Enzyme-assisted Solvent Extraction of High-yield *Paeonia suffruticosa* Andr. Seed Oil and Fatty Acid Composition and Anti-Alzheimer's Disease Activity

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Abstract: Enzyme-assisted solvent extraction (EASE) of *Paeonia suffruticosa* Andr. seed oil (PSO) was optimized by response surface methodology (RSM). The fatty acid composition and anti-Alzheimer's disease (AD) activity of PSO were analyzed. An enzyme mixture composed of cellulase and hemicellulase (1:1, w/w) was most effective in determining the extraction yield of PSO. The ideal extraction conditions were a pH value of 5.1, an enzymolysis time of 68 min, and a temperature of 50°C. The average extraction yield of PSO was 38.2 mL/100 g, 37.4% higher than that of untreated peony seed (27.8 mL/100 g). The fatty acid composition of PSO under optimal conditions for EASE was analyzed by gas chromatography-mass spectrometry (GC-MS). The predominant unsaturated fatty acids of PSO were determined to be more than 90.00%, including n-3 α -linolenic acid (43.33%), n-6 linoleic acid (23.40%) and oleic acid (23.59%). In this experiment, the anti-AD effect of PSO was also analyzed by performing learning and memory ability tests with *Drosophila*. PSO retarded the decrease in climbing ability in AD *Drosophila*. The 1% and 5% PSO groups were significantly different from the model group ($p < 0.05$). The smell short-term memory ability test revealed the number of *Drosophila* in barrier and barrier-free centrifuge tubes in each group. PSO feeding improved learning and memory in AD *Drosophila*, with the highest number entering the barrier-free centrifuge tube. The performance index (PI) measured by the Pavlov olfactory avoidance conditioning test also demonstrated the effect of PSO on the learning and memory abilities of *Drosophila*. The PI of the PSO group was significantly increased compared to that of the model group. HE-stained brain tissue sections of AD *Drosophila* showed higher neurodegenerative changes, while PSO significantly reduced neurodegenerative damage. These results indicated that PSO can significantly improve the cognitive function of AD *Drosophila* and may help to prevent AD.

Key words: *Paeonia suffruticosa* Andr. seed oil, enzyme-assisted extraction, response surface methodology, *Drosophila* with tau gene, cognitive improvement

1 Introduction

Paeonia suffruticosa Andr. (Peony) is a traditionally famous ornamental flower with medicinal value that is native to temperate Eurasia. It has been widely distributed and cultivated in many regions, such as China, New Zealand, Turkey, Europe, North America and others¹. According to the FAO's food balance sheets in 2013, the average global intake of vegetable oils is more than four times that of animal fats. There has been a search for low-fat healthy vegetable oils that can replace animal oils².

Abbreviations: PUFA, polyunsaturated fatty acid; EASE, enzyme-assisted solvent extraction; ALA, α -linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; AD, Alzheimer's disease; GC-MS, gas chromatography-mass spectrometer; BBD, Box-Behnken design; CI, climbing index; OCT, 3-octanol; CS+, conditioned stimulus; MCH, 4-methyl cyclohexanol; CS-, without electric shock stimulus; ANOVA, analysis of variance; R², R-Squared; adj R², Adjusted R-squared; RSM, Response surface methodology; MBs, Mushroom bodies.

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Previous studies revealed that peony seeds contain over 25% oil content and can be developed as an emerging oil crop resource for dietary supplementation. The yield per mu of peony seed is much higher than that of rapeseed, soybean and peanut, and the content of peony seed oil (PSO) can be up to twice that of soybean oil. Purple peony and 'Fengdan' were approved as new resource foods by the Ministry of Health of China in 2011.

PSO is rich in polyunsaturated fatty acids (PUFAs), and the total PUFA content of PSO is generally 80%~90%, of which α -linolenic acid (ALA) is approximately 37.72%~67.13%, linoleic acid is approximately 19.09%~34.90% and oleic acid is approximately 15.13%~27.73%³⁻⁵. PUFAs have many physiological functions and play a vital role in the normal growth, development, metabolism and health of organisms. The amount and proportion of n-3 and n-6 fatty acids in the brain have a significant effect on learning and memory⁶⁻⁸, and a lack of PUFAs in the brain may lead to a decline in learning and memory abilities⁹. PSO contains a higher content of α -linolenic acid, so PSO can effectively improve the content of n-3 fatty acids in edible oils¹⁰. It has been reported that ALA can improve memory and play an important role in the prevention and treatment of AD¹¹⁻¹³. ALA is the precursor of omega-3 PUFAs, which can be converted into docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in living organisms under the action of specific enzymes¹⁴. DHA and EPA are essential for maintaining normal brain function¹⁵. Dietary supplementation of a certain amount of DHA and EPA in one's diet may improve learning and memory abilities and prevent the formation of Alzheimer's disease (AD) to some extent¹⁶. PSO is an edible oil with a high content of α -linolenic acid, which can be used to study dietary supplements to prevent the formation of AD. At present, the main experimental animal model of AD is the rodent model¹⁷. However, the rodent model of AD has limitations, such as the requirements for a long time and high costs for development in the process of modeling. *Drosophila* models with abundant homologous genes are widely used in animal research on AD. At the same time, they have the advantages of requiring less time and lower costs¹⁸. Several *Drosophila* models with tau toxicity with widespread relevance to tau pathology have been applied to AD¹⁹⁻²¹.

Press extraction and solvent extraction methods are conventionally applied for oil extraction²². A series of methods have also been developed, including aqueous, aqueous-enzymatic and enzyme-assisted solvent extraction²³. Enzyme-assisted extracts have emerged recently as an eco-friendly approach for seed oil extraction, and they have been applied to obtain seed oil extracts from diverse plant materials²⁴⁻²⁶. Enzyme-assisted extraction can improve oil yield and prevent changes in oil functional components. The addition of specific enzymes during the seed oil extraction process can enhance oil yield because

the cell wall and lipid bodies can be broken under the mild action of enzymes²⁷. The enzymatic reaction is conducted under mild conditions, which prevents changes in the functional components of oil²⁸. Wang *et al.* studied the enzyme-assisted aqueous extraction of rice germ oil, and the optimum yield of seed oil extracted by the complex enzyme (alcalase and cellulase, 1:1) was 22.27%²⁹. The yield of rice germ oil was relatively low (9.78%) when no enzyme was added to the extraction system. Polman and G demonstrated that enzyme-assisted extraction of pecan oil could give (65.3%) a higher yield than mechanical pressing (57.2%)³⁰. Gai *et al.* used a green and effective enzyme-assisted water extraction process to obtain F. suspense seed oil under microwave conditions, and the oil extraction rate increased by 56%³¹. In recent years, some researchers have studied the extraction of PSO. The results of Liu *et al.* showed that the PSO yield was 28.85% with extraction by ultrasonic-assisted hexane³². Lin *et al.* studied ultrasonic-assisted extraction of PSO using petroleum ether as the solvent, and the yield was 33.90%³³. Sun *et al.* obtained the highest oil yield (34.49%) with an ethanol and hexane mixture agent microwave-assisted extraction method to extract PSO under optimum extraction conditions³⁴. To our knowledge, there are few reports on enzyme-assisted extraction of PSO.

In this study, an aqueous enzymatic extraction procedure using a mixture of two enzymes was optimized to extract oil from peony seeds. After the optimized enzyme-assisted pretreatment process parameters of PSO are studied, the extraction process of PSO with *n*-hexane will be optimized continuously. Then, the effects of PSO on the improvement of *Drosophila* cognitive impairment will be investigated, and the olfactory learning assay, smell short-term memory ability and ability of climb *Drosophila* will be analyzed. This study will provide a theoretical basis for improving the yield of PSO and guiding the large-scale production of PSO. Additionally, PSO can supply humans with functional food components or act as a specific preventative pharmaceutical against AD.

2 Materials and Methods

2.1 Materials and reagents

The peony seeds (*Paeonia suffruticosa* Andr.) were collected from Heze, Shandong province, China. Cellulase (≥ 400 U/mg) and endocellulase (≥ 400 U/mg) were purchased from Jining Hexin Co., Ltd. (Shandong, China). Hemicellulose (≥ 3.0 U/mg) was purchased from Sigma-Aldrich Co., Ltd. (Steinheim, Germany). Sucrose fatty acid ester was purchased from Hangzhou Ruilin Chemical Co., Ltd. (Hangzhou, China). 4-Methyl cyclohexanol and 3-octanol were purchased from Shandong West Asia's Chemical Industry Co., Ltd. (Shandong, China). Methylparaben was

purchased from Shanghai Jilan Biological Co., Ltd. Donepezil hydrochloride was purchased from Jiangsu Haosen Pharmaceutical Co., Ltd. (Jiangsu, China). *n*-Hexane and other reagents were purchased from Tianjin Zhiyuan Chemical Reagent Co., Ltd. (Tianjing, China). Maizena was purchased from the market.

2.2 *Drosophila* stocks and maintenance

Three *Drosophila* stocks were used in this experiment. Wild-type W¹¹¹⁸, elavc¹⁵⁵-Gal4 and *Drosophila* (BS28991) were obtained from the Core Facility of *Drosophila* Resources and Technology, Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The *Drosophila* lines for the experiment were acquired by using Gal4/UAS system hybridization. All *Drosophila* were reared and maintained on standard corn agar media supplemented with dry yeast granules at 24°C, 60%~70% relative humidity and 12 h light/12 h dark at constant temperature and humidity within an incubator.

2.3 Animal grouping

The Gal4/UAS system was used to overexpress UAS transgenes in *Drosophila*³⁵. Elavc¹⁵⁵-Gal4 (♀) hybridized with *Drosophila* (BS28991) (♂) was used to express the AD model of *Drosophila* (*Drosophila* with the tau gene). The wild-type control model of *Drosophila* was obtained by elavc¹⁵⁵-Gal4 (♀) hybridization with W¹¹¹⁸ (♂)³⁶. All *Drosophila* had the same genetic background due to gene hybridization. *Drosophila* were divided into six groups. The normal group was the wild-type control model. The model group was AD model flies fed standard *Drosophila* food; the positive group was fed donepezil in the diet. The other three groups of AD *Drosophila* were fed separately on different doses of PSO mixed in the diet. PSO mixed with the emulsifier (sucrose fatty acid ester) was added to standard corn agar media supplemented with dry yeast granules at final PSO concentrations of 0.2%, 1% and 5% (volume fraction).

2.4 Enzyme treatment

Fresh peony seeds were dried at 50°C for 12 h. Then, they were manually deshelled, pulverized and ground into powder. After passing through 40 mesh sieves, the peony seed powder was stored in a closed plastic bag at 4°C until the time of extraction. Five samples of peony seed powder (approximately 10 g) were mixed with 50 mL of distilled water (the pH was adjusted to 5 with 0.5 mol/L HCl). Subsequently, 1000 U of hemicellulase, cellulase, endocellulase, cellulase/hemicellulase (enzyme activity 1:1) and 1000 U of endocellulase/hemicellulase (enzyme activity 1:1) were added to create mixtures. The mixtures were then stirred with the enzyme solutions and kept for 60 min in a 50°C water bath for enzymatic treatment. Finally, the enzymatic reactions were terminated by quickly adding liquid nitro-

gen and then freeze drying.

2.5 PSO extracted by enzyme-assisted solvent extraction (EASE)

Freeze-dried peony seed powders treated with enzyme were mixed with *n*-hexane (1:10, *m/v*) and cultured by shaking for 12 h. Next, the *n*-hexane extract was separated from the seed powder via a vacuum filtration pump. The seed powders separated in the previous step were mixed with *n*-hexane and extracted again for 6 h. Finally, two filtrates were merged, and *n*-hexane was removed in a rotary evaporator at 50°C to obtain PSO. The experiment was carried out with three replications.

2.6 Optimization of PSO extraction process by EASE

2.6.1 Single factor experiment with EASE

Ten grams of peony seed powder was mixed with 50 mL of enzyme solution containing 1000 U. To optimize the pH value, the pH of the enzyme-powder mixture was adjusted to 3, 4, 5 and 6 with 0.5 mol/L NaOH and 0.5 mol/L HCl, respectively. Then, the mixture was kept in a 50°C water bath for 60 min for enzymatic treatment. To optimize the incubation time, the enzyme-powder mixture was stirred or hydrolyzed at 30 min, 60 min, 90 min and 120 min and incubated at 50°C in a water bath. The enzyme-powder mixture was incubated at temperatures of 30°C, 40°C, 50°C and 60°C for 60 min in a water bath. After incubation, all of the above transformations were terminated by quickly adding liquid nitrogen, and the treated mixtures were freeze-dried. Then, the freeze-dried mixtures were extracted according to section 2.5. PSO yields were calculated by the following formula to obtain the optimum extraction conditions:

$$Yield (mL/100 g) = V(mL)/m(g)$$

V, volume of the oil extracted from peony seeds;

m, mass of the peony seeds.

2.6.2 Box-Behnken design (BBD) for EASE

A BBD model for response surface analysis was utilized to obtain the optimum EASE conditions. Three factors (A, pH; B, time; C, temperature) at three levels were chosen to evaluate their combined effect. For each factor, the experimental range was selected based on the results of the single-factor experiments. The conditions of PSO extraction were optimized by Design-Expert® v. 10.0.7 analysis software, and the experimental factor-level code was derived, as shown in Table 1.

2.7 Composition of fatty acids in PSO

To determine the fatty acid composition of PSO and the effect of enzyme treatment, the oil sample was converted into fatty acid methyl esters and then analyzed by gas chromatography mass spectrometry (GC-MS). GC-MS analysis of PSO was carried out on an Agilent 7890A-7000B

Table 1 Experimental factors level code.

Level	Factor		
	A - pH	B - time (min)	C - temperature (°C)
-1	4	30	30
0	5	60	40
1	6	90	50

equipped with an HP-88 column (100 m × 0.25 mm i.d., 0.20-μm film thickness). Helium was used as the carrier gas at a flow rate of 1 mL/min, and analyses were performed in constant flow mode. The temperatures of the transfer line, ion source, and quadrupole were set at 280°C, 230°C, and 150°C, respectively, while the injector temperature was set at 250°C. The split ratio was 10:1, and the injection volume was 0.05 μL. The program temperature process started at 120°C for 1 min, increased at 10°C/min up to 175°C, and was maintained for 10 min. Then, the temperature was raised at 5°C/min up to 210°C for 5 min and 230°C for 5 min with a total run time of 37.5 min. The ionization mode had an electronic impact of 70 eV and a mass range of 30~500 m/z ³⁷⁾.

2.8 *Drosophila* learning and memory abilities test

2.8.1 *Drosophila* climbing assay

The *Drosophila* climbing assay was performed as previously described³⁸⁾. Twenty male *Drosophila* were transferred into the test vial to adapt for 5 min. After tapping the flies down to the bottom of the vial, the number of flies that climbed to the top of the vial within 10 s was counted as the climbing index (CI = A/account, where A is the number of flies that climbed to the top, and account is the total number of flies). The experiment was performed in a dark room with red light. Five trials were performed for each group to record the mean value of the whole number every five days until the flies were not able to complete the experiment.

2.8.2 *Drosophila* smell and short-term memory abilities test

First, the flies that fed for a month were starved in a dry sterilized empty bottle for 2 h. Next, the flies were placed in two 250 mL centrifuge tubes, which had approximately 10 holes in the lower half of each centrifuge tube. The two centrifuge tubes were placed into a large beaker. One of the centrifuge tubes contained bananas, which the flies were able to smell and eat. Another centrifuge tube contained bananas, but a layer of gauze was placed over them, and the flies were able to smell them but could not eat them. The number of flies entering the two tubes was recorded every half hour, and the average was obtained after performing the test five times.

2.8.3 Olfactory training

The olfactory assay was performed as previously de-

scribed³⁹⁾. Each group contained approximately 100 flies, and they were loaded into a training tube with a copper mesh cross-electrode followed by administration of fresh air for 90 s. One training cycle consisted of 60 s of 3-octanol (OCT) administration as the conditioned stimulus (CS+), which was paired with twelve 1.5 s pulses of 60 V for DC (direct current) electric shock. This was followed by an influx of fresh air for 45 s as the intertrial interval, subsequently followed by 60 s of 4-methyl cyclohexanol (MCH) administration without electric shock (CS-). After training, the memory retention test was performed with a Plexiglas T-maze in which both CS+ and CS- were introduced simultaneously in opposite directions with the same concentration of chemicals as provided in the training step. The trained flies of each group were introduced at the decision point and allowed to choose between CS+ and CS-. At the end of the training, the flies were collected and anesthetized. Memory performance indices were expressed as performance index (PI): $PI = (\text{Number CS-} - \text{Number CS+}) / (\text{Number CS-} + \text{Number CS+}) \times 100\%$. The overall PI was finally calculated as the average of the two PIs, one for each group.

2.9 Hematoxylin-eosin (HE)-stained *Drosophila* brain sections

Drosophila heads were cut off and fixed in 4% paraformaldehyde for 4 h at room temperature or overnight at 4°C. The fixed fly heads were flushed with running water for several hours. The samples were dehydrated with alcohol (70%~100%), made transparent in a mixture of pure alcohol and xylene for 30 min, and then cleared in xylene for 15 min twice. After being dehydrated, the samples were embedded in paraffin. With the help of a scalpel, the extra paraffin was carefully cut out from around the fly head tissue. The *Drosophila* brain tissues were cut on a hand-operated microtome with a section thickness of 10 μm~15 μm. The *Drosophila* brain sections were stained with HE. Finally, under an optical microscope, ten brain sections of *Drosophila* from each group were observed and compared. Then, the cavities and tangles of nerve fibers generated by neuron cell death were observed^{40, 41)}.

2.10 Statistical analysis

All analyses were performed with SPSS software package v. 21.0 for Windows. One-way ANOVA was used to determine the significant differences among the treatments (F-test at the 0.05 significance level), and Duncan's test was performed as a post hoc multiple comparison test.

3 Results and Discussion

3.1 The effect of different enzyme pretreatments on PSO yield

The EASE method for oil extraction from peony seeds is a good alternative because it is an environmentally friendly and green technology. We screened to identify an appropriate enzyme for pretreatment and optimized the extraction process conditions. Generally, oil droplets are surrounded by the cell wall, which contains various polysaccharides, such as hemicelluloses, starch, and pectin, in large amounts, reducing the extraction efficiency of conventional extraction techniques. Hence, we can use a mixture of cellulase, pectinase, and other enzymes to degrade these components, resulting in enhanced oil yield^{42–44}. Studies have shown that mixing diverse enzymes to work synergistically can enhance the extraction efficiency^{45, 46}.

The utilization of enzymatic pretreatment prior to solvent extraction can be attributed to the degradation of cellulose, pectin and protein, destroying the structural integrity of the seed cell wall and liberating more oils⁴⁷. F R Mohamed revealed that enzymatic hydrolysis of goldenberry pomace oil followed by *n*-hexane extraction can shorten the extraction time and improve extraction efficiency⁴⁸. Enzymatic treatment can further degrade the cellular structure of goldenberry pomace so that solvent extraction can allow more oil release. Compared with the effect of mixed enzymes, the extraction yield of a single enzyme is generally small. F R Mohamed investigated the combination of Pektinase L40 and Cellulase EC (1:1, w/w) and obtained the highest (42.1%) extracted goldenberry pomace oil under similar extraction conditions⁴⁹. According to **Table 2**, pretreatment with various combinations of enzymes has a significant influence on the PSO yield. The PSO yield without enzyme treatment was 27.8 mL per 100 g of peony seed powder. The mixed enzyme A (cellulase: hemicellulase = 1:1) group obtained the maximum PSO yield in all groups, reaching 31.0 mL/100 g. Compared to the treatment group lacking enzyme, the oil yield of the enzyme treatment group increased 11.5%. The test inferred that enzyme pretreatment prior to oil extraction can notably improve the PSO extraction yield.

3.2 Single factor experiments for pH, time and temperature

The pH, extraction time and temperature of the reaction are critical for the extraction of bioactive macromolecules⁵⁰. The effects of pH, time and temperature on the extraction yield of PSO are shown in **Fig. 1**. In general, all kinds of enzymes are active only within a suitable pH range⁵¹. The pH when the enzyme activity is the highest is the optimal pH of an enzyme. Enzyme activity will decrease gradually at pH values above or below this value. The three-dimensional shape of an enzyme changes due to the altered dissociation state of active groups. Thus, enzymes become denatured and unable to catalyze chemical reactions⁵². **Figure 1 (A)** shows the effect of pH on increased PSO yield. The variation in PSO yield at different pH levels demonstrated that the maximum enzyme activity was observed at pH 5. Under this condition, enzyme activity was maximized to accelerate the degradation of cellulose material of peony seeds. When the pH was over five, the chemical environment within the cell was not conducive to enzyme hydrolysis, and the enzyme activity was decreased.

In addition to pH, time and temperature were also vital parameters during oil extraction. As presented in **Fig. 1 (B)**, we can observe the effect of time on PSO yield. The enzymatic reaction took place between 30 min~60 min, and the enzymatic hydrolysis of peony seed was complete at 60 min. When the extraction time exceeded 60 min, the PSO yield decreased obviously. This result might be due to the depletion of substrates for the reaction or product-mediated inhibition of enzymes³¹. A more optimal enzymatic temperature led to a higher PSO yield, between 30°C and 50°C, as shown in **Fig. 1 (C)**. The PSO yield increased with increasing enzymatic hydrolysis temperature. However, the PSO yield was reduced beyond 50°C. Enzyme activity will be inhibited at elevated extract temperatures, which also causes the deactivation of proteins and other bioactive molecules⁵³. Therefore, we obtained a suitable range of extraction times and temperatures.

3.3 Optimization of EASE

3.3.1 Statistical analysis and model fitting

According to the results of the single-factor experiments,

Table 2 Yields of peony seed oil by different enzymatic pretreatment.

Number	Group	yield of PSO (mL/100 g)
1	No enzyme pretreatment	27.8
2	Hemicellulase treatment	30.0
3	Cellulase pretreatment	30.0
4	endo-cellulase pretreatment	27.0
5	Mixed enzyme A (Cellulase : Hemicellulase = 1: 1) pretreatment	31.0
6	Mixed enzyme B (endo-cellulase : Hemicellulase = 1: 1) pretreatment	29.4

Fig. 1 Effects of different pH(A), extraction time(B), and temperature(C) on PSO yield.

Table 3 Scheme and data results of Box-Behnken design.

Experiment no.	A: pH	B: time (min)	C: temperature (°C)	yield of PSO (mL/100 g)
1	6	60	50	34.4
2	5	30	30	34.5
3	6	30	40	34.0
4	6	60	30	34.1
5	5	30	50	35.0
6	4	60	30	32.0
7	4	30	50	31.0
8	5	90	30	35.5
9	4	60	50	34.0
10	6	90	40	35.8
11	5	90	50	36.5
12	4	30	40	31.0
13	5	60	40	37.0
14	5	60	40	38.8
15	5	60	40	38.9
16	5	60	40	38.6
17	5	60	40	38.7

the RSM was used to optimize the processing conditions of the PSO extraction. The following analysis shows the entire design of the present study, which consists of 17 experimental points (Table 3). All experiments were carried out at random to minimize any effect on the observed response

values. All trials were conducted in triplicate. Three replications at the center of the design were adopted to estimate the pure error of the sum of squares. By applying the multiple regression analysis on experimental data, a mathematical model describing the relationship of the response

Table 4 Variance analysis results of the response surface regression equation of peony seeds oil extraction.

Source of variation	Sum of Squares	DF	Mean Square	F-Value	P-Value	significance
Model	91.31	9	10.15	15.91	0.0007	**
A - pH	9.68	1	9.68	15.18	0.0059	**
B - time (min)	4.20	1	4.20	6.59	0.0371	*
C - temperature (°C)	1.80	1	1.80	2.83	0.1364	
AB	0.023	1	0.023	0.035	0.8564	
AC	0.72	1	0.72	1.13	0.3225	
BC	0.063	1	0.063	0.098	0.7634	
A ²	49.03	1	49.03	76.87	<0.0001	**
B ²	11.64	1	11.64	18.24	0.0037	**
C ²	7.82	1	7.82	12.25	0.0100	*
Residual	4.46	7	0.64			
Lack of Fit	1.97	3	0.65	1.05	0.4628	not significance
Pure Error	2.50	4	0.62			
Total	95.78	16				

variable and the test variables was derived according to the following second-order polynomial equation:

$$y = 38.40 + 1.10A + 0.73B + 0.47C - 0.25AB - 0.43AC + 0.13BC - 3.41A^2 - 1.66B^2 - 1.36C^2$$

The ANOVA results illustrated the response surface regression equation of PSO extraction (Table 4). The *p*-value was used as an indicator to determine the significance of each coefficient that may reveal the pattern of interactions among the variables. The smaller the *P* value, the more significant the corresponding coefficient. When $p < 0.05$, the parameters had a significant impact. R-squared (R^2) was used to determine the goodness of fit between the model and the data. The higher the R^2 value was, the better the model fitting data. R^2 had a value of 95.34%, thus indicating that the fitted model was highly significant. It was calculated by the ratio of 1 minus the sum of squared errors in the sum of squares. Adjusted R-squared (adj R^2) had a value of 89.34%, which was calculated by subtracting the ratio of the mean squared error from the sum of the mean squared error. This demonstrated that only 1.96% of the total variations could not be explained by the model. Table 4 shows that A, B, B², and C² had a significant effect (*p*-value less than 0.05) on the results, and C was not significant. The *p*-values of <0.0001 indicated that the model was highly significant and that A² had a highly significant effect (*p*-value less than 0.0001) on oil yield. The influence of each factor on the yield of PSO conformed to the following order based on F-values: A (pH) > B (time) > C (temperature).

3.3.2 Response analysis of PSO yield

The three-dimensional response surface plot for the effect of independent variables (pH, time and temperature)

on the PSO yield is presented in Fig. 2. Response surface maps can directly reflect the relationship between each factor and the response value as well as the interaction between each factor. A steeper slope of the response surface and a dense elliptical contour indicate a greater influence of the interaction between the two factors. However, the circular contour lines indicate that the interaction is not significant. Figure 2(A) shows that the contour lines are circular, and the interaction of pH and temperature is not significant. The steepness of the three-dimensional surface indicated that the PSO yield was affected by both A and B. This is consistent with the ANOVA results. When the temperature was fixed at 40°C, the pH was in the range of 4.5~5.5, and the time was in the range of 66~78 min, the PSO yield reached the maximum value. Figure 2(B) shows that the contour lines are oval, indicating that the interaction of pH and temperature had an impact on the PSO yield. When the extraction time was fixed at 60 min, the pH was in the range of 4.5~5.5 and the temperature was in the range of 35~45°C, the PSO yield reached the maximum value. Figure 2(C) shows that the contour lines are oval, indicating that the interaction of time and temperature had an impact on the PSO yield. When the pH was fixed at 5, the time was in the range of 66~78 min, the temperature was in the range of 35~45°C, and the PSO yield reached the maximum value. It was observed that the oil yield increased with pH and enzymatic hydrolysis time and then decreased after peaking. Furthermore, as the hydrolysis time and temperature increased, the PSO yield first increased and then decreased after peaking.

3.3.3 Validation of models

Based on the results of the RSM that investigated the

Fig. 2 3D response surface and contour plots effects: (A) pH and Time; (B) Temperature and pH; (C) Temperature and Time.

Fig. 3 The fatty acid profile of PSO by GC-MS analysis.

feasibility of the experimental results, the following parameters were selected as the conditions for the experiments: a pH value of 5.1, an enzymolysis time of 68 min, and an

enzyme solution temperature of 50°C. The procedure was repeated five times following the validation of the PSO average yield, which was 38.2 mL/100 g with a relative error of the theoretical value of 0.4%. This result was in agreement with the predicted values of the regression equation, and the extraction conditions had high reliability.

3.4 Analysis of PSO composition by GC-MS

According to the GC/MS conditions of PSO, the components of PSO extracted by EASE were analyzed (Fig. 3). By retrieving wiley7n.1 and consulting relevant literature on PSO, each chromatographic peak corresponding to the mass spectrum figure was analyzed. At the same time, the normalized method was used to calculate the relative proportion and peak area of each component (Table 5). The PSO samples detected five main fatty acids: palmitic acid, stearic acid, oleic acid, n-6 linoleic acid, and n-3 linolenic acid. The predominant unsaturated fatty acids of PSO were determined to be more than 90.00%, including n-3

Table 5 The results of the components of peony oil extracted from untreated and enzyme pretreated peony seeds.

Number	Compound	Chemical formula	Molecular weight	Relative amount
1	Palmitic acid	C ₁₆ H ₃₂ O ₂	256	7.43%
2	n-6 Linoleic acid	C ₁₈ H ₃₂ O ₂	280	23.40%
3	n-3 linolenic acid	C ₁₈ H ₃₀ O ₂	278	43.33%
4	Stearic acid	C ₁₈ H ₃₆ O ₂	284	2.24%
5	Oleic Acid	C ₁₈ H ₃₄ O ₂	282	23.59%

α -linolenic acid (43.33%), n-6 linoleic acid (23.40%) and oleic acid (23.59%). The n-6/n-3 ratio of PSO was 0.54, ranging between 0.4 and 1.63, and the content of ALA was greater than the standard state (38%). These results indicate that the PSO extracted by EASE conforms to the industry standard of Chinese peony seed issued in 2014.

3.5 The anti-Alzheimer's disease (AD) activity of PSO

In this study, we used the Gal4/UAS system to establish *Drosophila* expression in wild-type tau transgenic plants to study an experimental model of AD. The clinical manifestations of AD are cognitive impairment, including disorders in memory, attention and function. Language and visuospatial cognition were noticeable in the cognitive deficits of AD patients⁵⁴. Mushroom bodies (MBs) play a crucial role in smell learning and memory in *Drosophila*⁵⁵. The accumulation of tau in adult mushroom body neurons of the *Drosophila* brain can induce the impairment of learning and memory⁵⁶. Meanwhile, degenerating neurons and vacuoles were observed in the MBs. Alpha-linolenic acid deficiency caused a decrease in DHA in the hippocampus of the brain, damaging and shrinking the size of neurons⁵⁷. Tau protein is considered a target for the development of novel drugs aiming to treat AD⁵⁸. PSO was rich in ALA, which accounted for up to 40%⁵⁹. Chronic supplementation with ALA for elderly patients can prolong the development of learning and memory deficits⁶⁰. ALA also regulated the content of other fatty acids in the brain and increased the content of n-3 PUFAs, which may be an important reason for improving cognitive, learning and memory abilities⁶¹. ALA supplementation improved spatial learning and memory after stroke, which may correlate with higher survival of hippocampal neurons⁶².

3.5.1 Effect of PSO on improving the climbing ability of *Drosophila*

The initial sign of cognitive impairment in Alzheimer's patients is a barrier to the creation of new memories⁶³. The results pertaining to the climbing ability of *Drosophila* with the tau gene are shown in Fig. 4(A). After the fifth day of testing, the climbing ability of *Drosophila* in each group began to decline. Climbing ability in the AD group showed a significant decline on the 15th day. The climbing ability of *Drosophila* treated with PSO declined after 20

days. This means that treatment with PSO can improve the climbing ability of *Drosophila*. The climbing index of *Drosophila* treated with PSO was approximately twice that of the model group. The 1% and 5% PSO groups were significantly different from the model group (^b $p < 0.05$). Climbing behavioral experiments showed that *Drosophila* with the tau transgene fed PSO had better learning and memory abilities. These results demonstrated that PSO could improve and relieve the motor impairment of AD.

3.5.2 Effect of PSO on smell short-term memory of *Drosophila*

Figures 4B and 4C show the smell short-term memory ability of *Drosophila*. The number of *Drosophila* in the barrier and barrier-free centrifuge tubes in each group was observed, and the results showed that the numbers initially rose and then decreased. In the first hour, the number of *Drosophila* in the barrier centrifuge tube without an obstacle showed a rising trend and subsequently stabilized in 2.5 h. It was observed that *Drosophila* in each group entered the barrier-free centrifuge tube. The number of *Drosophila* in the donepezil hydrochloride group and the 5% PSO group changed the most rapidly, exhibiting higher numbers. After 1 h, the number of *Drosophila* in the model group was reduced and significantly lower than that in the other groups. The 1% and 5% PSO groups were significantly different from the model group (^b $p < 0.05$). We observed the number of *Drosophila* in each PSO-treated group that entered the barrier centrifuge tube. The number of *Drosophila* in the 5% PSO group was the lowest, with a minimal variation range, followed by the 1% PSO group, and the worst effect was observed in the 0.2% group. Experiments with olfactory short-term memory have shown that *Drosophila* can use their keen sense of smell to identify the direction of an odor.

3.5.3 Effect of PSO on the memory of *Drosophila* in olfactory conditioning

In this study, Pavlov olfactory avoidance conditioning was used to detect the cognitive function of *Drosophila*. This method has been widely used in the field of *Drosophila* behavior research and is a classic method to test the associative learning and memory ability of *Drosophila*. Electrical stimulation was used to punish *Drosophila* to determine whether they associated punishment with a par-

Fig. 4 (A) Climbing ability test of *drosophila*; (B) The variation of *drosophila* numbers entering the centrifuge tube and cannot eating food; (C) The variation of *drosophila* numbers entering the centrifuge tube and eating food; (D) Effects of PSO on the learning and memory of the *drosophila* with tau gene. ^a $p < 0.05$, Compared to normal group; ^b $p < 0.05$, Compared to model group.

titular smell. *Drosophila* in the model group had the lowest PI value, which proved that *Drosophila* with the tau gene had obvious learning and memory deficits. The PI value of the PSO group was significantly increased compared to that of the model group (Fig. 4(D)).

3.5.4 Morphology of *Drosophila* brain sections

Microscopic observation of 30-day-old *Drosophila* HE-stained brain tissue sections showed that cell body nerve degeneration was higher in the model group. *Drosophila* fed PSO had fewer vacuoles and nerve fiber tangles than those in the model group (Figs. 5A-5F). The results showed that normal *Drosophila* had better organization of the brain, no cavitation or nerve fiber tangles, and dense and uniform neurons. In the model group, vacuolar degeneration was significantly increased in the brain tissue of *Drosophila*, and nerve cell injury was increased with sparseness and unevenness. The hybrid experiment was successful in establishing a model to study AD. PSO could significantly reduce neurodegenerative lesions in the brain tissue of AD model *Drosophila*.

4 Conclusion

In this study, a moderate and effective method for extracting PSO was optimized. Compared with the reported extraction yield of PSO, the EASE method obtained a higher oil yield. The unsaturated fatty acid content of PSO was more than 90.00%, and the content of ALA (43.33%) was more than the standard state (38%). AD *Drosophila* was used to investigate improvements in learning and memory abilities and cognitive dysfunction conferred by PSO. This study provides a theoretical basis for the effect of PSO on improved cognitive impairment. PSO may become a new anti-AD resource food and has potential as a source of α -linolenic acid. Furthermore, the mechanism by which PSO improves cognitive dysfunction needs further study.

Ethics Statements

This study employed *drosophila* as model organism and has not involved any human subject.

Fig. 5 Photomicrographs of *drosophila* brain tissue (stained with H&E 400X). (A) The normal *drosophila* group; (B) The model *drosophila* group; (C) The group with low dose of PSO; (D) The group with middle dose of PSO; (E) The group with high dose of PSO; (F) The group with donepezil Hydrochloride Tablets. Among them, solid arrows point to cavitation and dotted arrows point to nerve fiber tangles.

Conflict of Interest

All authors declare that there are no conflicts of interest.

Author Contributions

Gang Wei: designed research, performed research, analyzed data, wrote the manuscript; **Zidong Zhang:** performed research, contributed analytic tools, analyzed data; **Dongmei Fu:** performed research, analyzed data; **Yuanyuan Zhang:** analyzed data, contributed analytic tools; **Weipeng Zhang:** analyzed data, contributed analytic tools; **Yuangang Zu:** project administration, funding acquisition; **Zhi Zhang:** funding acquisition, review the manuscript; **Lin Zhang:** Supervision, designed research, review modified the manuscript.

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