

Investigation of Polyphenolic constituents of *Diaphragma juglandis* Fructus and development of antioxidant instant tea

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Abstract *Diaphragma juglandis* (*D. juglandis*) Fructus is a brownish lamellar structure located between walnut kernels. As a traditional Chinese herbal medicine, it exhibits therapeutic effects including spleen-strengthening, kidney consolidation, astringency, diuresis, and heat-clearing. In this study, the polyphenolic constituents of *D. juglandis* and its functional instant tea were investigated in this study. Three polyphenolic compounds were isolated from 70% ethanol extract of *D. juglandis* Fructus as 2-methoxy juglone, regiolone and quercetin. The HPLC method was established for determination of the content of quercetin in the instant tea samples under the premise that the methodology was verified to meet the standard requirements. The preparation process of *D. juglandis* Fructus instant tea was optimized through single factor experiments and Box-Behnken design-response surface methodology and the optimal conditions were determined as follows: feed rate of 10 mL/min, inlet temperature of 151 °C, and β -cyclodextrin addition of 9%. Then the antioxidant activity of each sample was evaluated using DPPH and ABTS radical scavenging assays. The results showed that the quercetin had significant antioxidant activity. The instant tea group demonstrated superior antioxidant effects compared to the extracts group at 50, 200, and 800 μ g/mL ($P < 0.001$), likely due to the optimized spray-drying process, which might have enhanced the solubility, stability, and bioavailability of the active compounds. The results provided critical foundational data for the deep processing and comprehensive utilization of *D. juglandis* Fructus.

Keywords: *Diaphragma juglandis* Fructus; polyphenolic constituents; instant tea; antioxidant; process optimization

1 Introduction

Diaphragma juglandis (*D. juglandis*) Fructus, which is the dry wood diaphragm of walnut kernel, is one of the well-known by-products of walnut production [1]. *D. juglandis* Fructus is used in traditional Chinese medicine and has been used for treating several illnesses since ancient times. It is rich in a variety of bioactive components, such as phenolic acids, flavonoid, saponins, alkaloids, polysaccharides

and polyphenol substances [2-5]. The functional effect study of *D. juglandis* Fructus reveals that it has antioxidant, anti-microbial, antimutagenic, anti-inflammatory immunomodulatory and antiplatelet activity [6]. However, as a byproduct of walnut processing, *D. juglandis* Fructus has not been fully utilized. Therefore, systematic investigation of *D. juglandis* Fructus is warranted to facilitate further processing and comprehensive resource utilization.

The instant tea powder is mostly used for preparing the mixed tea beverage, bakery products, snacks, and other foods containing tea flavors [7]. The traditional

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drying method for instant tea is spray drying^[8]. The spray drying process method is carefully designed to preserve the integrity of tea's active ingredients, resulting in a powdered form of the final product^[9,10]. Instant tea exhibited a high retention of tea active ingredients, facilitating enhanced absorption by the human body and maximizing its various health benefits^[11]. Notably, instant tea is particularly well-suited for individuals with healthcare requirements. The demand for instant tea has increased on an annual basis, driven by its growing applications in the food industry^[12]. Instant tea has become a fast growing drinking product worldwide, as it's fully soluble, convenient to use and flexible to adjust the taste and components, comparing to the traditional leaf tea product^[9].

This study investigated polyphenolic compounds in *D. juglandis* Fructus and optimized instant tea preparation using response surface methodology (RSM). The antioxidant activities of isolated phenolic monomers, extracts and the instant tea of *D. juglandis* Fructus were carried out. The results established an important foundation and basis for the further application of *D. juglandis* Fructus, and also facilitated the reuse of walnut processing waste.

2 Materials and methods

2.1 Regents

D. juglandis Fructus was purchased from Yunnan Yangbi Wanbi Jiapin Agricultural By-products Development Co., Ltd.; quercetin was made by our laboratory with purity of 98%.

2.2 Extraction of active ingredients

5 kg of *D. juglandis* Fructus was extracted three times for 12 h at room temperature using a 70% ethanol solution, with a material-liquid ratio of 1:10. The filtrates were then concentrated using a rotary evaporator. After filtration, the combined filtrates were concentrated in distilled water, resulted in a total volume of 20 L of *D. juglandis* Fructus extract. This total extract was subsequently partitioned into equal

portions and subjected to sequential extraction using petroleum ether, ethyl acetate, and n-butanol, with each extraction lasting 6 hours. The resulted extracts were then concentrated under reduced pressure and evaporated to dryness on a water bath. The structures of three polyphenolic compounds were elucidated as 2-methoxy juglone, regiolone and quercetin by using nuclear magnetic resonance spectroscopy.

2.3 Research on the quality of *D. iaphragma juglandis* Fructus instant tea and examination of processing technology

2.3.1 The main points of operation of the preparation process of instant tea from *Diaphragma juglandis* Fructus

2.3.1.1 Extract preparation

Selected 50.0 g of dry and mold-free *D. juglandis* Fructus was directly crushed, with 500 mL (material-liquid ratio of 1:10) of 70% ethanol solution at room temperature impregnation, 3 h / times, repeated twice, filtration, the combined filtrate concentrated under reduced pressure to the dip paste-like, placed in a water bath to completely evaporate dry.

2.3.1.2 Mixed excipients blending

0.5 g of edible gelatin was dissolved in 500 mL of water, resulting in an aqueous gelatin solution with a concentration of 1 mg/mL. This solution was then added to the concentrated solution of *D. juglandis* Fructus, stirred thoroughly, and placed in a water bath at 60 °C for 10 min. After treatment, the mixture was allowed to cool adequately. Subsequently, 500 mL of β -cyclodextrin aqueous solution was added. The resulting clarified aqueous solution of *D. juglandis* Fructus was obtained by filtration for further use.

2.3.1.3 Spray drying

Based on the results of single-factor experiments, response surface methodology (RSM) was employed

to optimize the extraction process. The factors selected for optimization included the feed rate (A), inlet temperature (B), and the amount of β -cyclodextrin addition added (C), with the sensory score as the dependent variable.

2.3.2 Chromatographic conditions

Agela C₁₈ column (4.6 mm $\times$ 250 mm, S-5 μ m): isocratic elution was used, and the mobile phase was selected to be methanol-0.1% phosphoric acid (45:55); the column temperature was set at 35 °C; the flow rate was set to be 1.0 mL/min; the detection wavelength of UV was 370 nm; the injection volume was 20 μ L.

2.3.3 Preparation of control solution

The quercetin was weighed accurately and dissolved in methanol to obtain 0.1 mg/mL quercetin control solution.

2.3.4 Preparation of test solution

The extract preparation procedure mirrored that outlined in section “3.1.1”. For auxiliary material mixing, detailed in “2.3.1.2”, 500 mL of an 8% β -cyclodextrin aqueous solution was introduced. Instant tea was then produced via spray drying at 150 °C and 10 mL/min. A 50 mg instant tea sample was dissolved in 10 mL methanol and volume adjusted. Ethyl acetate of equal volume was added, followed by a two-hour, twice-repeated extraction. The resultant extract was evaporated to dryness on a water bath. Subsequently, 10 mg of the dried extract was dissolved in 10 mL methanol, volume adjusted, and thoroughly mixed. The final test solution was filtered through a 0.22 μ m microporous filter membrane.

2.3.5 Methodological examination

2.3.5.1 Linearity study

Solutions of quercetin control were prepared at concentrations of 0.001, 0.005, 0.01, 0.05, and

0.1 mg/mL. Appropriate amounts of the control were dissolved and shaken with methanol in volumetric flasks. Chromatographic determination of these solutions was conducted, and the corresponding peak areas were recorded. Correlation coefficients and linear regression equations were obtained by plotting standard curves with the mass concentration of quercetin control (mg/mL) as the horizontal coordinate (X) and the chromatographic peak area as the vertical coordinate (Y).

2.3.5.2 Precision test

0.05 mg/mL of the control solution was injected six times following the chromatographic conditions outlined in section “2.2.4”. The peak area obtained after each injection was determined, and the relative standard deviation (RSD) value was calculated.

2.3.5.3 Stability test

Following the procedure outlined in “2.3.4”, a test solution was prepared with a concentration of 5 mg/mL of instant tea sample. The sample was injected into the system at 0, 2, 4, 6, 8, 10, 12, and 24 hours, respectively, under the chromatographic conditions specified in “2.3.2”. Subsequently, the peak area was measured and recorded, and the relative standard deviation (RSD) was calculated.

2.3.5.4 Repeatability test

With reference to the operation described in “2.3.4”, 6 test solutions of instant tea samples at a concentration of 5 mg/mL were prepared in parallel, and analyzed by the chromatographic conditions in “2.3.4”, the peak areas were measured and recorded, and the RSD was calculated.

2.3.5.5 Sample recovery test

Instant tea samples with known quercetin content were each supplemented with 0.025, 0.050, and 0.075 mg of quercetin control, respectively. Following the

procedure outlined in “2.3.4”, three test solutions were prepared. Subsequently, the samples were analyzed using the chromatographic conditions specified in “2.3.2”. Each test solution was then injected three times, and the peak area after each injection was determined. Finally, the recovery and relative RSD were calculated.

2.3.6 One-factor investigation of spray drying processing of instant tea from *Diaphragma juglandis* Fructus

$$Y = \frac{\text{Sensory evaluation score}}{\text{Maximum sensory evaluation score}} \times 50\% + \frac{\text{quercetin content}}{\text{Maximum quercetin content}} \times 30\% + \frac{\text{Flour collection rate}}{\text{Maximum powder collection rate}} \times 20\%$$

2.3.6.2 Analysis of feeding speed

The extract preparation procedure mirrored that outlined in “2.3.1”, while the mixing method of auxiliary materials was detailed in “2.3.2”, with the addition of 500 mL of 8% β -cyclodextrin aqueous solution. The machine’s inlet temperature was set at 150 °C, and the powder was spray-dried at feed rates of 8, 10, and 12 mL/min, respectively, resulting in three portions of instant tea. Separate assessments of powder collection rate and sensory evaluation were conducted for each instant tea portion. Test solutions were prepared according to the procedure described in “2.3.4”, and analyzed under the chromatographic conditions specified in “2.3.2”. The chromatographic peak area obtained after each injection was measured and recorded, and the quercetin content in each instant tea sample was subsequently calculated and compared.

2.3.6.3 Analysis of inlet temperature

Under conditions of 8% β -cyclodextrin addition and a feed rate set at 10 mL/min, the powder was spray dried at temperatures of 140 °C, 150 °C, and 160 °C, respectively. The remaining steps followed the protocol outlined in “2.3.6”, yielding three samples of instant tea. Analysis method remained consistent with the previously described approach.

2.3.6.1 Establishment of evaluation indexes

The evaluation of the spray drying process of *D. juglandis* Fructus instant tea was based on three main indices: quercetin content, powder collection rate, and sensory evaluation score. Optimization was achieved by calculating the composite score (Y). The sensory evaluation score accounted for 50% of the weight, while the quercetin content and powder collection rate accounted for 30% and 20% of the weight, respectively.

2.3.6.4 Analysis of β -cyclodextrin additions

Each portion of the extract was supplemented with 500 mL of β -cyclodextrin aqueous solution, with mass fractions of 6%, 8%, and 10%, respectively. Under the process conditions of spray drying into powder, with a feed rate of 10 mL/min and an inlet temperature of 150 °C, the remaining steps were carried out following the method outlined in “2.3.6.1” for preparation, resulting in three samples of instant tea. The determination method remained consistent with the previously described approach.

2.3.7 Response surface experimental design

The design of response surface analysis test was carried out in Design-Expert 12 software. Three factors, feed rate (A), inlet temperature (B), and β -cyclodextrin addition (C), were determined by a one-way test with three levels for each factor. A Box-Behnken analytical model test set was established to optimize the spray drying processing conditions using the quercetin content, powder collection rate and sensory evaluation score in the instant tea from split logs as the response values of the composite score (Y). Two-dimensional contour planes and 3D interaction surface plots of the effects of the three factors, A , B and C , on the composite score of the quality of instant tea with split logs were plotted using Design-Expert

12 software to reflect the strength of the interaction between the factors.

2.3.8 Research on the antioxidant activity of *D. juglandis* Fructus

2.3.8.1 Determination of DPPH radical scavenging rate

A 35 µg/mL DPPH solution was prepared, and the four samples: Vc, quercetin, *D. juglandis* extracts and its instant tea were dissolved in water to obtain 1 mg/mL solutions. Subsequently, dilutions of the samples were made to achieve concentrations of 800, 400, 200, 100, and 50 µg/mL. Similarly, a 1 mg/mL solution of Vc was prepared and diluted to concentrations of 800, 400, 200, 100, and 50 µg/mL to serve as the control solution. In a 96-well plate, 100 µL of the DPPH working solution and 100 µL of the different concentrations of the sample solutions or Vc solution were added to each reaction well. The blank control wells received 100 µL of anhydrous ethanol. Following mixing, the reactions were conducted at room temperature under light protection for 30 minutes. Subsequently, the absorbance values of each well were measured using a spectrophotometer at an absorption wavelength of 517 nm. Antioxidant capacity was quantified as the scavenging rate (%) using the following formula:

$$\text{Scavenging Rate (\%)} = \frac{A_0 - A_i}{A_0} \times 100\%$$

A_0 : absorbance value of blank control group

A_i : absorbance value of the sample solution to be tested or control Vc solution

2.3.8.2 Determination of ABTS free radical scavenging rate

Preparation of ABTS working solution: The

ABTS working solution is prepared by mixing the detection solution, ABTS solution, and reagent three application solution in a ratio of 76:5:4, stored in the dark, and used within 30 minutes. Prior to the formal experiment, the ABTS stock solution is diluted approximately 30 times with anhydrous ethanol to ensure a difference in absorbance of 0.68 at 734 nm compared to the blank control. The preparation of the sample solutions and the Vc control solution follows the method described in “2.3.6.2”. In a 96-well plate, 10 µL of the diluted ABTS working solution and 10 µL of the sample solutions at various concentrations or the Vc reference solution were sequentially added. Anhydrous ethanol serves as the blank control. After mixing, the reaction is allowed to proceed in the dark at room temperature for 6 minutes, and the absorbance values at 405 nm were measured for each well. The antioxidant capacity is expressed as the ABTS radical scavenging rate (%). Antioxidant capacity was quantified as the scavenging rate (%) using the following formula:

$$\text{Scavenging Rate (\%)} = \frac{A_0 - A_i}{A_0} \times 100\%$$

A_0 : absorbance value of blank control group

A_i : absorbance value of the sample solution to be tested or control Vc solution

3 Results and discussion

3.1 Methodological examination

The results of the linear investigation are shown in Fig. 1. The regression equation between the mass concentration (X) and the chromatographic peak area (Y) of quercetin was calculated as $Y = 5.0 \times 10^6 X - 6719.4$, a correlation coefficient (R^2) of 0.9995. These results indicate that quercetin exhibits good linearity ($R^2 > 0.999$) over the concentration range of 0.001–0.1 mg/mL.

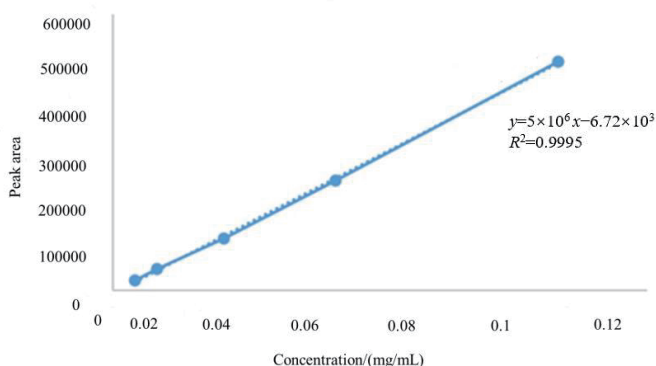


Fig. 1 Standard curve for the determination of quercetin content

3.2 Single-factor investigation of spray-drying processing of instant tea from *Diaphragma juglandis Fructus*

As can be seen from Fig. 2A, under the condition of unchanged inlet temperature and β -cyclodextrin addition, with the increase of inlet speed from 8 mL/min to 10 mL/min, the comprehensive score of the instant tea logs increased; while the inlet speed continued to increase to 12 mL/min, the comprehensive score of the instant tea logs decreased.

As shown in Fig. 2B, when the inlet temperature and the amount of β -cyclodextrin remained unchanged, increasing the feed rate from 8 to 10 mL/min led to a higher comprehensive score for the instant tea. However, further increasing the feed rate to 12 mL/min caused the comprehensive score to decline.

β -Cyclodextrin acts as a drying aid by promoting uniform droplet formation. As shown in Fig. 2C, increasing the β -cyclodextrin addition from 6% to 8% led to a higher composite score, while further increasing it to 10% resulted in a declining trend.

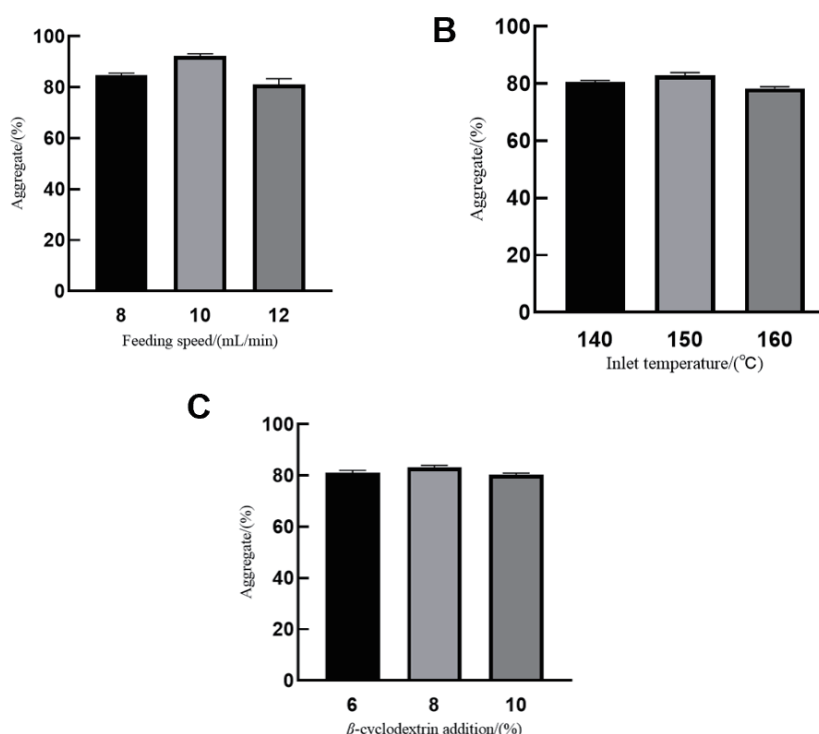


Fig. 2 Effects of process parameters on the quality of instant tea of *D. juglandis Fructus*. (A) Influence of feed rate on instant tea quality; (B) Effect of inlet temperature on instant tea quality; (C) Impact of β -cyclodextrin addition on instant tea quality

3.3 Modeling and significance test

As shown in Table 1, the quadratic regression model for the composite score of instant tea from *D. juglandis* Fructus is: $Y = 90.16 + 0.9300A + 0.3587B + 2.20C + 0.8250AB - 1.22AC + 1.15BC - 3.34A^2 - 6.27B^2 - 2.24C^2$. ANOVA results (Table 2) show that the model is highly significant ($P < 0.0001$). The lack-of-fit term is not significant ($P = 0.0811 > 0.05$), suggesting a good model fit. The model's determination coefficient (R^2) was 0.9894, indicating that over 98% of the variability in the experimental

data can be explained and predicted by this model.

From the P -values, factor C has a highly significant impact on the composite score, factor A has a significant effect, and factor B 's effect is not significant. Interaction terms AB , AC , and BC all significantly influence the composite score, and the quadratic terms A^2 , B^2 , and C^2 also show significant effects. Factor C exhibits the largest F -value, followed by factor A and then factor B . Overall, considering both P -values and F -values, the relative influence of each factor on the composite score is $C > A > B$.

Table 1 Box-Behnken Response Surface Test design and results

Test number	A: Feeding speed (mL/min)	B: Inlet temperature /°C	C: β -cyclodextrin addition/%	Y: Composite score/%
1	0	0	0	90.30
2	0	0	0	89.60
3	-1	0	1	87.74
4	0	1	1	84.63
5	0	-1	-1	80.98
6	0	0	0	90.00
7	-1	-1	0	79.64
8	0	1	-1	78.87
9	0	0	0	90.15
10	-1	0	-1	79.94
11	0	0	0	90.77
12	1	0	1	86.78
13	-1	1	0	79.23
14	1	-1	0	80.23
15	1	1	0	83.12
16	1	0	-1	83.86
17	0	-1	1	82.13

Table 2 Response Surface Modeling ANOVA results

Source of variance	SS	df	σ	F -value	P -value
model	315.78	9	35.09	72.66	< 0.0001
A-Feeding speed	6.92	1	6.92	14.33	0.0068
B-Inlet temperature	1.03	1	1.03	2.13	0.1876
C- β -Cyclodextrin addition	38.85	1	38.85	80.46	< 0.0001
AB	2.72	1	2.72	5.64	0.0493
AC	5.95	1	5.95	12.33	0.0098

(to be continued)

Continued Table 2

Source of variance	SS	df	σ	F-value	P-value
BC	5.31	1	5.31	11.00	0.0128
A ²	46.99	1	46.99	97.31	< 0.0001
B ²	165.44	1	165.44	342.59	< 0.0001
C ²	21.19	1	21.19	43.88	0.0003
Residual	3.38	7	0.4829		
Lack of Fit	2.65	3	0.8831	4.83	0.0811
Pure Error	0.7309	4	0.1827		
Cor Total	319.16	16			
$R^2 = 0.9894$					

3.4 Response surface analysis

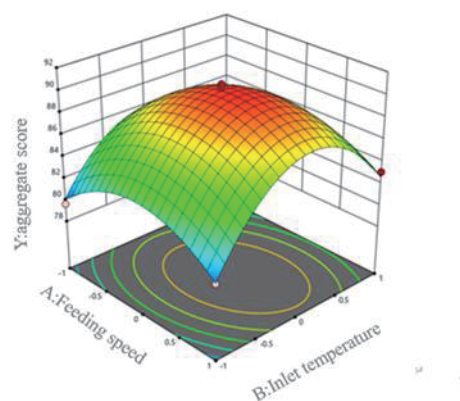
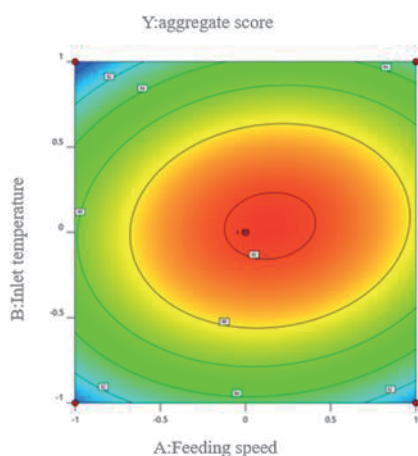
A Box-Behnken experimental design was employed to optimize the spray-drying parameters for the instant tea from *D. juglandis* Fructus. Quercetin content, powder collection rate, and sensory evaluation score were combined into a single comprehensive score (Y) as the response value. The experimental design and corresponding results are shown in Table 3.

As shown in Fig. 3, dense contour lines in the

2D contour plots and near-elliptical shapes suggest a strong interaction effect among the factors. Similarly, the larger curvature and steeper slopes observed in the 3D surface plots indicate more pronounced interactions between factors A, B, and C. Specifically, the contour lines for AC and BC appear closer to ellipses, implying that $AC > BC > AB$ in terms of interaction strength. These findings are consistent with the ANOVA results.

Table 3 Box-Behnken Response Surface Test factor levels

Level	A: Feed rate/(mL/min)	B: Inlet temperature/°C	C: β -cyclodextrin addition/%
-1	8	140	6
0	10	150	8
1	12	160	10



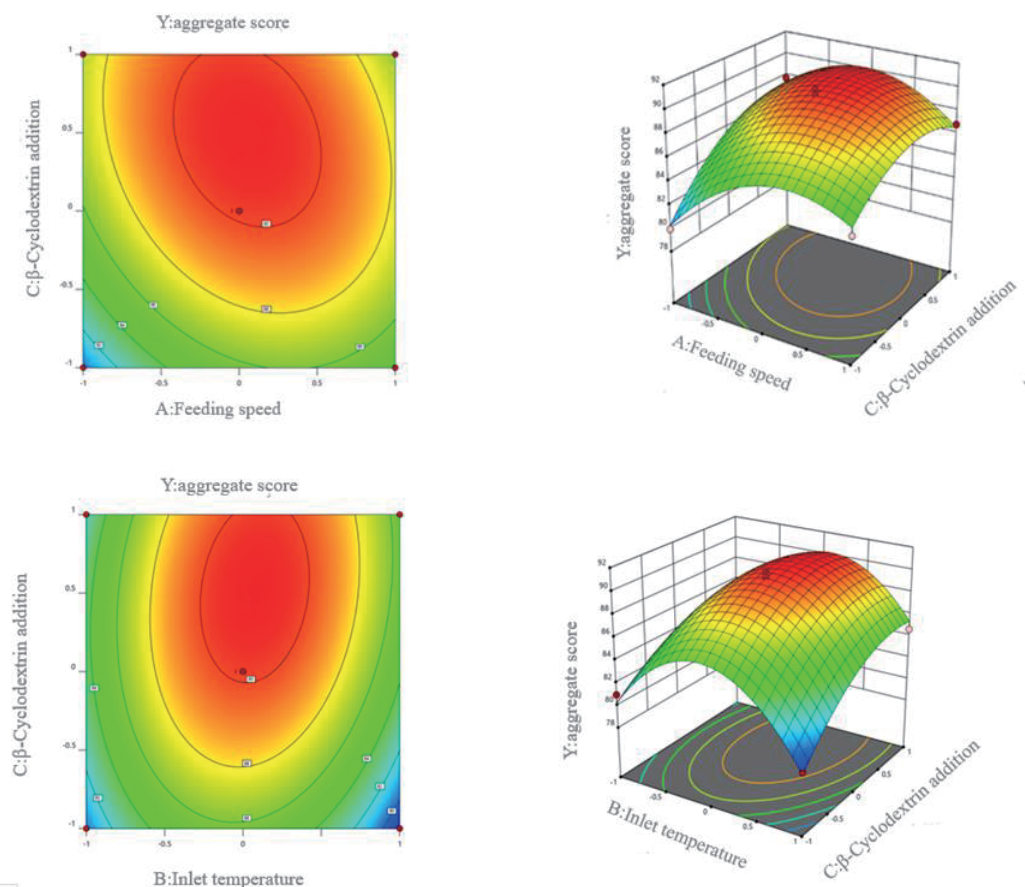


Fig. 3 Response surface test results

3.5 Optimal process analysis

Based on the Design-Expert 12 analysis, the maximum comprehensive score for instant tea was achieved at a feed rate of approximately 10.12 mL/min, an inlet temperature of 150.78 °C, and a β -cyclodextrin addition of 8.99%, with a predicted composite score of 90.75%. For practical production, these parameters were adjusted to a feed rate of 10 mL/min, an inlet temperature of 151 °C, and a β -cyclodextrin addition of 9%. Under these conditions, using 5 g of *D.*

juglandis Fructus and repeating the process three times, the comprehensive score averaged 90.73% with an RSD of 0.45% (Table 4). These results confirm that the model is valid, the process is reliable, and the findings are consistent.

As shown in Table 4, the instant tea achieved a comprehensive score of 90.73% under the optimized spray-drying process, with an RSD below 2%, indicating that this process is stable and yields a high quercetin content.

Table 4 Determination of the comprehensive score of instant tea with split logs under the optimal process

Serial number	Aggregate score/%	Average value/%	RSD/%
1	91.1	90.73	0.45
2	90.8		
3	90.3		

3.6 The antioxidant activity of *Diaphragma juglandis* Fructus

Excessive production of free radicals, including reactive oxygen and nitrogen species, can induce oxidative damage to biomolecules and precipitate various chronic diseases, such as cardiometabolic disorders, neurodegenerative diseases, and cancer [18]. Zhang [19] reported a dose-dependent relationship between the antioxidant capacity of *D. juglandis* Fructus and its concentration, with higher levels yielding greater antioxidant efficacy.

Flavonoids exert antioxidant effects through multiple mechanisms, including direct scavenging of reactive oxygen species (ROS). This capacity stems from the o-dihydroxy structure and the 3,4-double bond conjugated with oxygen-containing functional groups in

the heterocyclic ring. Additionally, hydroxyl substituents on the aromatic ring effectively neutralize ROS [20].

3.6.1 DPPH radical scavenging rate

As shown in Fig. 4, within a certain concentration range, the DPPH radical scavenging rates of the four tested samples exhibited a dose-dependent relationship. Among them, quercetin exhibited a scavenging rate comparable to that of Vc and significantly different from that of the extracts and instant tea ($P < 0.001$), suggesting stronger antioxidant activity. Notably, at 100 $\mu\text{g/mL}$ and 200 $\mu\text{g/mL}$ ($P < 0.001$), the scavenging rate of the instant tea is significantly higher than that of the extracts, indicating that the instant tea markedly outperforms the extracts in DPPH radical scavenging activity.

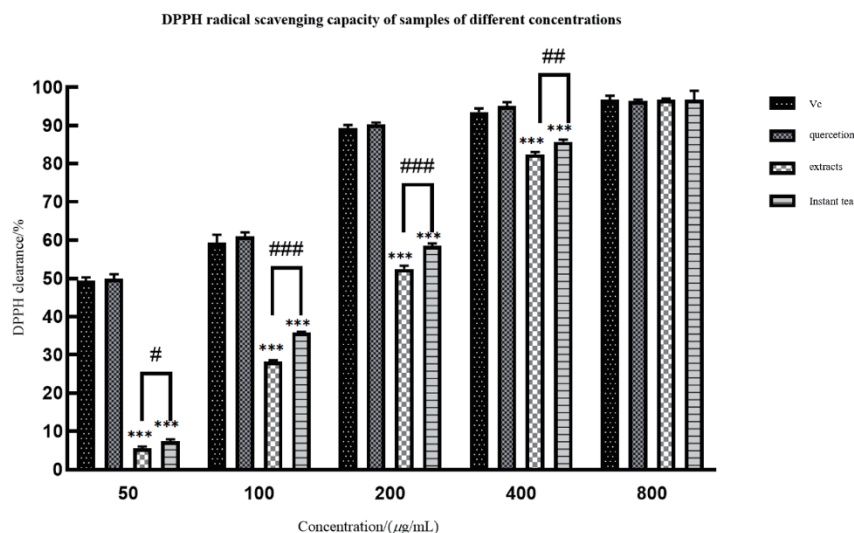


Fig. 4 DPPH radical scavenging capacity of samples of different concentrations. Compared with Vc, *** $P < 0.001$; Compared with instant tea and extracts, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$

3.6.2 ABTS free radical scavenging rate

As shown in Fig. 5, the ABTS radical scavenging rates of all four tested samples increase with concentration. At 50 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$, the scavenging rates of quercetin, the extracts, and instant tea significantly differ from that of Vc ($P < 0.01$). However, at 800 $\mu\text{g/mL}$, the differences become non-significant. It was worth mentioning that, the instant

tea demonstrating significantly enhanced capacity at 50, 200, and 800 $\mu\text{g/mL}$ compared to extracts ($P < 0.01$), indicating that the instant tea exhibits superior ABTS radical scavenging capacity. It may be due to instant tea has the characteristics of less loss of nutrients in the product, low moisture content, fine and uniform particle size, and good solubility, which makes the bioactivity of instant tea higher than that of extract [21].

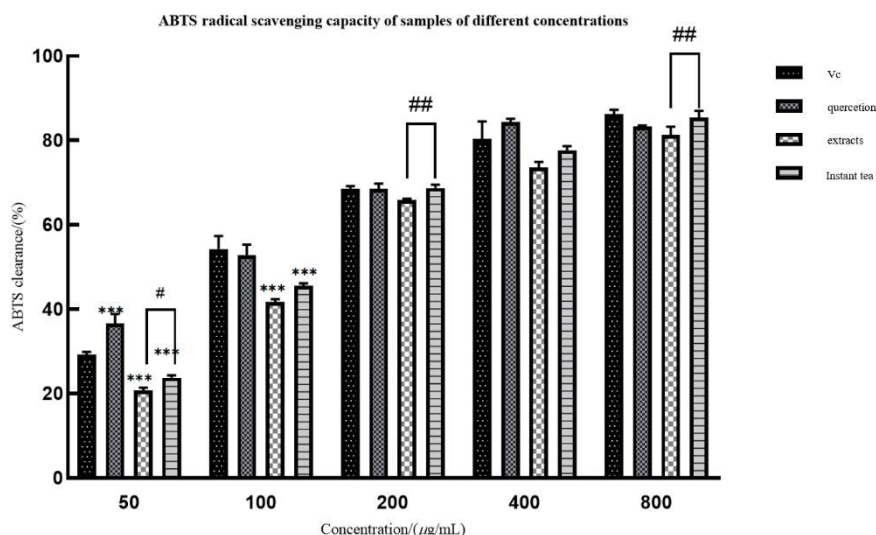


Fig. 5 ABTS free radical scavenging ability of samples with different concentrations. Compared with Vc, *** $P < 0.001$; Compared with instant tea and extracts, # $P < 0.05$, ## $P < 0.01$

4 Conclusion

In conclusion, three polyphenolic compounds were isolated from *D. juglandis* Fructus, among which quercetin was predominant. The antioxidant assessment demonstrated that quercetin possesses remarkable antioxidant capacity. Instant tea exhibited superior antioxidant activity over extract owing to its uniform particle size, high solubility, and enhanced stability. These results provided a theoretical foundation and experimental basis for the potential development of *D. juglandis* Fructus instant tea and its application in functional food product. Furthermore, it is also conducive to the comprehensive utilization of walnut waste and plays an environmental protection role.

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