

# Optimization of Process Parameters and Kinetic Model of Enzymatic Extraction of Polyphenols from *Lonicerae Flos*

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## ABSTRACT

**Objective:** To optimize and verify the cellulase extraction of polyphenols from honeysuckle and provide a reference for enzymatic extracting polyphenols from honeysuckle. **Materials and Methods:** The uniform design was used According to Fick's first law and kinetic model, fitting analysis of the dynamic process of enzymatic extracting polyphenols was conducted. **Results:** The optimum enzymatic extraction parameters for polyphenols from honeysuckle are found to be 80% (v/v) of alcohol, 35:1 (mL/g) of liquid-solid ratio, 80°C of extraction temperature, 8.5 of pH, 6.0 mg of enzyme levels, and 130 min of extraction time. Under the optimal conditions, the extraction rate of polyphenols was 3.03%. The kinetic experiments indicated kinetic

equation  $\ln \frac{C_{\infty}}{C_{\infty} - C_0}$  had a good linear relationship with t even under the

conditions of different levels of enzyme and temperature, which means fitting curve tallies well with the experimental values. **Conclusion:** The results of quantification showed that the results provide a reference for enzymatic extracting polyphenols from honeysuckle.

**Key words:** Cellulase, kinetic model, *Lonicerae Flos*, polyphenols, uniform design

## SUMMARY

- *Lonicerae flos* (*Lonicera japonica* Thunb.) is a material of traditional Chinese medicine and healthy drinks, of which active compounds mainly is polyphenols. At present, plant polyphenols are the hotspots contents of food, cosmetic and medicine, because it has strong bioactivity. Several traditional methods

are available for the extraction of plant polyphenols including impregnation, solvent extraction, ultrasonic extraction, hot-water extraction, alkaline dilute alcohol or alkaline water extraction, microwave extraction and Supercritical CO<sub>2</sub> extraction. But now, an increasing number of research on using cellulase to extract active ingredients from plants. Enzymatic method is widely used for enzyme have excellent properties of high reaction efficiency and specificity, moderate reaction conditions, shorter extraction time and easier to control, less damage to the active ingredient. At present, the enzymatic extraction of polyphenols from honeysuckle and dynamic had not been reported. In this study, using cellulase to extract polyphenols from honeysuckle is first applied. Moreover, uniform design was used to optimize process and kinetic model of extraction was established to analyze the characteristics of enzymatic extraction, in order to improve the yield of polyphenols from honeysuckle and make maximum use of *Lonicerae flos*, which provide references for industrial production.

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## INTRODUCTION

*Lonicerae Flos* (*Lonicera japonica* Thunb.) is a material of traditional Chinese medicine and healthy drinks, of which active compounds mainly is polyphenols, including phenolic acids and flavonoids, such as chlorogenic acid, isochlorogenic acid, and luteoloside.<sup>[1-4]</sup> At present, plant polyphenols are the hotspots contents of food, cosmetic, and medicine because it has strong antioxidant activity and bioactivity of anti-aging, treating cardiovascular and cerebrovascular disease, hypolipidemic, anti-cancer, anti-radiation.<sup>[5-10]</sup> Several traditional methods are available for the extraction of plant polyphenols including impregnation, solvent extraction, ultrasonic extraction, hot-water extraction, alkaline dilute alcohol or alkaline water extraction, microwave extraction, and supercritical CO<sub>2</sub> extraction.<sup>[11-14]</sup> However, now, an increasing number of research on using cellulase to extract active ingredients from plants, which has been carrying on since 1970s in our country, although cellulase was found in the snail's digestive juices by Seilliere in 1906 at the earliest.<sup>[15,16]</sup> The enzymatic method is widely used for enzyme have excellent properties of high reaction efficiency and specificity, moderate reaction conditions, shorter extraction time and easier to control, less damage to the active ingredient.<sup>[17-23]</sup>

Active ingredients of natural product mainly exists in the cytoplasm, which cause that a few active ingredients were left in the cell with

the barrier of plant cell walls, which effects the extraction rate.<sup>[24-26]</sup> Enzymolysis method improve extraction rate through cellulase hydrolyze cellulose of plant cell walls to reduce the resistance of the mass transfer, which benefit dissolution of active ingredients.

At present, the enzymatic extraction of polyphenols from honeysuckle and dynamic had not been reported. In this study, using cellulase to extract polyphenols from honeysuckle is first applied. Moreover, uniform design was used to optimize process and kinetic model of extraction was established to analyze the characteristics of enzymatic extraction, in order to improve the yield of polyphenols from honeysuckle and make maximum use of *Lonicerae Flos*, which provide references for industrial production.

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## MATERIALS AND METHODS

### Plant material and reagents

*Lonicerae Flos* were purchased from Kunfa Chinese Medicine Yinpian co., Ltd. (Henan China), sifted 40 meshes in order to prepare honeysuckle powder. The gallic acid reference substance was obtained from National Institute of Control of Pharmaceutical and Biological Products. In addition, dipotassium hydrogen phosphate, potassium sodium tartrate, and ferrous sulfate were domestically analytical grade.

### Apparatus

Thermostat Water Bath (H-6, Honghua Equipment Co., Jiangsu, China), Rotary Evaporator (RE-52CS), and Water Circulating Vacuum Pump (Yingyuyu Equipment Co., Gongyi, China). Ultraviolet-visible spectrophotometer (UV1101, Tianmei Equipment Co., Shanghai, China).

### Extraction procedure

Powders of Honeysuckle powder (1.000 g) were accurately weighed and placed in the erlenmeyer flask, which was extracted with 60 min extraction time, 50°C extraction temperature, 60% v/v ethanol, 1:50 g/mL solid-liquid ratio, 8.0 mg amount of enzyme, 5 of pH. 30 mL of distilled water was used to wash residue after vacuum filtration. Then both filtrate and scrubbing solution were transferred into 100 mL volumetric flasks and diluted to the mark.

### Preparation of reagents and reference solution

Ferrous tartrate solution: 1.0000 g ferrous sulfate and 5.0000 g potassium sodium tartrate were dissolved in distilled water and set the volume to 1 L. Phosphate buffer: Disodium hydrogen phosphate 23.377 g were dissolved in distilled water to 1 L. Potassium phosphate 9.07 g were dissolved in distilled water to 1 L. Then maxing disodium hydrogen phosphate solution 85 mL and potassium phosphate solution 15 mL to prepared phosphate buffer.

Accurately weighed gallic acid reference substance (13.0 mg) were dissolved in distilled water and diluted to the mark to prepare 0.520 mg/mL standard solution.

### Selection of absorption wavelength

- Determination of the wavelength of reference substance: According to the previous operation, the maximum absorption of reference solution was at the wavelength of 540 nm when scanning from 400 nm to 700 nm
- Determination of the wavelength of the sample: According to the previous operation, the maximum absorption of water extract of the sample was at the wavelength of 540 nm when scanning from 400 nm to 700 nm.

**Table 1:** Factors and levels

Number	Factor	Minimum	Maximum
X <sub>1</sub>	Liquid-solid ratio	10 (10:1)	65 (65:1)
X <sub>2</sub>	Temperature	25	80
X <sub>3</sub>	Time	20	130
X <sub>4</sub>	Ethanol concentration	25 (%)	80 (%)
X <sub>5</sub>	Amount of enzyme	2.0	13.0
X <sub>6</sub>	pH	3	8.5

### Preparation of the standard curve

Accurately weighed gallic acid reference substance (13.0 mg) were dissolved in distilled water and diluted to the mark to prepare 0.520 mg/mL standard solution. Drawing gallic acid standard solution 0.5 mL, 1.0 mL, 1.5 mL, 2.0 mL, 2.5 mL, 3.0 mL into 6 volumetric flasks (25 mL), respectively. After adding 5 mL water and 5 mL ferrous tartrate solution to every flask, phosphate buffer (pH 7.5) was also added till the mark to prepare for the following operations. With blank reagent for reference, the absorbance of the solution were measured at 540 nm, and drew a standard curve, which amount of gallic acid was as the abscissa and the absorbance values of the gallic acid solution was as the ordinate. Statistics regression displayed the standard curve equation is  $A = 0.4936x - 0.0055$ ,  $R^2 = 0.9996$ , which indicated the amount of gallic acid has a good linear relationship with absorbance value in the range of 0.26 ~ 1.56 mg.

### Trial design of extracting polyphenols

Experiments were made by using  $U_{12}^{*12^{10}}$  uniform design with a yield of polyphenols as index and factors, and their levels were shown in Table 1. Honeysuckle weighing 1.000 g were extracted in every experiment, of which liquid-solid ratio/(mL/g) (X<sub>1</sub>), temperature/°C (X<sub>2</sub>), time/min (X<sub>3</sub>), ethanol concentration/% (X<sub>4</sub>), amount of enzyme/mg (X<sub>5</sub>) and pH (X<sub>6</sub>) were equally divided into 12 levels, respectively [Table 1].

### Statistical analysis

All statistical analyses were carried out using Uniform Design 3.0 software (Provide by The ministry of agriculture of special economic animal and plant and product quality supervision, inspection and test center.) to establish mathematical models and regression analysis, through, which we can find out the significant factor of extraction rate of polyphenols from honeysuckle and determine the optimum extraction parameters for polyphenols.

### Determination of the extraction rate of polyphenols from honeysuckle

Honeysuckle powder weighing 1.000 g was placed into erlenmeyer flask and extracted under designed conditions. After filtrating, 30 mL of distilled water were used to wash residue, and the erlenmeyer flask then transferred into 100 mL volumetric flask and diluted to the mark. Sample solution drawing 3.5 mL was placed into a volumetric flask and diluted to the mark with phosphate buffer (pH 7.5) after adding 1.5 mL distilled water, 5 mL ferrous tartrate solution. Absorbance values of the solution were determined at 540 nm with a blank reagent for reference in order to calculate the amount and the extraction rate of polyphenols.

$$Z = \frac{x \div \frac{3.5}{V} \times 10^{-3}}{m} \times 100$$

Z is the extraction rate of polyphenols (%), x is the amount of total polyphenols of 3.5 mL sample solution (mg), V is the constant volume of extracts (mL) and m is the quality of honeysuckle (mg) in the formula.

### Kinetic model experiments

Temperature factor: Honeysuckle powder weighing 1.000 g was added to 6.0 mg enzyme and 30 mL 60% (v/v) ethanol in order to be thermostatic extracted after preheating to the setting temperature (30°C, 40°C, 50°C). According to the above conditions, sample solution were prepared respectively, which were under different extraction time of 5 min, 10 min, 15 min, 20 min, 25 min, 30 min, respectively. The extraction rate of polyphenols were determined and calculated on the basis of the same of operation

Amount of enzyme factor: 1.000 g Honeysuckle powder were accurately weighed and thermostatic extracted at 40, after added the setting amount

of enzyme (2.0, 6.0, 10.0 mg) and 30 mL 60% (v/v) ethanol. According to the above conditions, sample solution were prepared respectively, which were under different extraction time of 5 min, 10 min, 15 min, 20 min, 25 min, 30 min respectively. The extraction rate of polyphenols were determined and calculated on the basis of the same of operation.

Polyphenols concentration formula is  $C = \frac{y + 0.0055}{0.4936} \div \frac{3.5}{100}$  (mg/mL), of which  $y$  is the extraction rate and  $C$  is polyphenols concentration.

## RESULTS AND DISCUSSION

### Uniform design and experimental results

Optimization experiments were made by using  $U_{12}^{*12^{10}}$  uniform design, which 12 trials were conducted in order to make factors and levels are repeated, to improve the accuracy and reliability of the trial. Mixed-level uniform design table was conducted using Uniform Design 3.0 software. In addition, the design program and experimental results were shown in Table 2.

### The establishment of regression model and variance analysis

All statistical analyses were carried out by quadratic polynomial stepwise regression method using Uniform Design 3.0 software. Moreover, the regression equation was  $Y = 9.72 \times 10^{-2} + 3.67 \times 10^{-3} X_2 + 1.62 \times 10^{-2} X_3 + 2.06 \times 10^{-3} X_4 - 1.31 \times 10^{-5} X_3^2 - 7.91 \times 10^{-4} X_1 X_3 X_6$ , wherein  $Y$  is extraction rate of polyphenol, %;  $X_1$  is liquid-solid ratio, mL/g;  $X_2$  is temperature, °C;  $X_3$  is time, min;  $X_4$  is ethanol concentration, % (v/v);  $X_5$  is amount of enzyme, mg;  $X_6$  is pH. Regression analysis was resolved,

**Table 2:** Uniform design scheme and experimental results

Factor (%)						Absorbance values
$X_1$	$X_2$	$X_3$	$X_4$	$X_5$	$X_6$	
1 (10)	2 (30)	6 (70)	8 (60)	9 (10.0)	10 (7.5)	0.350
2 (15)	4 (40)	12 (130)	3 (35)	5 (6.0)	7 (6)	0.257
3 (20)	6 (50)	5 (60)	11 (75)	1 (2.0)	4 (4.5)	0.465
4 (25)	8 (60)	11 (120)	6 (50)	10 (11.0)	1 (3)	0.428
5 (30)	10 (70)	4 (50)	1 (25)	6 (7.0)	11 (8)	0.441
6 (35)	12 (80)	10 (110)	9 (65)	2 (3.0)	8 (6.5)	0.517
7 (40)	1 (25)	3 (40)	4 (40)	11 (12.0)	5 (5)	0.296
8 (45)	3 (35)	9 (100)	12 (80)	7 (8.0)	2 (3.5)	0.419
9 (50)	5 (45)	2 (30)	7 (55)	3 (4.0)	12 (8.5)	0.415
10 (55)	7 (55)	8 (90)	2 (30)	12 (13.0)	9 (7)	0.407
11 (60)	9 (65)	1 (20)	10 (70)	8 (9.0)	6 (5.5)	0.514
12 (65)	11 (75)	7 (80)	5 (45)	4 (5.0)	3 (4)	0.505

**Table 3:** Analysis of variance

Source	Quadratic sum	DOF	Variance	Variance ratio
Regression	$7.38 \times 10^{-2}$	5	$1.48 \times 10^{-2}$	$F=75.54$
Residue	$1.17 \times 10^{-3}$	6	$1.95 \times 10^{-4}$	
Sum	$7.50 \times 10^{-2}$	11		

**Table 4:** Partial regression sum of squares

U (i)	$X_2$	$X_3$	$X_4$	$X_3^2$	$X_1 X_3 X_6$
	$3.8 \times 10^{-2}$	$1.49 \times 10^{-3}$	$1.46 \times 10^{-2}$	$2.27 \times 10^{-3}$	$1.27 \times 10^{-3}$

then the results of variance analysis were shown in Table 3 and partial regression sum of squares was shown in Table 4.

The regression equation was significant for multiple correlation coefficient of 0.9922 ( $R = 0.9922$ ), significance level of 0.05 ( $\alpha = 0.05$ ), inspection value of 75.54 ( $F_t = 75.54$ ), critical value ( $F [0.05, 5, 6] = 4.387$ ) and  $F_t > F (0.05, 5, 6)$ . According to the contribution to regression, equation items were sorted, which was based on descending order of partial regression sum of squares. The sequence was as follows,  $X_2, X_4, X_3^2, X_3, X_1 X_3 X_6$ , which was temperature, ethanol concentration, time, interaction with liquid-solid ratio, time and pH respectively, whereas the influence of the amount of enzyme was not significant. According to the regression equation, the optimum process of extraction was  $X_1 = 65 (65:1)$ ,  $X_2 = 80$ ,  $X_3 = 130$ ,  $X_4 = 80$ ,  $X_6 = 8.5$  respectively, and the expected absorbance value was 0.533, which meant the theoretical extraction rate of polyphenols was 3.12%.

### Validation experiments

Validation experiments were performed under the optimized conditions of the above screening: 3 copies of honeysuckle weighing 1.000 g and making it sift 18 mesh were performed 3 times experiments. In consideration of saving energy and economy, extracts were prepared under the conditions of 80% (v/v) of alcohol, 35:1 (mL/g) of liquid-solid ratio, 80°C of extraction temperature, 8.5 of pH, 6.0 mg of the amount of enzyme, 130 min of extraction time. Absorbance values of polyphenols were 0.518, 0.516 and 0.520 respectively, which the mean was 0.518 and the extraction rate of polyphenol was 3.03% that was 0.09% difference with uniform test prediction 3.12%. Therefore, the experimental value was compared well with the theoretical value.

### Effect of the amount of enzyme and temperature on dynamic of polyphenols extraction from honeysuckle

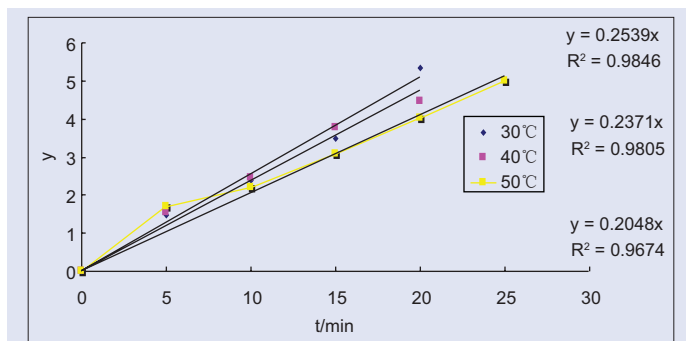
At present, there are two major categories about dynamics math models of the extracting natural drug, which are experience model and the model of basing on mass transfer theory. Experience model is not involved in internal extraction mechanism, a black box model, which is simple and practical.<sup>[27]</sup> The model of basing on mass transfer theory is related to an internal mechanism, which is a wide application, but the process is complicate to established. According to the practical application, we chose the model of basing on Fick's law and experience,

which is  $\ln\left(\frac{C_\infty}{C_\infty - C}\right) = Ft + \ln\left(\frac{C_\infty}{C_\infty - C_0}\right) (F > 0)$ . The experiment was

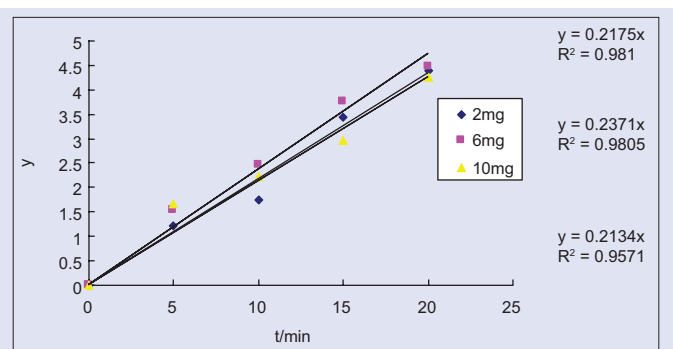
designed and verified by using experimental data of enzymatic extraction of polyphenols from honeysuckle. The drug powder were without soaking before extraction, so  $C_0 = 0$  and the model was simplified to  $\ln\left(\frac{C_\infty}{C_\infty - C}\right) = Ft (F > 0)$ .

The relationship between extraction time and polyphenols concentration under different temperature and amount of enzyme was investigated. The result showed that the factor of temperature 30°C, 40°C, 50°C corresponded to equilibrium concentration that were 0.244, 0.250, 0.254 mg/mL respectively and equilibrium time that were 25, 30, 30 min respectively. Moreover, the factor of amount of enzyme 2.0, 6.0, 10.0 mg corresponded to equilibrium concentration that were 0.235, 0.250, 0.245 mg/mL respectively and equilibrium time that were 25, 30, 30 min respectively. The results were shown in Figures 1 and 2, and Table 5.

From Figures 1 and 2, and Table 5, we can know that  $\ln\left(\frac{C_\infty}{C_\infty - C_0}\right)$  had good linear relationship with  $t$  even under the conditions of different levels of enzyme and temperature and most of  $R^2$  were above 0.98, which indicated it was feasible that extraction rate was described by Fick's law,



**Figure 1:** The relationship between  $y$  and  $t$  under different temperature conditions ( $y$  is  $\ln(\frac{C_{\infty}}{C_{\infty}-C})$ ,  $t$  is time)



**Figure 2:** The relationship between  $y$  and  $t$  under the conditions of different amount of enzyme ( $y$  is  $\ln(\frac{C_{\infty}}{C_{\infty}-C})$ ,  $t$  is time)

**Table 5:** Kinetic parameters

Amount of enzyme/mg	Temperature/°C	$C_{\infty}$ (mg/mL)	$F$	$R^2$
6.0	30	0.244	0.2539	0.9846
6.0	40	0.250	0.2371	0.9805
6.0	50	0.254	0.2048	0.9674
2.0	40	0.235	0.2175	0.9810
6.0	40	0.250	0.2371	0.9805
10.0	40	0.245	0.2134	0.9571

$\ln(\frac{C_{\infty}}{C_{\infty}-C}) = Ft$ , is feasible. As the temperature increases,  $F$  decreases, that is, the ratio of the solute concentration gradient decreasing rate reduction. The experiments indicated that with increasing temperature, limited ingredient dissolute easier in the range of 30~50°C while the extraction yield of polyphenols increased modestly.  $F$  was the result of dissolution and damage of temperature of polyphenols. Cellulase hydrolyze cellulose of plant cell walls to reduce the resistance of the mass transfer, which benefit dissolution of limited ingredients. Enzymolysis method is efficient, so the equilibrium time of extracting polyphenols required for short, which just need less 30 min. With increasing amount of enzyme, whether  $C_{\infty}$  or  $F$ , are the maximum when 6.0 mg of the amount of enzyme, which is agree with the result of the single-factor experiment. In conclusion, Fick's law,  $\ln(\frac{C_{\infty}}{C_{\infty}-C}) = Ft (F > 0)$  can well explain the process of enzymatic extraction.

## DISCUSSION ON MECHANISM

Cellulase generally contains the endoglucanases ( $C_1$  enzyme), dextran exonuclease ( $C_x$  enzymes) and  $\beta$ -glucosidase (cellobiose carbohydrases) of three main components of the induction-type composite Enzymes, the degradation of the cell wall by these three major components completed together. The  $C_1$  enzymes from hydration, acting on the surface of the insoluble fiber, cellulose crystallinity chain cracking, resulting in the end portion of the cellulose molecules of the long chain free and exposed, so that the cellulose easy hydration; after  $C_1$  enzyme may be formed  $C_x$  desired a new free end,  $C_x$  enzyme can be adsorbed on the cellulose molecules above, cut from the key internal arbitrary position of  $\beta$ -1, 4-glycosidic linkage, the cellulose molecules break for cellobiose and celotriose; Finally, these the product is cleaved by cellobiase decomposition is glucose. Cellulase synergy order is not absolute, but

$C_1$ ,  $C_x$  and cellobiase must also exist to natural hydrolysis of cellulose. Is now generally believed that the natural cellulose is first opened in the role of the enzyme in nonhydrolyzable Solutions chain factor or extract hydrogen between the cellulose chains and the hydrogen bonds within the chain, and the formation of disordered noncrystalline cellulose, and then the three enzymes. Synergistic effect hydrolysis of fiber dextrin and glucose.

Plant cell wall can maintain plant cell morphology and prevent cells from bursting in a low permeable environment. Cellulose is a major component in plant cell wall, which is the main barrier of limited ingredient dissolution. Therefore, the cell wall will lost the original function after cellulase hydrolyze cellulose of plant cell walls for cellobextrin and glucose. Moreover, the plant cells burst when solvent penetrated into them, which benefited the dissolution of active ingredients and spread to the main solution the proliferation in a low osmotic pressure environment, because of the loss of protection from cell wall.

## CONCLUSION

(1) Optimization parameters of cellulase extraction of polyphenols from honeysuckle were found to be 80% (v/v) of alcohol, 35:1 (mL/g) of liquid-solid ratio, 80°C of extraction temperature, 8.5 of pH, 6.0 mg of the amount of enzyme, 130 min of extraction time. Under the optimal conditions, the extraction rate of polyphenols was 3.03% (2) The dynamic of polyphenols extraction from honeysuckle consistent with Fick's first law. As the temperature increases,  $F$  decreases, that is, the ratio of the solute concentration gradient decreasing rate reduction. With respect to the amount of enzyme 2.0 mg and 10.0 mg, whether  $C_{\infty}$  or  $F$ , are the maximum when 6.0 mg of the amount of enzyme (3) The extraction mechanism of polyphenols from honeysuckle showed that cellulase can hydrolyze cellulose of plant cell walls for cellobextrin and glucose, which lead to the loss of the cell wall to maintain the function of the plant cell morphology. The plant cells burst when solvent penetrated into them, which benefited the dissolution of active ingredients and spread to the main solution the proliferation in a low osmotic pressure environment (4) Polyphenol content of honeysuckle at different parts, different harvest periods, and different season polyphenol content are not the same. Moreover, different parts of honeysuckle of the corresponding extraction method may be different. Hence in this study, the optimization enzymatic extraction parameters of polyphenols from honeysuckle are reliable, stable and available in practice. In addition, the fitting equation of kinetic model fitting equation can provide a reference for honeysuckle plant resources in enlarging application engineering.



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Nil.

## Conflicts of interest

There are no conflicts of interest.

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