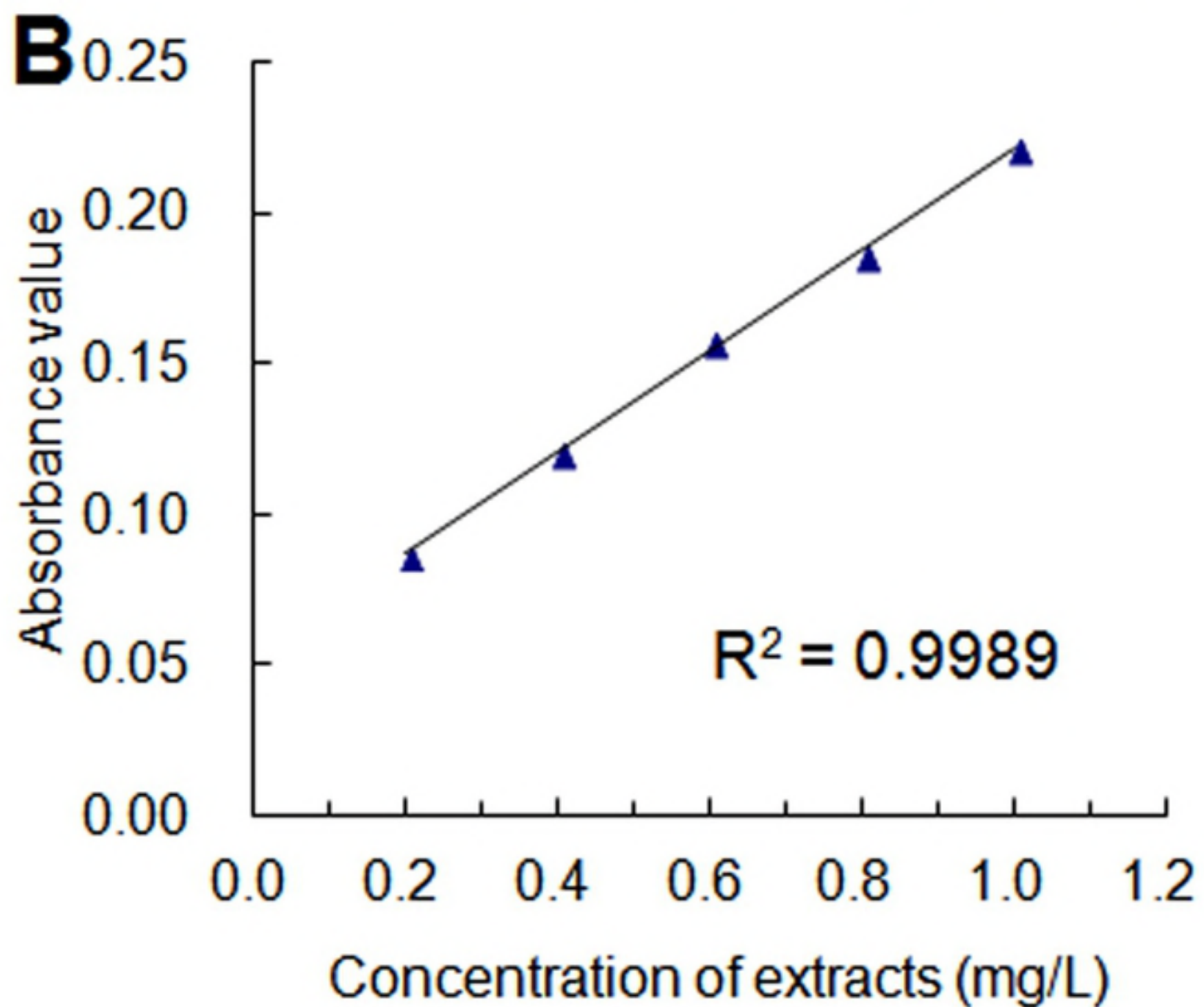
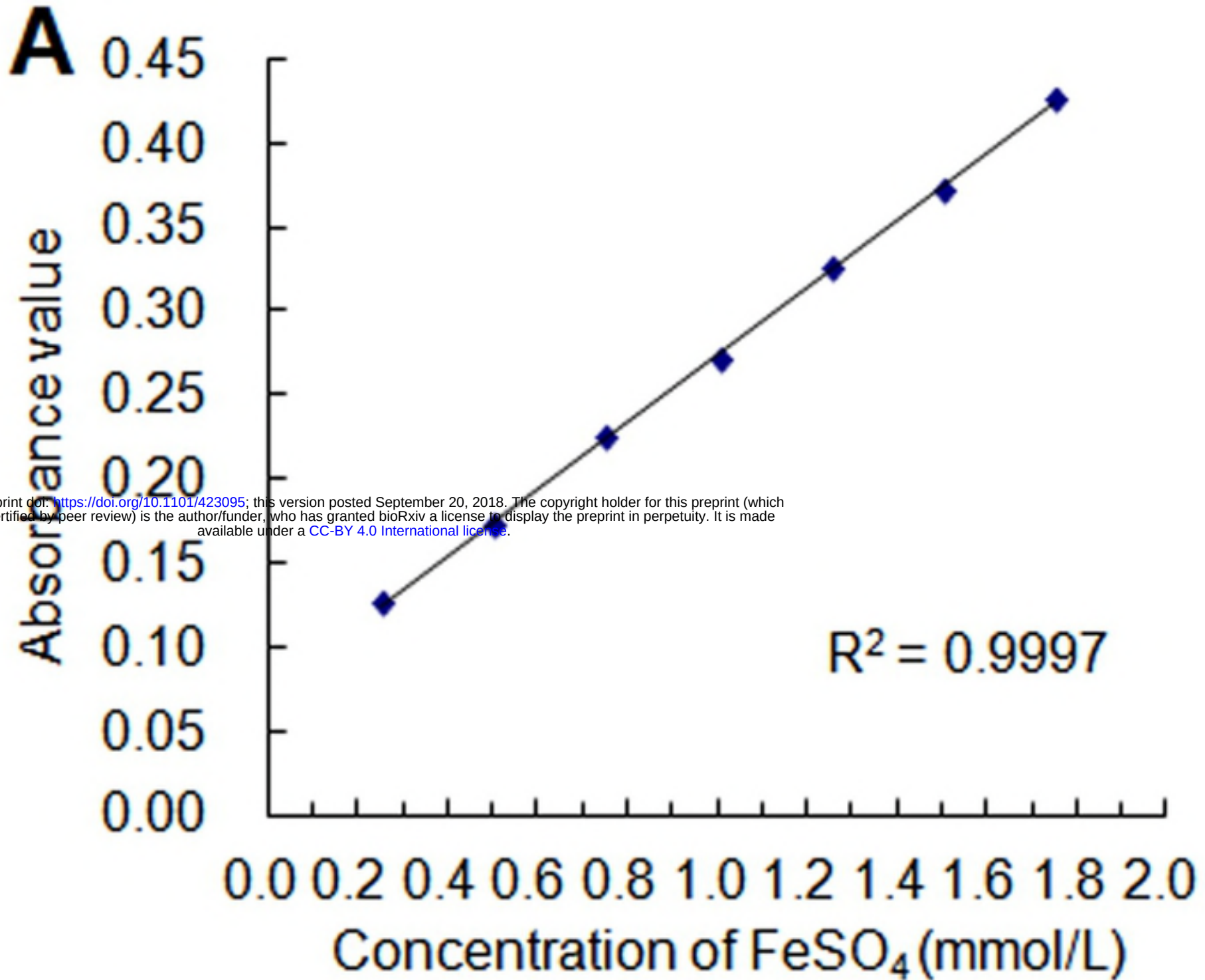
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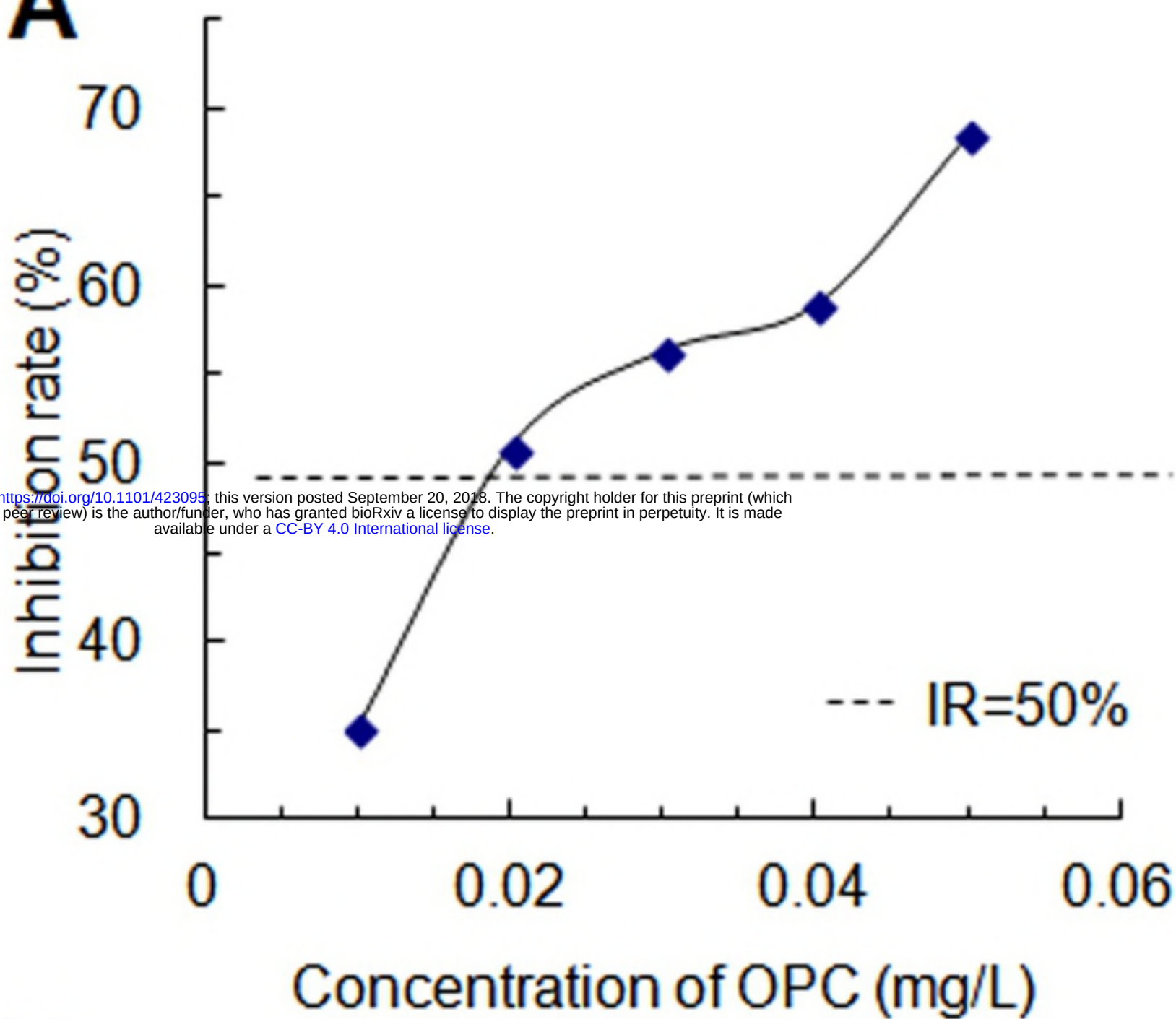
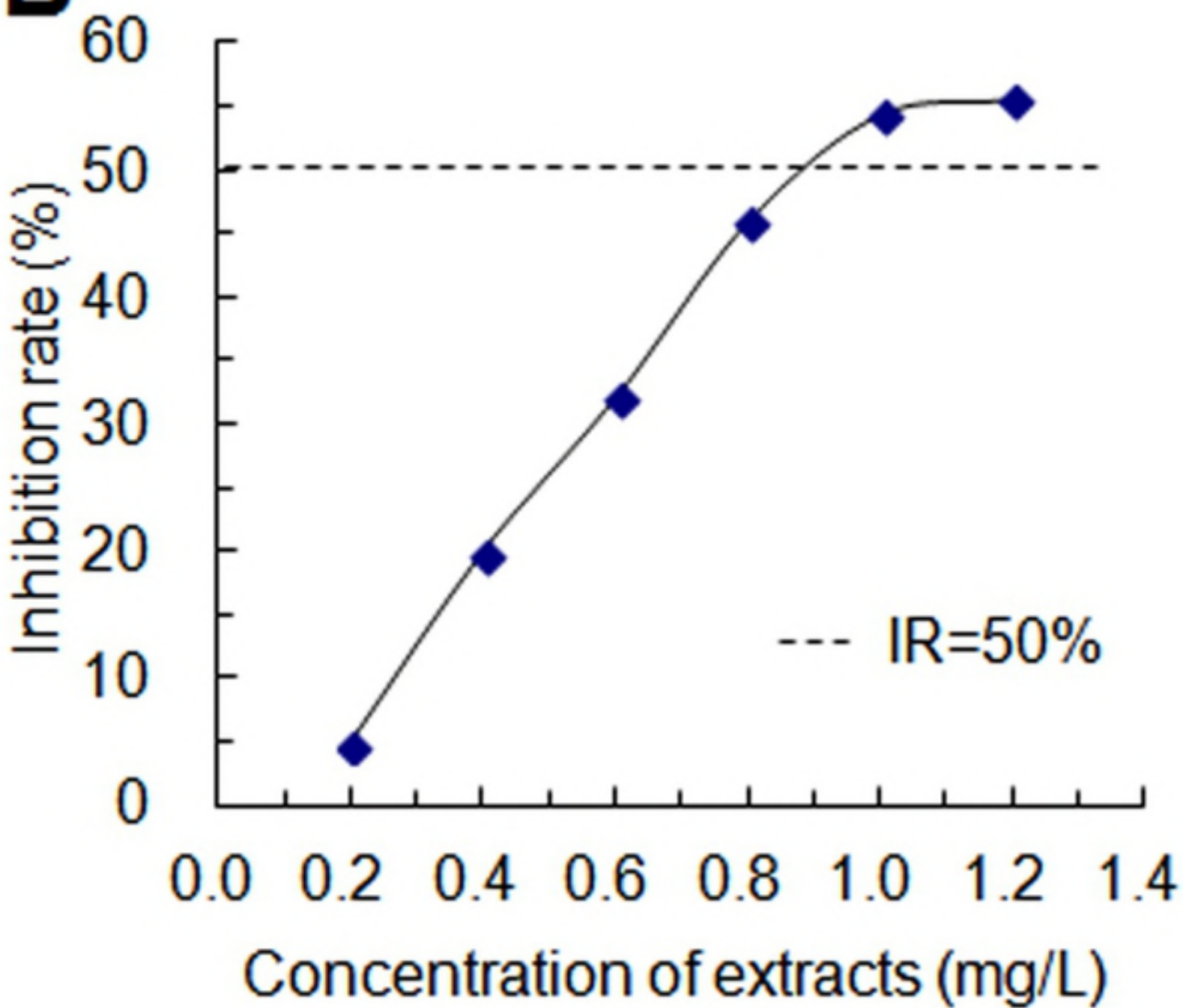
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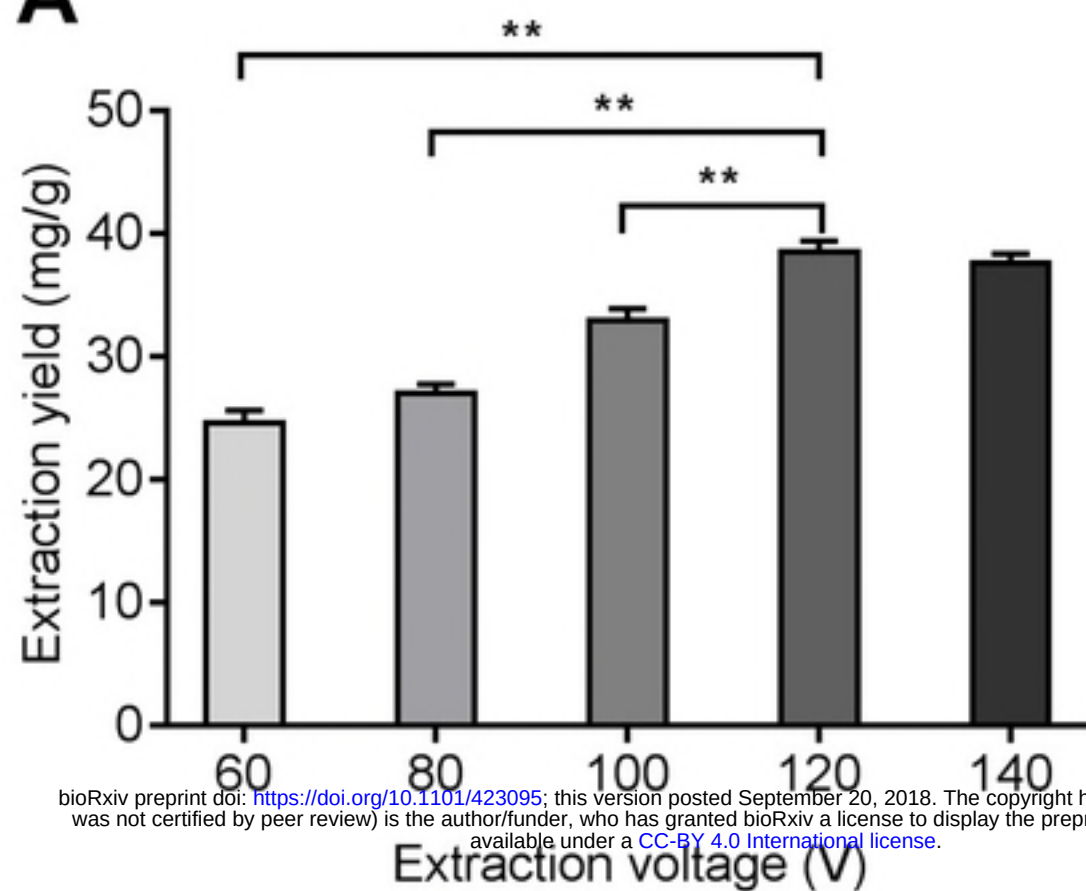
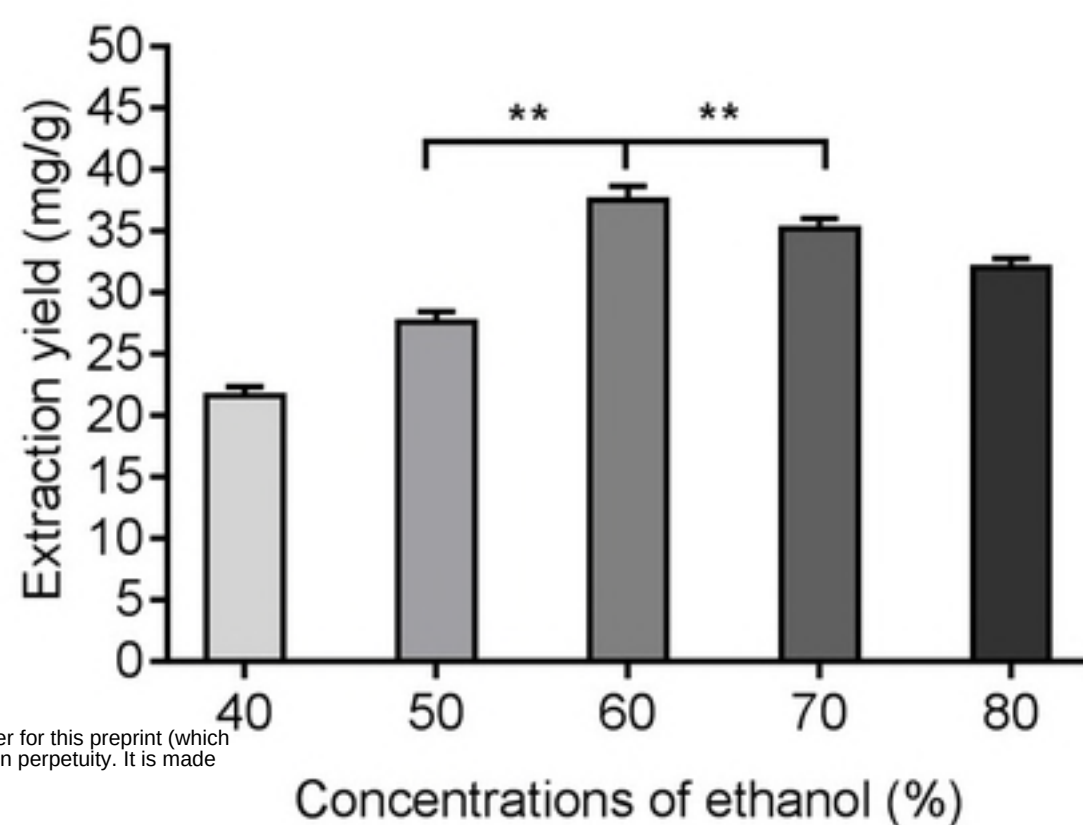
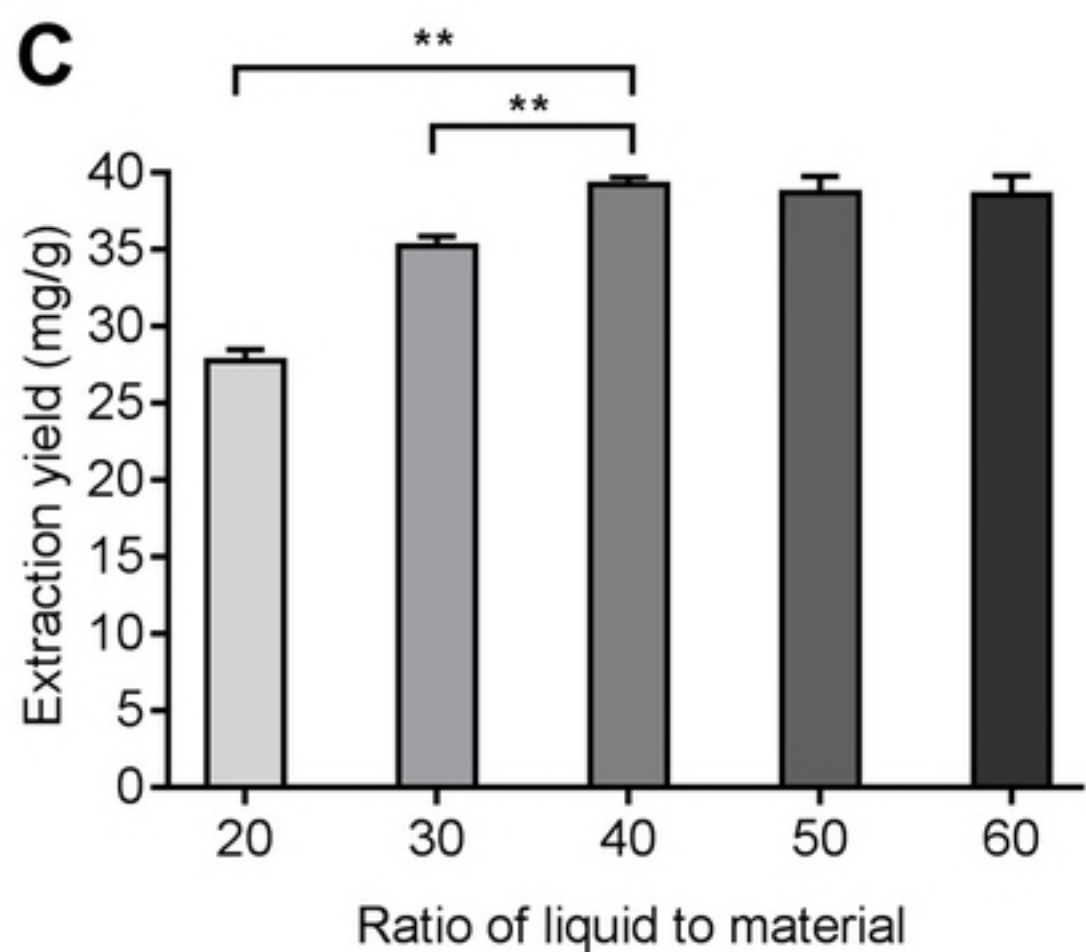
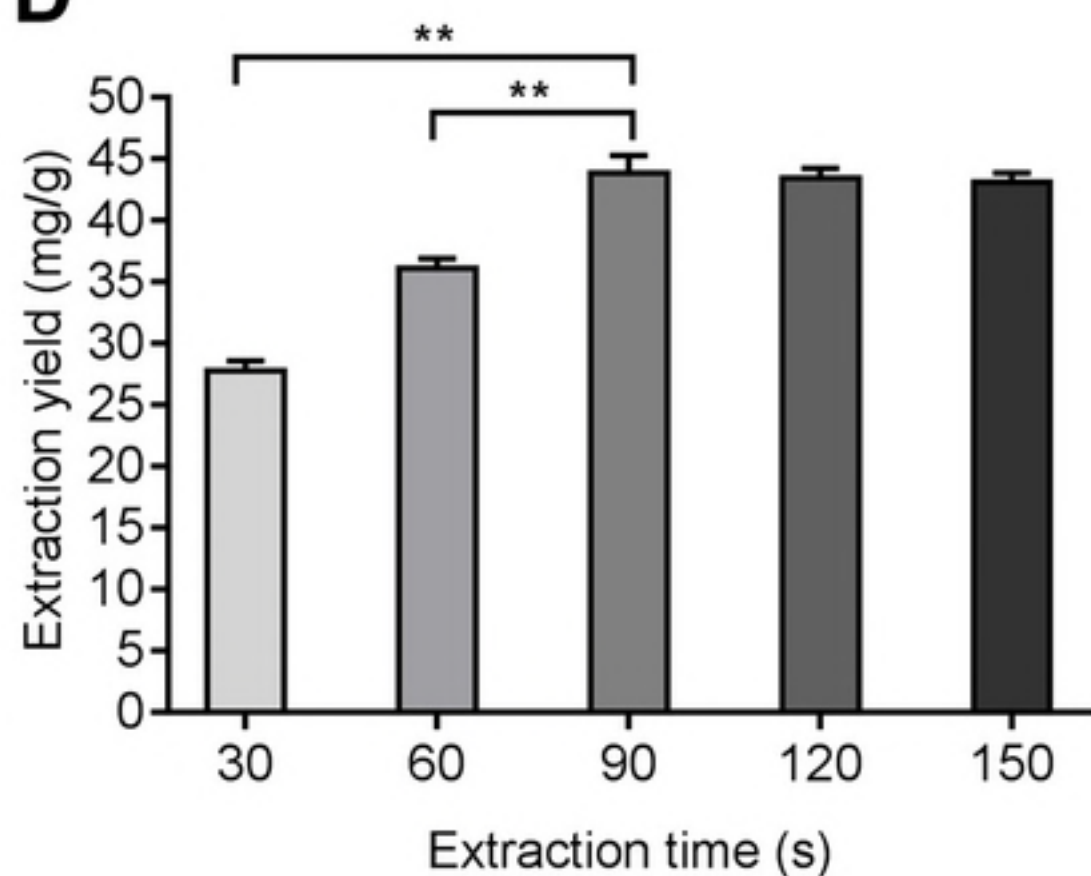
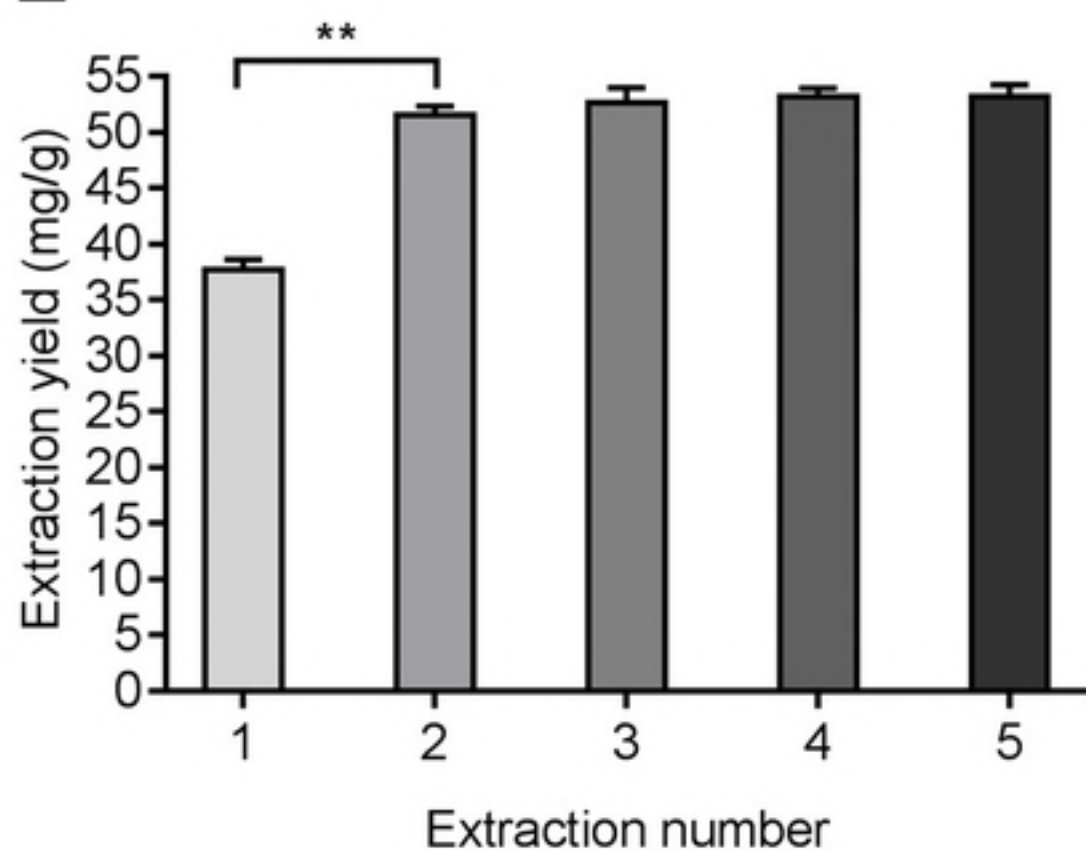
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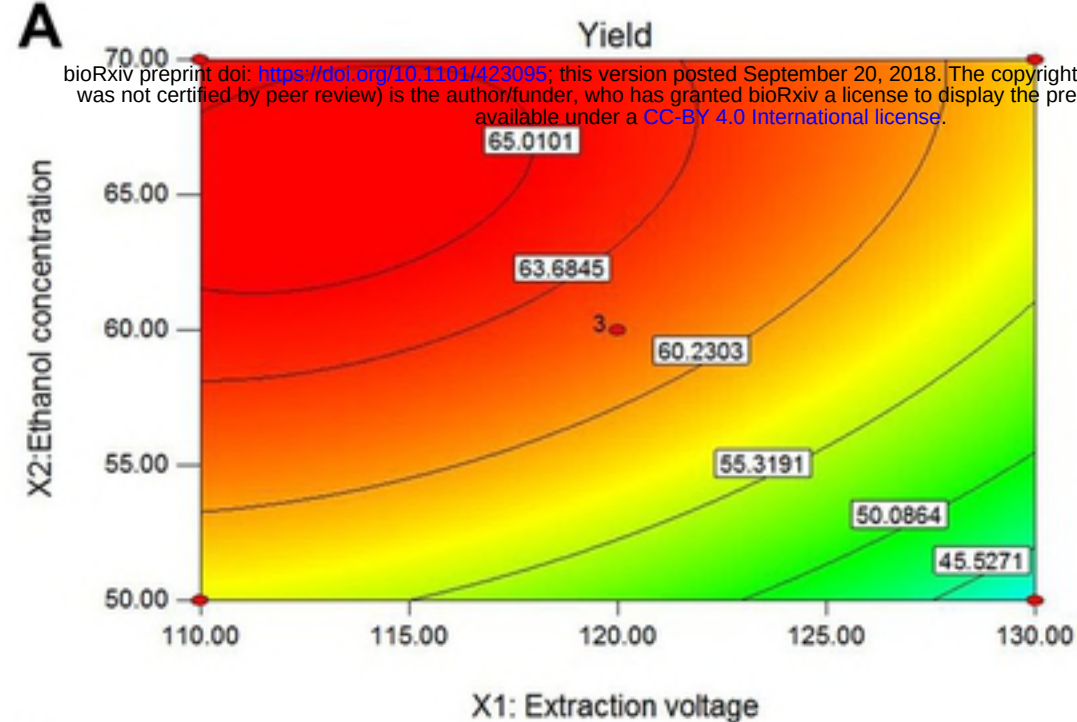
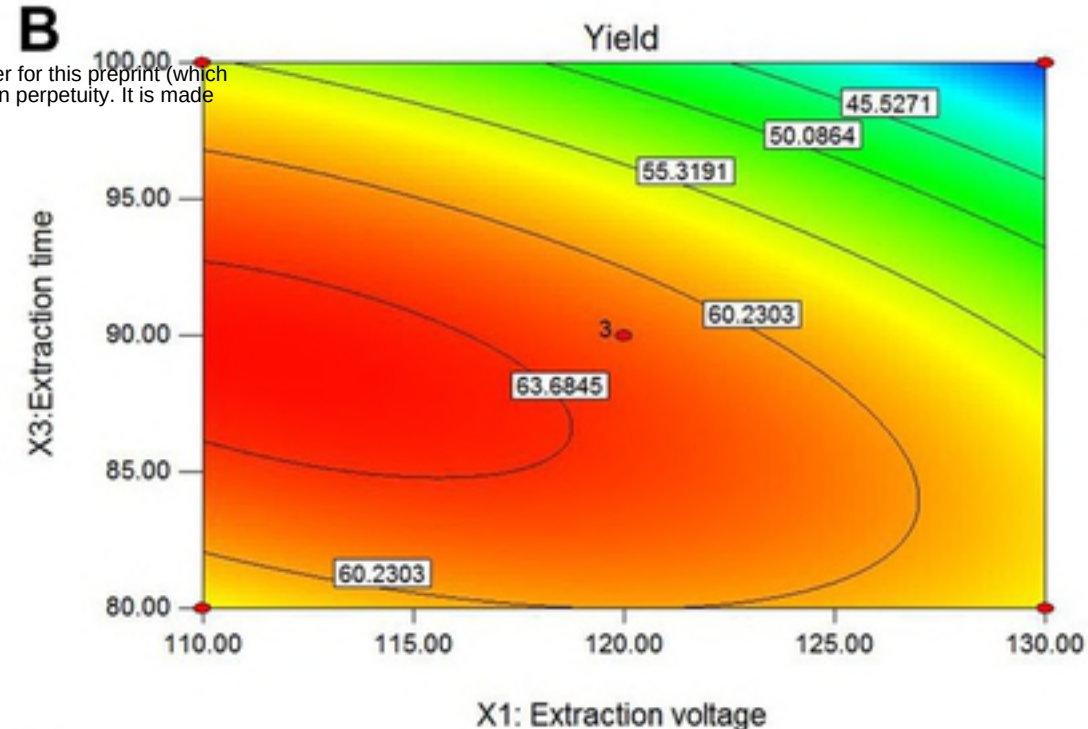
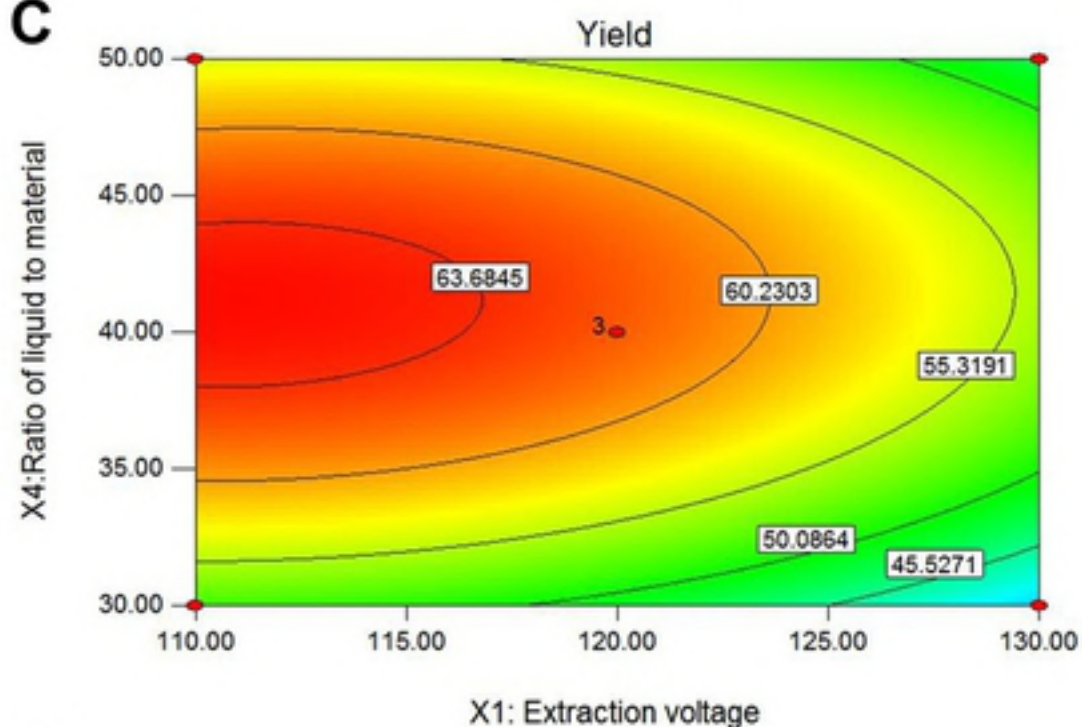
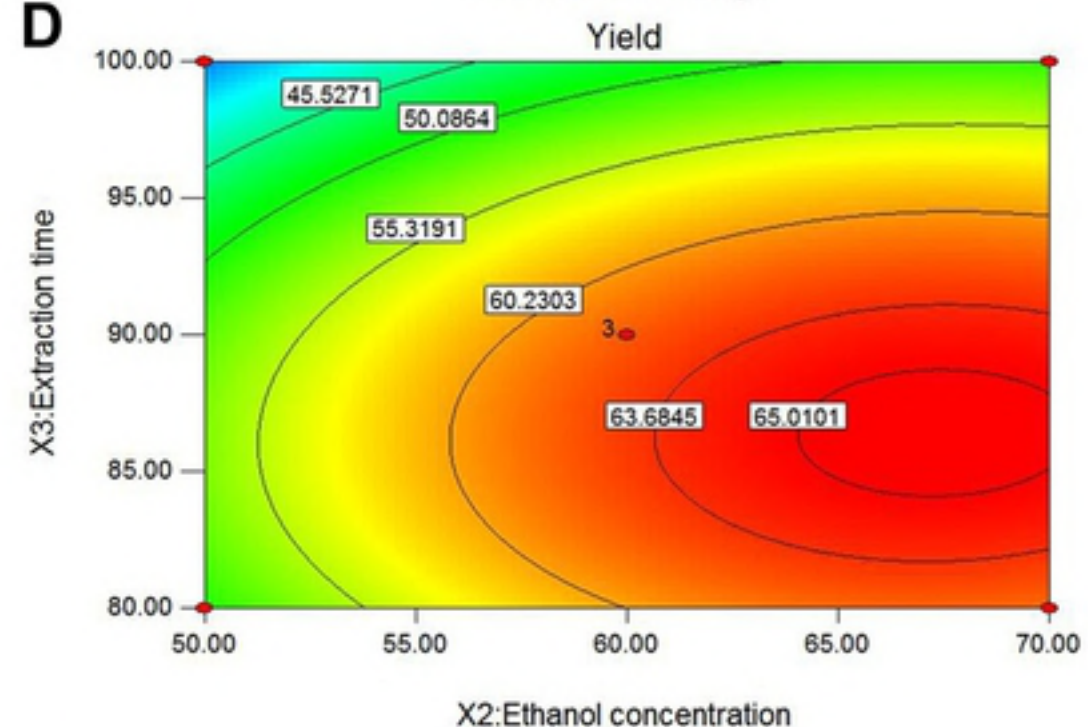
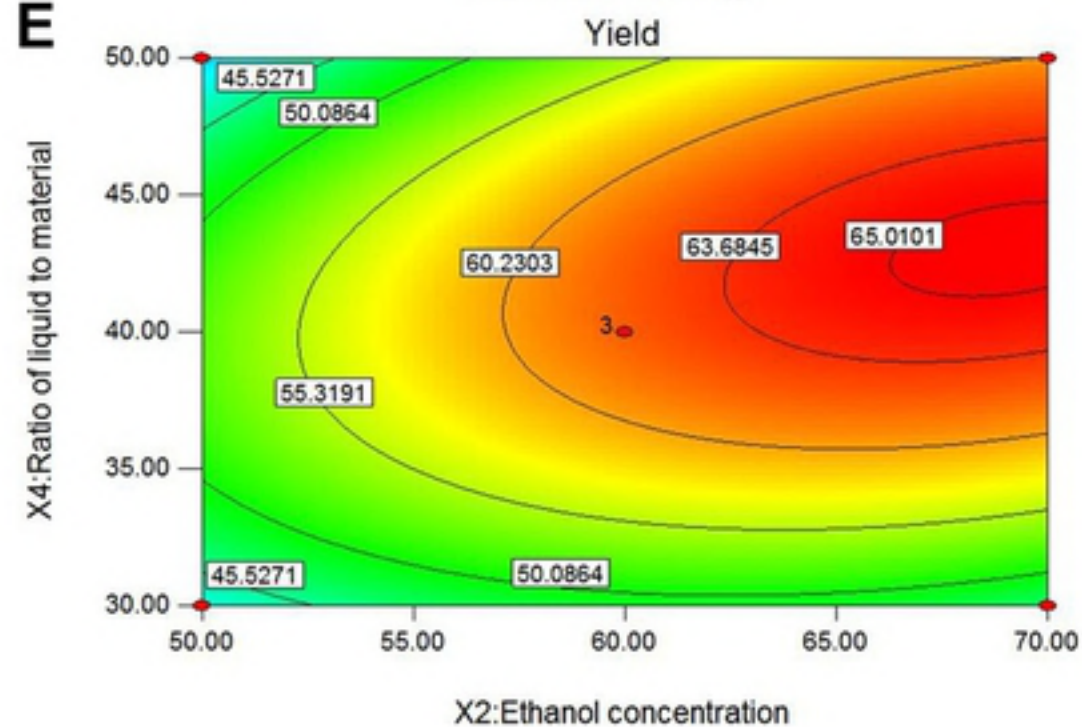
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