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ORIGINAL ARTICLE

Novel Method of Preparation and Activity Research on Arctigenin from Fructus Arctii

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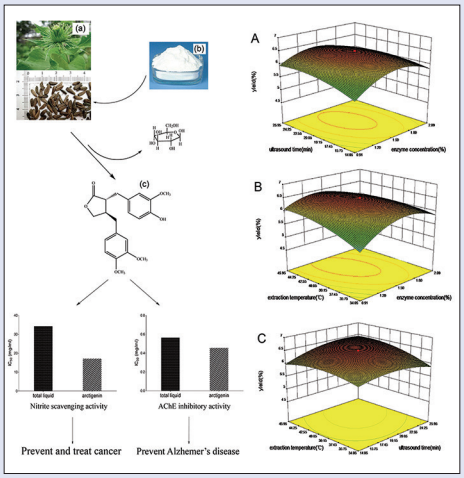
ABSTRACT

Background: Arctigenin has many pharmacological activities with clinical significance and is derived from *Arctium lappa* L. However, the present extraction method is inefficient and does not have meaningful industrial production. **Objective:** A new method to directly prepare arctigenin was established by combining enzyme-assisted extraction and central composite design. Arctigenin's further pharmacological activity was also surveyed *in vitro*. **Materials and Methods:** β-D-Glucosidase, a food-grade enzyme, was added directly to the fruits of *A. lappa* L. to hydrolyze the arctiin to arctigenin, and the obtained samples were subsequently subjected to ethanol (30%, v/v) extraction. The pharmacological activity of the extraction and arctigenin was determined by inhibiting acetylcholinesterase (AChE) and scavenging nitrite. **Results:** The factors investigated include the enzyme concentration (0.5%–2.5%), ultrasound time (10 min^{-3} 0 min), and extraction temperature (30°C–50°C). From the analysis of the results by Design-Expert (V8.0.6), the optimal extraction conditions were obtained: enzyme concentration (1.4%), ultrasound time (25 min), and extraction temperature (45°C). The highest yield of arctigenin, obtained under the optimal conditions was 6.39%, representing an increase of 28.15% compared to the reference extraction without enzyme processing. The IC_{50} values of the extraction and arctigenin, respectively, for inhibiting AChE were 0.572 mg/ml and 0.462 mg/ml, and those for nitrite-scavenging were 34.571 mg/ml and 17.49 mg/ml. **Conclusions:** The results demonstrate that using an enzyme directly in the production is an effective means for extracting arctigenin from Fructus arctii. The extraction has the activities of inhibiting AChE and scavenging nitrite, probably because there has arctigenin in it. It is implied that the extraction and arctigenin could contribute to human health in clinical applications.

Key words: Arctigenin, enzyme-assisted extraction, Fructus arctii, inhibiting acetylcholinesterase activity, nitrite-scavenging activity, response surface

SUMMARY

- The new method of adding enzyme directly to the preparation of arctigenin was carried out instead of preparing arctigenin by two-step method
- Three factors affecting the efficiency of preparation were analyzed and discussed include the enzyme concentration, ultrasound time, and extraction temperature by central composite design
- This new method of preparing arctigenin improved the yield significantly than other methods
- Arctigenin has remarkable pharmacological activities of inhibiting acetylcholinesterase and scavenging nitrite.



Abbreviations used: AChE: Acetylcholinesterase, CCD: Central composite design, TCM: Traditional Chinese medicines, AD: Alzheimer's disease, DTNB: 5,5′-dithiobis-(2-nitrobenzoic acid), DMSO: Dimethyl sulfoxide, ATCI: Acetylthiocholine iodide.

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INTRODUCTION

Arctium lappa L., also known as east ginseng[1] or ginseng, is native to Asia,[2] and it is a fleshy root and biennial herbaceous plant that belongs to the Compositae.[3] Fructus arctii, the dry fruits of *A. lappa* L., has many names in Chinese reports.[4] According to the basic theory of traditional Chinese medicine, its medicinal property is cold, and flavor is bitter, and its meridian distribution belongs to the lungs and stomach. It has many functions such as evacuating wind chill, ventilating the lungs, promoting eruption, detoxification, and relieving sore throat.[5] The main chemical compounds of Fructus arctii are arctiin and arctigenin. Arctiin exhibits low activities, but it can be absorbed by the human body through metabolization into arctigenin.[6] Arctigenin possesses many pharmacological activities such as anti-inflammatory,[7] antiviral,[8,9] anti-HIV-1,[10] antitumor,[11,12] and anti-leukemia. However, the content of arctigenin in Fructus arctii is 0.13% ~ 1.0%, whereas arctiin is 2.64% ~ 8.02%.[13]

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The most common extraction method of arctigenin from Fructus arctii is two-step method, in which arctiin was extracted from Fructus arctii first, and then β-D-glucosidase or snailase is added to the extraction solution to hydrolyze the arctiin into arctigenin.[14,15] Alternatively, a supercritical CO_2 extraction method can be used to extract the crude arctigenin, and it is purified by a degreasing treatment.[16] One-step preparation method has also been reported which is to regulate the acidity of the ethyl acetate extraction solution to pH 4.0–5.0 to prepare arctigenin.[17] All of these methods depend on a large energy consumption, long extraction time, and environmental disruption, making them not conducive to industrial production.

B-Glucosidase (EC 3.2.1.21) is an important component in the cellulolytic enzyme system and can catalyze the hydrolysis of the β-glucose glycoside bond generated by an aromatic base or hydrocarbyl with glycoside.[18] In the present study, it is used to hydrolyze the glycosidic bond of arctiin to obtain arctigenin. The difference of this study is adding the enzyme to the samples directly to prepare the arctigenin in one step, and the hydrolysis procedure is shown in Figure 1.

Central composite design (CCD) has been commonly used in recent years, integrating mathematical and statistical methods of experimental design.[19,20] It is a method depicting effect factors to examine the response surface and selecting a better effect area on the response surface to launch the range of independent variable values that provides the best experimental condition of the optimization method.[21] This is a simple, easy, and intuitive method and widely used in the extraction of traditional Chinese medicines. CCD was used in the experiment of this paper. The optimization process of the response surface methodology (RSM) includes selecting a reliable experimental design to fit the linear or nonlinear fitting, establishing the mathematical relationship between the effect and factors, ensuring the credibility of the model by statistical tests, and optimizing the conditions of the best procedure.[22,23]

Nitrite could form strongly carcinogenic nitrosamine compounds in human body, especially in the role of gastric juice and induce digestive system cancer, chronic toxicity, and teratogenic risk.[24] Hence, the proper clearance of nitrite will prevent cancer in a certain sense. The worst concentration of nitrite is as a preservative widely used in meat, milk, and other foods although there is also natural content in vegetables.[25] Hence, looking for nitrite scavengers is necessary.

Alzheimer's disease (AD) is the most important type of dementia.[26] Due to the complex pathology of AD, many hypotheses have been proposed including cholinergic neuron system dysfunction. The theory is that the decrease of cholinergic neurons in the hippocampus and cerebral cortex of AD patients' brains causes the neurotransmitter acetylcholine in the synaptic gap to decrease.[27] Acetylcholinesterase (AChE) plays a key role in the nerve conduction process and is an enzyme that has a close relationship with acetylcholine. If the activity of the enzyme is inhibited, a cholinergic effect can be produced, thereby increasing the level of acetylcholine.[28] According to this theory, AChE inhibitors have been applied in clinical settings such as tacrine, rivastigmine, galantamine, and donepezil. Unfortunately, the effectiveness of these drugs has been reported to be limited.[29] Amazingly, the new AChE inhibitor Huperzine A, which was extracted from the natural plant *Huperzia serrata*, has been clinically proven to be effective and compared to tacrine and donepezil, possesses a longer duration of action and higher therapeutic index and exhibits minimal peripheral cholinergic side effects at therapeutic doses.[30,31] The extraction from natural plants is thus an effective way to develop safe and efficient AChE inhibitors. This paper investigates the inhibitory effect on the activity of AChE of natural plant extractions under an optimization process to provide reference for the further development of AChE inhibitors.

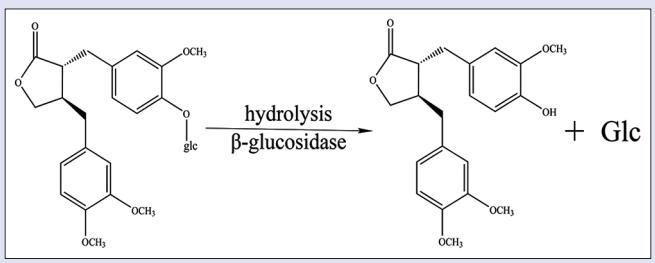

Figure 1: Hydrolysis of arctiin by β-glucosidase

MATERIALS AND METHODS

Chemicals and reagents

β-glucosidase (food grade), arctiin standards (Lot: 120605), and arctigenin standards (Lot: 120509) were purchased from Chengdu Biological Technology Co., LTD. (Chengdu, China). HPLC grade methanol was purchased from Fisher Scientific (Shanghai, China). Analytical grade methanol and anhydrous ethanol were purchased from Beijing Chemical Works (Beijing, China). Purified water was purchased from Hangzhou Wahaha Group Co., LTD (Hangzhou, China). SPECTRA MAX190 (United States) acetylthiocholine iodide (ATCI, corresponding to 1 mM diluted in phosphate-buffered saline [PBS]), AChE (0.28 U/mL) (C3389 from electric *Electrophorus electricus* [electric eel], Type VI-S lyophilized powder, 200–1000 units/mg protein, store: −20), 5,5'-dithiobis-(2-nitrobenzic acid) (DTNB), Huperzine A (sigma), PBS buffer (PH = 8), and dimethyl sulfoxide (DMSO) were purchased from Sigma (USA). Ninety-six-well culture plates were purchased from Falcon, $V_{C,}$ 1 mM $NaNO_{2,}$ and 0.4% sulfanilic acid.

Plant material

Fructus arctii, fruits of *A. lappa* L., was purchased from the Anguo market (Anguo Henan province, China) and was collected from Jilin Province, China. The samples were dried at 60 to constant weight and ground into fine powder and then packed in a dryer and stored at room temperature.

Arctigenin assay

The arctigenin content in Fructus arctii was determined by HPLC at room temperature. Before analysis, the sample solutions were filtered through a 0.22 μm membrane filter, and 10 μL of each sample was injected in the C_{18} column. The mobile Phase A was water and mobile Phase B was methanol (HPLC grade). The parameters of the chromatography are as follows: sensitivity of AUFS, 1.0; column, Alltima HP C_{18}, 250 mm length, 4.6 mm internal diameter; wavelength, 280 nm; flow rate, 1.0 mL/min; run time, 10 min; elution, methanol: and Water, 55:45.

Extraction methods

Extraction procedure

The pretreated samples (0.5000 g) were taken in a 50 mL conical flask, and β-glucosidase was added in different amounts. It was extracted by 30 mL water under ultrasonic for a set time and temperature and then cooled to room temperature. After that, 15 mL ethanol (90%, v/v) was added to the solution. The sample solutions were filtered through a 0.22 μm filter and stored at room temperature until analysis. Reference experiments, without enzyme, were performed under the same conditions as the enzyme-assisted extraction procedure.

Experimental design of single factor

The effects of the three factors including enzyme concentration (0.5%–5%), ultrasound time (10–240 min), and extraction temperature (25°C–50°C)

were first studied as single factors. The experimental design was as follows: one factor was varied while the other two factors were fixed in each experiment. The evaluation standard is the determination of the arctigenin extraction yield for the effect of each factor.

Central composite design in experiments

The factors of enzyme concentration (X_1), ultrasound time (X_2), and extraction temperature (X_3) were optimized by a CCD (Design-Expert Software, Version 8.0.6) were purchased from Stat-Ease, Inc. (Mfinneapolis, USA) for the best yield of arctigenin extraction (y). The three factors, on the basis of the single-factor tests, enzyme concentration (X_1: 0.5%–2.5%), ultrasound time (X_2: 10–30 min), and extraction temperature (X_3: 30–50), were the selected independent variables, which were to be optimized in this design, and the coded and actual levels of the three variables are shown in Table 1. The arrangement of the design is shown in Table 2 through the software. Overall, a total of 14 + 3 center points = 17 runs of experiments were designed by the software. They were conducted in a randomized order in the hope of minimizing the effects of extraneous factors on the observed responses.

Nitrite scavenging activity

Weigh 20 mg Arctigenin in the centrifuge tube and add 40 mg cholesterol and phospholipids 120 mg. Then, 5 mL dichloromethane was added to dissolved, and the solvent was evaporated at 30. Then, add 5 mL two sodium hydrogen phosphate-citric acid buffer (pH = 3) into the centrifuge tube and stir it into a mixed spin liquid 4 mg/ml liposome solution and filter and dilute the sample in the experiments. In this experiment, 40 mg extraction was added 1 mL two sodium hydrogen phosphate-citric acid buffer (pH = 3) and finally diluted the concentration as 40, 8, 1.6, 0.32, 0.064, and 0.0128 mg/mL by two sodium hydrogen phosphate-citric acid buffer (pH = 3). Sample (40 uL) was added to 20 μL of 1 mM $NaNO_2$ and mixed. The mixture was then adjusted to a volume of 40 μL using distilled water and adjusted to disodium hydrogen phosphate and citric acid buffer solution (PH = 3). The reaction solution was incubated at 37 for 1 h. Then, 40 μL of 0.4% sulfanilic acid was added to 20 μL of the reaction mixture solution. Shaking it up thoroughly, in 5 min, 20 μL of 0.2% N-1-naphthyl oxalic acid amine hydrochloride and 120 μL distilled water were mixed for the reaction. The absorbance of the mixture was measured on a microplate reader (Spectra Max 190, United States) at 405 nm after the mixture was incubated for 15 min at room temperature and the reaction reached equilibrium. A blank was prepared according to the above method but by adding 40 μL of buffer solution instead of the sample. The interpretation of the result was performed using the following formula: Nitrite scavenging activity (%) = (A–B)/A*100% where A is the absorbance of the blank group and B is the absorbance of the sample group.

Acetylcholinesterase inhibitory activity

The AChE enzymatic activity was measured by Ellman's test using arctigenin as a positive control. The plant extract and arctigenin were dissolved by DMSO, and they were both samples. Next, 20 μL of samples with different concentrations, 140 μL of 200 mM phosphate buffer (pH = 8), and 15 μL of an enzyme solution containing 0.28 U/mL were incubated for 20 min at 4. Subsequently, 10 μL of 1 mM DTNB and 10 μL of a solution of ATCI 1 mM were added. The absorbance of the mixture was measured on a microplate reader (SpectraMax 190, United States) at 405 nm within 30 min after the mixture was incubated for 20 min at 37 and the reaction reached equilibrium. Because the samples were both dissolved in DMSO, there are both positive and negative controls. The former took 20 μL DMSO instead of the sample solution, and the latter took the PBS buffer instead of the sample solution. A control sample mixture was also prepared, using phosphate buffer instead of the extract. The inhibition was calculated in the following way: I% = $(A_0–A_i)/A_0 \times 100\%$ (A_0 = A [negative-negative control] A_i = A [sample-control sample]). The extraction concentration providing 50% inhibition (IC_{50}) was obtained by plotting the inhibition percentage against the extract solution concentrations.

Table 1: Factors and levels of central composite design

	factors		
leavels	X1 (enzyme concentration)/%	X2 (ultrasound time)/min	X3 (extraction temperature)/℃
-1.682	0.5	10	30
-1	0.9	14	34
0	1.5	20	40
1	2.1	26	46
1.682	2.5	30	50

Table 2: Arrangement and results of central composite design

No.	X_1	X_2	X_3	Yield/%
S1	1.5	20	40	6.489
S2	1.5	20	40	6.505
S3	1.5	20	40	6.442
S4	0.9	14	34	5.133
S5	0.9	14	46	5.548
S6	0.9	26	34	5.322
S7	0.9	26	46	6.392
S8	2.1	14	34	5.765
S9	2.1	14	46	5.468
S10	2.1	26	34	5.548
S11	2.1	26	46	5.696
S12	0.5	20	40	4.898
S13	2.5	20	40	5.071
S14	1.5	10	40	5.792
S15	1.5	30	40	5.898
S16	1.5	20	30	5.709
S17	1.5	20	50	6.013

RESULTS

Methodology assay of arctigenin content

Weigh accurately 0.004 g of arctigenin into a 10 mL volumetric flask and dilute with methanol. Shake until blended as a standard solution at a concentration of 0.4 mg/ml. Pipette the standard solution, using the above optimum chromatography condition, and the injection amounts were 2, 4, 8, 10, 12, and 20 μL. Plot with the injection amount on the horizontal axis and the peak area on the vertical. The result showed that arctigenin has a good linear relationship in the area of 0.8 to 8 mg. The regression equation was Y = 263014X-50253, r = 0.9997. According to the extraction procedure method used to prepare the test solution, upon continuously injecting five times, the relative standard deviation (RSD) was 1.03%. Weigh 0.200 g arctigenin powder to carry out the experiment of sample recovery and five parallel experiments. Add arctigenin standard substance and measure the test solution. The average recovery was 101.38, and the RSD was 0.86%.

Arctigenin extraction of reference experiment

According to the results of the reference experiment without enzyme, the yield of arctigenin is 4.98%. The assays were tested in triplicate.

Effect of enzyme on arctigenin extraction

The yields of the arctigenin extraction are shown as percentage of the amount of arctigenin extracted from the total amount of *A. lappa* L. in the fruits. The assays were performed with an ultrasound time of 30 min and extraction temperature of 35 to evaluate the yield of arctigenin with enzyme concentrations of 0.5, 1.0, 1.5, 2, 2.5, and 5.0%, and the results are shown in Figure 2a. Figure 2a indicates that β-glucosidase with different amounts shows different effects on the arctigenin production, and the production was the best with an enzyme concentration of 1.5%, and then the extraction yield declined with higher enzyme concentrations, so the enzyme concentration of 1.5% was selected as the superior condition. It can be interpreted that the desired compound hydrolysis occurred at higher enzyme doses. Hence, β-glucosidase with a 1.5% amount was used in further research.

Effect of ultrasound time on arctigenin extraction

The factor of ultrasound time is quite important in the extraction procedure. The extraction yield of arctigenin as affected by different ultrasound times from 10 to 240 min is shown in Figure 2b when the other extraction conditions were fixed at enzyme concentration 1.5% and extraction temperature 35. The results show that the arctigenin production was increased when the ultrasound time ranged from 10 to 30 min, and at the time of 30 min, the maximum production reached 6.3%. The yield of arctigenin showed a dramatic decrease as the time increased, and it increased to the ultrasound time of 200 min and then decreased again to 240 min. It can be explained that part of the arctigenin hydrolysis occurred with the long ultrasound time under these fixed conditions. Therefore, the ultrasound time adopted in the further study ranged from 10 to 30 min.

Effect of temperature on arctigenin extraction

The yield of the arctigenin as affected by the extraction temperature was also investigated as an important factor, and the extraction conditions were set at 25, 30, 35, 45 and 50, whereas the other extraction variables (enzyme concentration and ultrasound time) were fixed at 1.5% and 30 min. The results show that the yield of arctigenin was increased sharply as the temperature ranged from 25 to 35, and then the yield decreased at temperatures beyond 35, but the yield increased when the extraction temperature was more than 40. The maximum yield, 6.50%, was obtained at the temperature of 35, as shown in Figure 2c. The reasons for this result may be an increase in the arctigenin solubility and an improved extraction speed at higher temperature and better enzyme activities at the suitable temperature. However, extremely high temperatures may lead to other disadvantages such as an increased energy cost, acceleration of solvent volatilization, and enhancement of impurity extraction. Therefore, the extraction temperature used in the present work was selected in the scope of 30–50.

Optimization of the procedure by response surface methodology

Statistical analysis and the model fitting

The sum of 17 experiments was statistically designed by the Design-Expert software using different combinations of the physical parameters and carried out to optimize the independent variables (enzyme concentration, ultrasound time, and extraction temperature) with their combined effects on the arctigenin extraction yield. The yields of the arctigenin extraction under different conditions are shown in Table 2, and the data were analyzed by the software. The maximum production of arctigenin (6.505%) was obtained under the experimental conditions of an enzyme concentration 1.5%, ultrasound time 20 min, and extraction temperature 40°C. As a result, the equation for the relationship between the arctigenin yield and the independent variables is

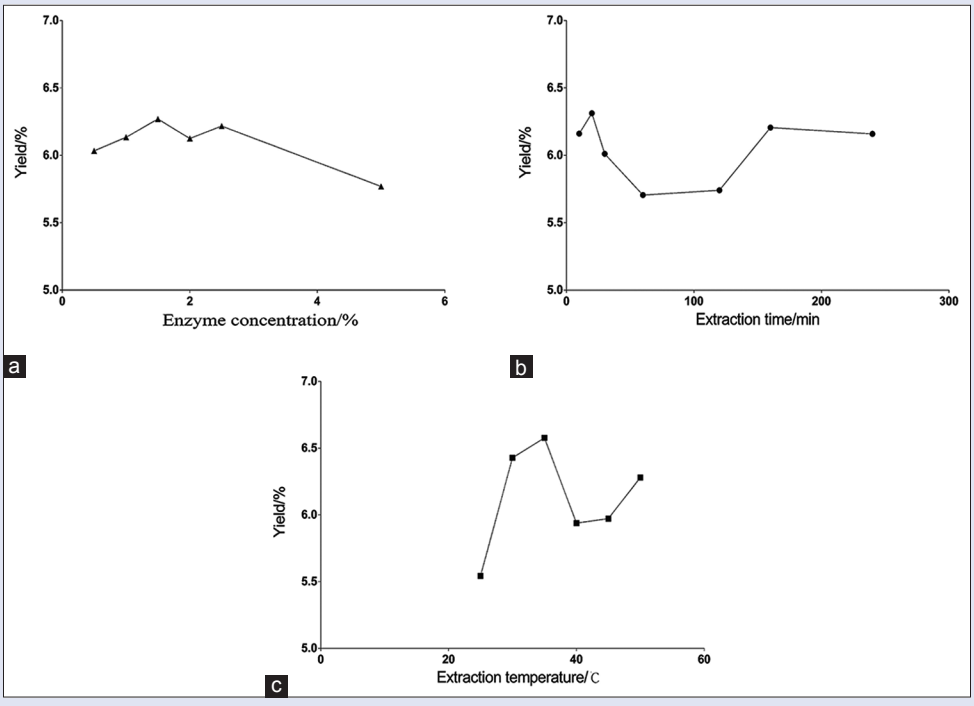

Figure 2: Effects of extraction parameters on the arctigenin yield . (a) Effect of enzyme concentration on the yield of arctigenin. (b) Effect of ultrasound time on the yield of arctigenin. (c) Effect of extraction temperature on the yield of arctigenin

$Y = -10.615 + 7.372 \times_1 + 0.142 \times_2 + 0.475 \times_3 - 0.036 \times_1 \times_2 - 0.058 \times_1 \times_3 + 0.004 \times_2 \times_3 - 1.431 \times_1^2 - 0.006 \times_2^2 - 0.005 \times_3^{2[1]}$ where Y is the arctigenin yield and X_1, X_2, and X_3 are the actual values of the enzyme concentration, ultrasound time, and extraction temperature, respectively.

The corresponding *F*-test and *P* value of the regression model were performed to investigate the statistical significance, and the results of the analysis of variance are shown in Table 3 for the response surface quadratic model. The *F*-value of the model is 31.19 and its $P < 0.0001\%$, which indicates that the model is significant with little noise. Values of Prob > $F < 0.01$ indicate that model terms are significant. The significant model terms are X_2X_3, X_1X_2, X_1X_3, X_2X_3, X_1^2, X_2^2, and X_3^2 in this case. The lack of fit with a value of 17.83 is significant relative to the pure error, and the lack of fit *F*-value implies little chance, 0.054, that the results could be explained by noise. The value of the determination coefficient (R^2 = 0.9757) of the quadratic regression model indicated that most of the total variation could be explained by the model. The Pred R^2 of 0.9444 and the Adj R^2 of 0.8162 are in reasonable agreement. A ratio of Adeq Precision <4 is desirable, and it is used to measure the signal-to-noise ratio. Thus, it is an adequate signal, with a ratio of 16.295. The experimental values are highly precise and have good reliability, with a low value of the coefficient of variation (C.V.% =2.06).

Analysis of the response surface

Response surface plots were constructed over the independent variables (enzyme concentration, ultrasound time, and extraction temperature) to investigate their combined effects on the arctigenin yield and obtain the optimum conditions. As shown in Figure 3, one variable was held constant at the central value, whereas the other two variables varying within the investigative range in the present experiment were regarded as continuous variables, and the corresponding yields of arctigenin were obtained. In the figure, the maximum predicted values were indicated in the smallest range by the contour diagram of the surface, and the maximum predicted value and independent variable values were obtained by the equations.

As expected, the enzyme concentration (X_1) and ultrasound time (X_2) showed quadratic effects on the arctigenin yields. The arctigenin yield increased dramatically when the enzyme concentration (X_1) rose from 0.9% to 1.5%, as shown in Figure 3a, but beyond 1.5%, the extraction yield declined as the amount increased; the maximum yield was achieved from 0.9%–2.1%. Likewise, the ultrasound time (X_2) possessed a similar effect on the arctigenin yield to that of the enzyme concentration (X_1), and the maximum yield was achieved from 14 to 26 min. In Figure 3b and c, the effects were the same as the interactive effects of the enzyme concentration and ultrasound time, and the maximum yield was obtained at extraction temperatures (X_3) ranging from 34 to 46. The optimization of the procedure was performed by Design-Expert software, and the results showed that the maximum yield was achieved when the enzyme

Table 3: Analysis of variance

Sum of Source	Squares	df	Mean Square	*F*- Value	*P*-value
Model	3.92	9	0.44	31.19	< 0.0001
X_1-enzyme amount	0.010	1	0.010	0.73	0.4213
X_2-extraction time	0.11	1	0.11	7.84	0.0266
X_3-extraction temperature	0.25	1	0.25	17.90	0.0039
X_1X_2	0.13	1	0.13	9.35	0.0184
X_1X_3	0.33	1	0.33	23.91	0.0018
X_2X_3	0.15	1	0.15	10.83	0.0133
X_1^2	2.88	1	2.88	206.62	< 0.0001
X_2^2	0.46	1	0.46	32.82	0.0007
X_3^2	0.43	1	0.43	31.01	0.0008
Residual	0.098	7	0.014		
Lack of Fit	0.096	5	0.019	17.83	0.054
Pure Error	2.145E-003	2	1.072E-003		
Cor Total	4.02	16			

$R^2 = 0.9757$, Adj $R^2 = 0.9444$, Pred $R^2 = 0.8162$, Adeq Precision =16.295, C.V.% =2.06

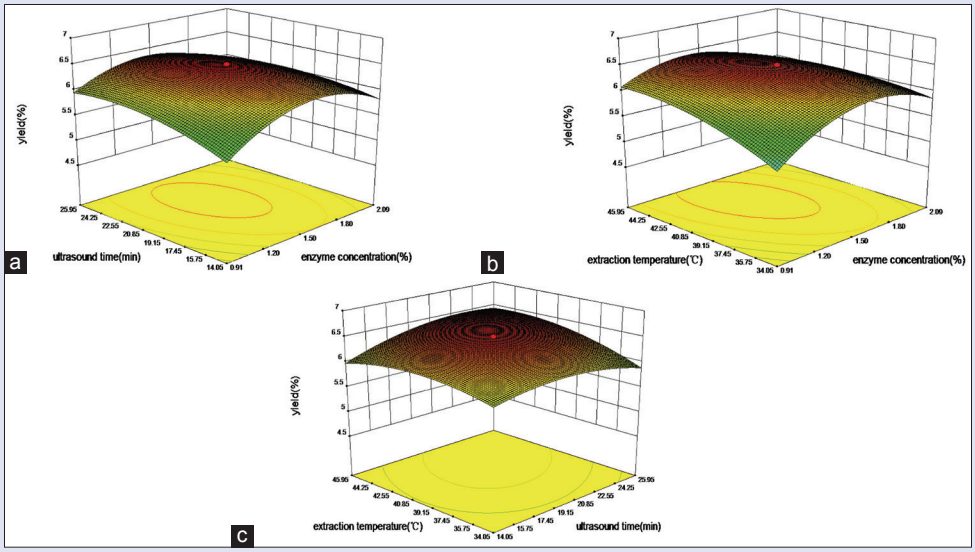

Figure 3: Response surface plots showing the effects of the independent variables tested on the yield of arctigenin extraction (a) Enzyme concentration and ultrasound time. (b) Enzyme concentration and extraction temperature. (c) Ultrasound time and extraction temperature. For each reponse surface plot the levels for the factors are held at their central values

concentration, ultrasound time, and extraction temperature were 1.41%, 25 min, and 45, respectively.

Verification of predictive model

According to the optimization, the best condition for the procedure of the extraction of arctigenin from Fructus arctii is as follows: enzyme concentration (X_1) 1.41%, ultrasound time (X_2) 25 min, and extraction temperature (X_3) 45°C. Under this procedure, a maximum response of 6.53% was predicted by the model. For the validation of the results, triplicate confirmatory experiments were conducted under the optimal procedure, and the average extraction yield was 6.39%, which indicated that the model was appropriate for the extraction process.

Production and separation of arctigenin

A large quantity production of arctigenin was performed using the best extraction procedure obtained from the above results. A large ultrasonic cleaner (power 40 kHz and acoustical power 2000 w) was used for producing the arctigenin. Approximately 10 kg pretreated powder (60 mesh) was taken in the ultrasonic cleaner extracted with 125 L water and 200 g β-glucosidase and then ultrasound 25 min at 45°C temperature. After that, ethanol (95%, v/v) was added to the extraction solution to make a lower concentration ethanol (30%, v/v) solution, extraction 12 h, and ultrasound 1 h again in the same conditions. The extraction solution was filtrated, and the remaining material was extracted again with 10 and 8 times the volume of ethanol (30%, v/v). Finally, all the arctigenin-containing solvent was completely evaporated.

Take part of the dried extraction (70 g) and mix with 105 g silica gel. The sample was then chromatographed on a silica gel (100–200 mesh) column, and 500 mL eluent was formulated as a fraction. Elution was performed using a solvent mixture of chloroform/methanol with a 40:1 ratio. In the 12th, 13th, and 14th fractions, there were canary yellow jellies and crystals after evaporating, and 22 g pure arctigenin was obtained by the recrystallization treatment. This experiment indicates that the preparation procedure is practicable.

Nitrite scavenging activity

The samples with different concentrations were studied to determine their ability of scavenging nitrite, and the results are shown in Figure 4. With extraction with the increase of concentration, the nitrite scavenging ability increased significantly. With a concentration of 40 mg/mL, the removal rate is close to 60%. Arctigenin's nitrite scavenging activity also has a significant concentration dependence. At the highest concentration, the highest removal rate is achieved. The IC_{50} value of arctigenin is 17.49, and it shows some nitrite scavenging activities. The data are presented in Figure 5.

Acetylcholinesterase inhibitory activity

The samples with different concentrations were studied to determine their ability of AChE inhibition, and the results are shown in Figure 6. In this study, the AChE inhibitor activity of the samples was found to increase dose dependently with the increasing concentration of the sample, and the results expressed as IC_{50} values were calculated from the regression equations obtained from the activities of the samples at different concentrations. The data are presented in Figure 7.

DISCUSSION

In recent years, the extraction methods of arctigenin from Fructus arctii have exhibited notable progress. Lu Kongxu's work[32] indicated that extraction with 50% ethanolic, using a heating reflux method for an hour and 8 times of menstruum dosage, obtained a highest extraction rate of arctigenin of 4.07%. Ye Shengqiang *et al.*[33] put 5% hydrochloric acid into nonfat dry Fructus arctii powder and performed backflow extraction with 50% ethanol for 5 h, and the extraction rate of arctigenin was 1.1%. Li Hongfu *et al.*[34] used alkali extraction and acid precipitation to extract the arctigenin, and the extraction rate was 1.3%. No matter what method mentioned above is used, these methods depend on a large energy consumption, long extraction time, and environmental disruption, making them not conducive to industrial production. In particular, the extraction rates are low. Compared with previously published data, the arctigenin extraction yield from the fruits of *A. lappa* L. can be increased significantly by the use of β-D-glucosidase. The method of adding enzyme directly to the preparation of arctigenin is novel and effective, and it can also reduce the environmental impact and cost savings. Furthermore, the commercial food-grade enzyme preparations with comparatively low cost are suitable for production in industry than the cost of normal extract. The optimum values of the factors in the extraction procedure are also studied by the appropriate method of RSM.

In the nitrite scavenging experiment, when arctigenin is provided at a concentration of 2 mg/ml, the nitrite scavenging rate was approximately 40%. Thus, the highest concentration of arctigenin was obtained at 4 mg/ml. When arctigenin is provided at a concentration of 0.125 mg/ml, the changes of its nitrite clearing activity were not clearly, so the lowest concentration of arctigenin was 0.125 mg/ml. Pilot experiments

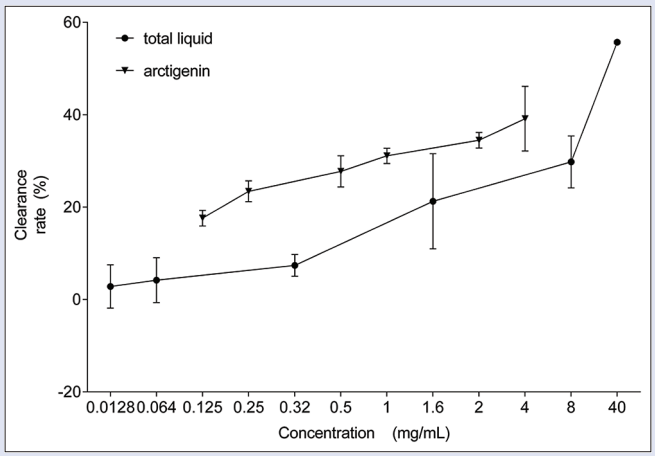

Figure 4: Results of nitrite scavenging activity

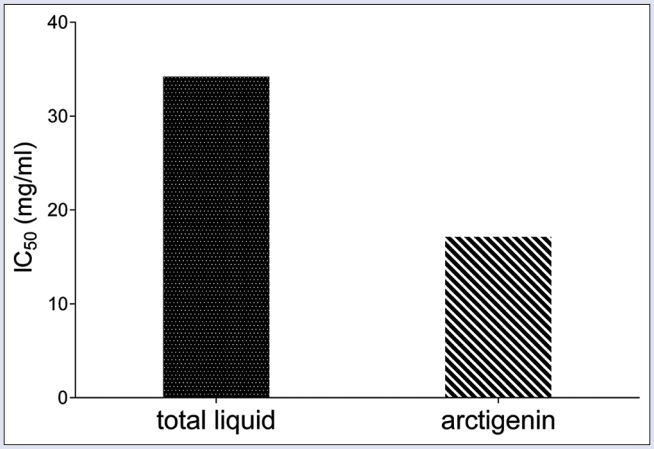

Figure 5: Results of nitrite scavenging activity (IC_{50})

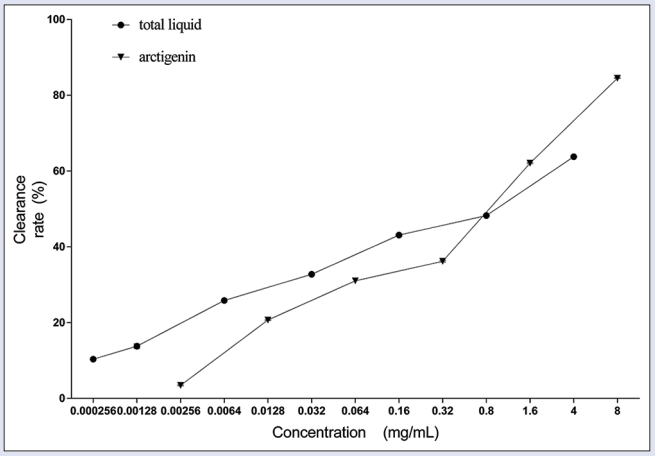

Figure 6: Results of acetylcholinesterase enzyme inhibitory activity

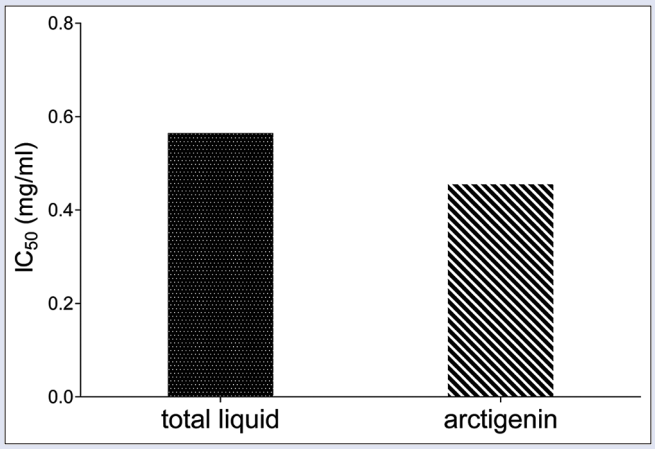

Figure 7: Results of acetylcholinesterase enzyme inhibitory activity (IC_{50})

showed that when the concentration of the extracting solution was 0.064 mg/ml, the nitrite clearing function was not obvious. Thus, the lowest concentration of extraction was 0.128 mg/ml. Because arctigenin has the defects of low water solubility, liposomes were prepared to overcome that and this created a conducive environment to combine with nitrite.

In the AChE inhibiting experiment, the concentrations of DTNB and ATCI were improved. First, we performed a study with the concentration on the basis of the data in the related literature. The concentration of ATCI is 0.075 M, and that of DTNB is 0.01 M. Next, we observed in this condition that the absorbance of the blank control was 3.110. It was easy to see that we did not obtain satisfactory data. Fortunately, we obtained an appropriate concentration through experiment. We rejoiced in surprise upon realizing that the absorbance value of the blank control was approximately 0.075 when the DTNB and ATCI concentrations were reduced, the concentration was 1 mM. At this time, the results are close to the absorbance values of only DMSO and PBS, and the other sample and control data are reasonable. In conclusion, we determined that the concentrations of DTNB and ATCI were both 1 mM.

From Figures 4 and 5, we can see that the extraction has the activities of inhibiting AChE and scavenging nitrite. The arctigenin obviously has better activity. Thus, we have reason to believe that the extraction has the activities of inhibiting AChE and scavenging nitrite because of the large amount of arctigenin in it. It is implied that the extraction and arctigenin would be beneficial to humans in clinical applications.

Nitrite is widely used as a food additive, especially in meat production.[35] Consuming an excessive amount of nitrite could cause a long list of injuries in public health. When nitrite reacts with the secondary amines and amides in the stomach, nitrosamine, a strong carcinogen, will be synthesized.[36] Furthermore, nitrite interferes with the oxygen transport system in the body by converting hemoglobin to methemoglobin in the blood,[37] and there are many serious health hazards associated with that. At present, AD has become a threat to elderly life after cardiovascular disease, and many academics are devoted to looking for drugs that exhibit an AChE enzyme inhibitory activity. Encouragingly, the results for the AChE enzyme inhibitory activity show that arctigenin can be developed for AChE inhibitor drugs, and it is very helpful for the treatment of AD. Arctigenin is also a potential compound that can be used in the treatment of many types of cancer. As a result, the exploitation of Fructus arctii to provide a source of natural arctigenin could not only bring considerable economic benefits but also contribute to health.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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