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Isolation, Purification, and Anti-Aging Activity of Melanin from *Lachnum singerianum*

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Abstract Intracellular melanin from *Lachnum singerianum* YM296 (LIM) was obtained by the method of microwave-assisted extraction. On the basis of single factor experiment, the optimum microwave-assisted extraction conditions of LIM using Box–Behnken design were as follows: NaOH concentration, 1.05 mol/L; ratio of raw material to liquid ratio, 1:14.72 (g/mL); microwave time, 118.70 s; and microwave power, 320 W. Under these conditions, the extraction yield of LIM was 11.08 %, and 40.43 % higher than that of extraction by alkali extraction and acid precipitation. The results showed that microwave-assisted extraction could increase the extraction yield of LIM. Three homogeneous components were fractionated from LIM by Sephadex G-15 column. LIM-a was the main homogeneous component, accounting for 75.7 % of the total content of the homogeneous components. Aged mice model were induced by injecting D-galactose, and the aged mice were given LIM-a of different dosages, respectively. The results showed that LIM-a can significantly increase body weight, thymus indexes, and spleen indexes of the aged mice; effectively elevate the levels of SOD, GSH-PX, and CAT; and decrease the level of MDA in mice liver homogenate, brain homogenate, and serum, indicating that LIM-a had significant anti-aging activity.

Keywords *Lachnum singerianum* melanin · Microwave-assisted extraction · Fractionation · Anti-aging activity

Introduction

Aging is the degenerative change of body tissue and organ functions in the life cycle along with age, which is the result of the comprehensive effects of many physiological and pathological processes [1]. Research shows that one of the important reasons leading to the aging of human body is that, the surplus free radicals produced at the time of a series of

Highlights The optimum microwave-assisted extraction conditions of *Lachnum singerianum* were obtained. The yield of melanin (LIM) under the optimal conditions was 11.08 %. Three homogeneous components were fractionated from *L. singerianum* intracellular melanins. LIM-a was the main homogeneous component. LIM-a had significant anti-aging activity.

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oxidative metabolism of the organism, excess free radicals may attack the cell membrane, nucleic acid, protein, enzymes, and other biomacromolecules by peroxidation resulting in the severe damage of cell functions and eventually aging and death [2]. Reducing the generation of free radicals, clearing away the aging metabolites, and enhancing the activities of antioxidant enzymes, which turn out to be effective ways for anti-aging [3]. Therefore, seeking for natural free radical scavengers without toxic side effects, developing anti-aging drugs are important significance to human health protection.

Melanins are polyphenol polymer with irregular structure, which are formed by the oxidation of polyhydric phenols [4]. Melanins are produced by some animals, plants, and microorganisms [5–7], and have antioxidant, antitumor, antiviral, antiradiation, liver injury protection, and other biological activities [8–11]. Generally, melanins are soluble in alkaline solutions but may precipitate as sediment in acid solutions. Therefore, the alkali solution and acid isolation is one of the commonly used methods to extract natural melanins [12]. However, the foresaid method takes long time and shows low extraction efficiency [13], so, in recent years, ultrasonic-assisted extraction, microwave-assisted extraction, and enzymatic methods have been used to extract the melanin [14–16], aiming to shorten the extraction time and increase the melanin yield.

Production of melanin by microbial fermentation is prone to industrialization, which has the advantages of less input. *Lachnum* is a class of saprophytic fungi, which can produce a large number of melanins with antiradiation and antioxidant activities under submerged culture conditions [17, 18]; however, melanins from *Lachnum* having anti-aging activity is unclear. This study is intended to obtain the optimum microwave-assisted extraction conditions of LIM, obtain the homogeneous component of *L. singerianum* YM296 melanins (LIM-a), and evaluate its anti-aging activity.

Materials and Methods

Strains

Lachnum specimens were collected from Huangshan Mountain (Anhui China) and they were identified as *L. singerianum* by morphological observation method [19]. The strain was isolated using the spore shooting methods [20], which was preserved in Microbial Resource and Application Laboratory of the Hefei University of Technology.

Experimental Animals

Sixty Kunming mice (30 male and 30 female), weighed 20±2 g, were purchased from the Experimental Animal Center of Anhui Medical University (Certificate number: No. 1 license of the Medical Laboratory Animal of Anhui). The animals were kept at 23±2 °C with humidity of 55±5 % and cultured in a 14:10 h light–dark cycle.

Reagents and Instruments

Sephadex G-15 (Pharmacia, USA); superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), and glutathione peroxides (GSH-PX) kits (Jiancheng Bioengineering Institute, Nanjing, China) and D-galactose (Sigma, USA); other reagents were all analytical reagents, which were purchased from Shanghai Zhenqi Chemical Reagent Co., Ltd. (Shanghai, China).

WD780BS microwave oven (Galanz, China), 752 spectrophotometer (Shanghai Precise Scientific Instruments Co., Ltd), AR1140 electronic analytical balance (Ohaus International Trade Co., Ltd), TDL-50B desk centrifuge (Shanghai Anting Scientific Instrument Factory), and 5-L magnetic stirring glass fermentation tank (Oriental Bioengineering Equipment Co., Ltd, Zhenjiang, China).

Acquisition of *L. singerianum* YM296 Mycelium

L. singerianum YM296 with the diameter of 6 mm was inoculated in 250-mL flask (with 150 mL liquid) using the puncher, stirred at the rate of 160 r/min, and incubated at 27 °C for 4 days; 5-L automatic fermentation tank was used to ferment the seed solution. Fermentor conditions—stirring speed, 160 r/min; fermentation temperature, 27 °C; and fermentation time, 8 days. Fermentation medium was prepared with 20 % potato extract, 2 % glucose, 0.5 % yeast extract, 0.003 % tyrosine, and 0.028 % magnesium sulfate. The fermentation broth was drawn and filtrated to obtain the mycelium, which was dried in an oven at 50–55 °C for 24 h.

Microwave-Assisted Extraction of LIM

The mycelium (0.5 g, on dry wt basis) was mixed with 20 mL NaOH solution. After being put in the microwave oven for some time, the mixture was centrifuged at 3,800×*g* for 10 min. The supernatant was obtained and diluted 20 times, and the absorbance at 520 nm value was determined. The greater the absorbance value is, the higher the yield is. The single factor experiment was conducted to examine the effects of the concentration of NaOH solution, microwave power, microwave time, solid–liquid ratio on the yield of melanin by changing only one parameter at a time, leaving the others unchanged. The NaOH concentrations were 0.5, 1.0, 1.5, 2.0, and 2.5 mol/L; microwave powers were 160, 320, 480, 640, and 800 W; microwave times were 30, 60, 90, 120, and 150 min; and solid–liquid ratio were 1:10, 1:15, 1:20, 1:25, and 1:30 g/mL, respectively.

According to the results of the single factor experiment, appropriate NaOH concentration, microwave time, and solid–liquid ratio were selected for Box–Behnken experiment and the experimental results were verified. The experiment was repeated three times, and the average of absorbance values was used. Using multiple regression analysis with Design Expert 8.0 software, the Box–Behnken experimental results were fitted into the quadratic polynomial model $Y=\beta_0+\alpha_1 A+\beta_1 B+\gamma_1 C+\alpha_1\beta_1 AB+\alpha_1\gamma_1 AC+\beta_1\gamma_1 BC+\alpha_{11}A^2+\beta_{11}B^2+\gamma_{11}C^2$, and F test was employed for analysis of variance to evaluate the significance of the quadratic polynomial model and its regression coefficient.

Preparation of the Homogenous Melanin

The intracellular melanins of *L. singerianum* YM296 (LIM) were purified according to the method of Ye et al. [21]. The extracted melanins were fractionated by the method of Olennikov et al. [22] with slight modification. LIM (15 mg) was dissolved in 1 mL 0.5 % $NaHCO_3$ solution for chromatography with Sephadex G-15 column (1.6 cm×60 cm), with 0.5 % $NaHCO_3$ solution as eluant, injection volume of 1 mL, and flow rate of 200 μL/min. The samples were partly collected with each tube containing 4 mL. The absorbance values of the samples were measured at 520 nm, and the elution curve was made by absorbance vs. tube number. The main component was collected, concentrated under reduced pressure and dialyzed against distilled water for 48 h using dialysis membrance (MWCO 200), and freeze-dried to obtain the homogeneous component of LIM (for subsequent experiments).

Anti-Aging Activity

Grouping and Treatment

Healthy KM mice were weighed after a 3-day adaptation to the environment. The mice were allowed free access to food and water. In addition, D-gal was injected intraperitoneally at a dose of 200 mg/kg body weight every day. After injection for six consecutive weeks, the mice became thin, weak, and languished, showing obvious characteristics of aging, which indicated that the D-gal aging model was successfully established [23].

Fifty D-gal-induced aged mice (25 male and 25 female) were randomly divided into five groups (10 mice in each group) as follows: D-gal model control group (0.5 % sodium carboxymethyl cellulose saline), positive control group (vitamin C, 100 mg/kg bw/day), low-dose LIM-a group (50 mg/kg bw/day), medium-dose LIM-a group (100 mg/kg bw/day), and high-dose LIM-a group (200 mg/kg bw/day). Ten normal mice (not treated with D-gal) were taken as the normal control group (0.5 % sodium carboxymethyl cellulose saline). All LIM-a with corresponding concentrations were dissolved in 0.5 % sodium carboxymethyl cellulose suspension. Mice of all groups were weighed and recorded, respectively. Each mouse was given 0.2 mL drug by gavage for 30 days continuously.

Determination of Anti-Aging Indexes

The mice were weighed and killed on the second day after last gavage. The blood was collected by eye enucleation and the serum was separated by centrifugation for 5 min at 5,000 rpm. The serum was reserved at 4 °C. Spleens and thymuses of mice were taken and weighed, and the organ indices were calculated. Weight growth rate (%)=[(final weight−initial weight)/initial weight]×100; organ index (%)=(organ weight/body weight)×100. Livers and brain tissues of the mice were washed with precooled normal saline (4 °C), and dried with filter paper and weighed. The liver or brain homogenates at 10 % (*w*/*v*) concentration was prepared by homogenizing 200 mg of liver or brain in 2.8 mL of cooled normal saline (4 °C), centrifuged at 5,000 rpm for 5 min, and the supernatants were collected and used for enzymes and MDA analysis. Determination of SOD, GSH-PX, CAT activities, and MDA content in serum, liver homogenate, and brain homogenate were performed in accordance with the kit instructions.

Statistical Analysis

All data were processed with DPS V6.55 statistical software and represented as mean± standard deviation. The *t* test analysis was used to analyze the differences between groups. Differences were considered statistically significant when $P<0.05$ and very statistically significant when $P<0.01$.

Results and Discussion

Extraction Technology of LIM

The singlefactor experiment showed that the optimum microwave-assisted extraction conditions of LIM were as follows: 1:15 (g/mL) solid–liquid ratio, 320 W microwave power, 120 s microwave time, and 1.0 mol/L NaOH concentration.

The appropriate NaOH concentration, microwave time, and solid–liquid ratio were selected based on the single factor experiment to conduct the Box–Behnken experiment (Table 1), and Design-Expert 8.0 was used to analyze the data, as shown in Table 2. The NaOH concentration (A) had the greatest effect on the absorbance value, followed by the solid–liquid ratio (B) and the microwave time (C).

The regression equation is:

$$Y = 0.32 + 0.012A - 6.875 \times 10^{-3}B + 3.375 \times 10^{-3}C - 2. \times 000 \times 10^{-3}AB - 0.013AC + 7.750 \times 10^{-3}BC - 0.061A^2 - 0.063B^2 - 0.059C^2 \quad (1)$$

Variance analysis was carried out to the regression coefficient and experimental results of the quadratic polynomial model and the results were shown in Table 3. F_{model}=49.70 and P_{model}<0.0001, indicating that Eq. (1) was significant; the lack of fit probability P_{lose} (0.1155) was greater than 0.05, suggesting that no lack of fit factor existed; the determination coefficient R^2=0.9846, implying that Eq. (1) could well reflect the true relation between the selected parameters and the response values [24]. In addition, the variable coefficient CV (4.47 %) <5 %, indicating that Eq. (1) had a good reproducibility [25]. The NaOH solution concentration and LIM yield showed significant linear effects (P<0.5) and greatly significant quadratic effect (P<0.1). The solid–liquid ratio, microwave time, and LIM yield showed poor linear effects but significant quadratic effects. The interaction between NaOH solution concentration and duration was obvious.

The quadratic polynomial regression model was analyzed by using Design-Expert 8.0. A factor of the regression equation was fixed to the zero level, and the response surface analysis chart of the other two factors was drawn (Fig. 1). The optimum extraction conditions of LIM were as follows: 1:14.72 (g/mL) solid–liquid ratio, 1.05 mol/L NaOH solution concentration, and 118.70 s microwave time. Under the above conditions, the predicted highest absorbance value of the extracting solution was 0.329. Verification test was conducted under the extraction conditions of 1.00 mol/L NaOH solution concentration, 1:15 (g/mL) solid–liquid ratio and 118 s microwave duration, the obtained solution absorbance value was 0.317, basically fitting the predicted maximum response value of the model, indicating that the model was reasonable and effective. Under these conditions, the LIM yield was 11.08 %, increasing by 40.43 % compared to that of obtained by alkaline extraction and acid precipitation.

Acquisition of the Homogenous Melanin

After elution by Sephadex G-15 column, three eluted components (LIM-a, LIM-b, and LIM-c) of LIM were obtained (Fig. 2). Among them, LIM-a was the main homogeneous component accounting for 75.7 % of the total content of the homogeneous components.

Table 1 Analytical factors and levels for Box–Behnken design

Level	Factor		
	NaOH concentration (mol/L)	Solid–liquid ratio (g/mL)	Microwave time (s)
−1	0.5	1:10	90
0	1.0	1:15	120
1	1.5	1:20	150

Table 2 Box–Behnken design and observed responses

No.	*A* C_{NaOH} (mol/L)	*B* Solid–liquid ratio (g/mL)	*C* Microwave time (s)	*Y* Absorbance
1	0.5	1:10	120	0.207
2	1.5	1:10	120	0.212
3	0.5	1:20	120	0.176
4	1.5	1:20	120	0.189
5	0.5	1:15	90	0.170
6	1.5	1:15	90	0.234
7	0.5	1:15	150	0.191
8	1.5	1:15	150	0.203
9	1.0	1:10	90	0.210
10	1.0	1:20	90	0.194
11	1.0	1:10	150	0.186
12	1.0	1:20	150	0.201
13	1.0	1:15	120	0.322
14	1.0	1:15	120	0.320
15	1.0	1:15	120	0.314
16	1.0	1:15	120	0.316
17	1.0	1:15	120	0.325

Anti-Aging Activity of LIM-a

The weight growth rate of mice in the normal control group was higher than that of the mice in the model control group ($P<0.01$) (Table 4). The weight growth rates of mice in LIM-a groups

Table 3 Estimated regression coefficients for the quadratic polynomial model and the analysis of variance for the experimental results

Source	Sum of squares	*df*	Mean square	*F* value	Pr>*F*	Significance
Model	0.055	9	6.085×10^{-3}	49.70	<0.0001	**
A	1.104×10^{-3}	1	1.104×10^{-3}	9.02	0.0198	*
B	3.781×10^{-4}	1	3.781×10^{-4}	3.09	0.1223	
C	9.112×10^{-5}	1	9.112×10^{-5}	0.74	0.4168	
AB	1.600×10^{-5}	1	1.600×10^{-5}	0.13	0.7284	
AC	6.760×10^{-4}	1	6.760×10^{-4}	5.52	0.0411	*
BC	2.402×10^{-4}	1	2.402×10^{-4}	1.96	0.2040	
A^2	0.016	1	0.016	127.25	<0.0001	**
B^2	0.016	1	0.016	134.67	<0.0001	**
C^2	0.015	1	0.015	120.03	<0.0001	**
Residual	8.570×10^{-4}	7	1.224×10^{-4}			
Lack of Fit	7.778×10^{-4}	3	2.593×10^{-4}	13.09	0.1155	
Pure Error	7.920×10^{-5}	4	1.980×10^{-5}			
Cor Total	0.056	16				
$R^2=0.9846$		$R^2_{adj}=0.9648$			C.V.%=4.47	

*5 % significance level; **1 % significance level

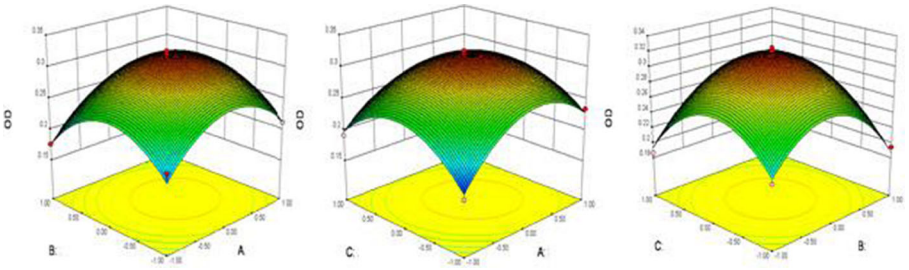

Fig. 1 Response surface curve for sensory score showing the interaction among NaOH concentration (**a**), solid–liquid ratio (**b**), and microwave time (**c**)

and the positive control group (Vc) were all higher than those of the D-gal model control group ($P<0.01$), which indicated that LIM-a could significantly increase the weight growth rate of mice that had been reduced by D-gal.

Thymus and spleen are important immune organs in the body, which can participate in immune responses to regulate the cellular immune function by lymphocytes and take part in the whole aging process of body fundamentally [26]. Degradation of immune function is the main reason resulting in body aging, and the improvement of immune functions can retard the aging progress [27]. Compared with the normal control group, the thymus index and spleen index of mice in the model control group decreased significantly, indicating that the D-gal oxidative damage had resulted in the immune organ atrophy of mice (Table 4). After gavaging with melanin, the thymus indices of mice in the low-, middle-, and high-dose LIM-a groups increased by 17.80, 25.65, and 26.70 %, respectively, being close to the normal level when compared to those of the model control group [28]. The spleen indices of mice of the low-, middle-, and high-dose LIM-a groups increased by 10.23, 27.41, and 35.09 %, respectively, compared to those of the model control group. These results indicated that the low-, middle-, and high-dose melanin could significantly enhance the thymus and spleen indices of mice,

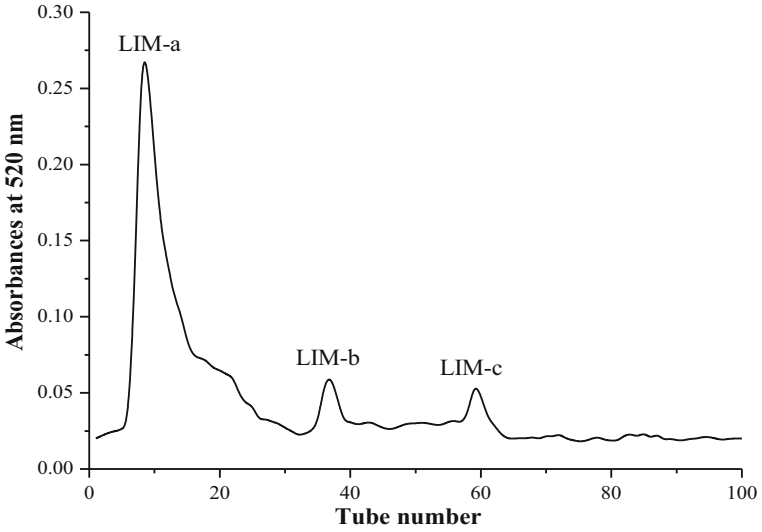

Fig. 2 The elution curve of LIM-a by Sephadex G-15 column

Table 4 Effects on weight growth rate and organ index of aged mice

Groups	Weight growth rate (%)	Thymus index (mg/g)	Spleen index (mg/g)
Normal control group	3.97±0.91	2.48±0.21	6.32±0.25
Model control group	1.45±0.31[*]	1.91±0.22[**]	3.42±0.23[*]
LIM-a (50 mg/kg)	2.50±0.22[*,***]	2.25±0.43[****]	3.77±0.21[*,****]
LIM-a (100 mg/kg)	2.91±0.39[*,***]	2.40±0.37[****]	4.33±0.29[*,***]
LIM-a (200 mg/kg)	3.48±0.42[*,***]	2.42±0.28[****]	4.62±0.31[*,***]
Positive control group (VC)	2.73±0.53[*,***]	2.32±0.16[****]	3.81±0.24[*]

[*] $p<0.01$, compared with normal control group

[**] $p<0.05$, compared with normal control group

[***] $p<0.01$, compared with D-gal model control group

[****] $p<0.05$, compared with D-gal model control group

facilitate the recovery from thymus and spleen damage, and fight against the aging-resulted weakening of immune function.

Free radicals and oxygen-derived free radicals are widespread in organisms; they are necessary for keeping body healthy when at low concentration, regulating signal transmission between cells, and participate in cellular immunity [29]. However, excessive free radicals can seriously

Table 5 Effects on SOD (U/mg protein), GSH-Px (U/mg protein), CAT (U/mg protein), and MDA (nmol/mg protein) of aged mice

Parameters	Normal control group	Model control group	LIM-a (50 mg/kg)	LIM-a (100 mg/kg)	LIM-a (200 mg/kg)	Positive control group
Liver						
SOD	31.92±2.36	27.31±1.27[*]	32.35±2.07[**]	33.25±1.58[**]	41.03±1.55[*,**]	38.01±2.84[*,**]
GSH-Px	104.26±6.32	93.01±3.11[*]	100.21±3.65[***]	102.72±2.49[***]	111.80±2.62[**]	106.87±2.52[**]
CAT	2.77±0.39	2.32±0.14[*]	2.48±0.18	2.76±0.15[**]	3.02±0.19[**,****]	2.71±0.21[**]
MDA	2.31±0.17	3.34±0.26[*]	2.73±0.25[**,****]	2.32±0.11[**]	1.90±0.12[*,**]	2.72±0.14[**]
Brain						
SOD	74.30±1.29	67.43±3.56[*]	70.78±1.91	73.77±1.26[**]	83.46±1.84[*,**]	76.32±0.47[**,****]
GSH-Px	122.86±6.99	104.05±5.19[*]	113.04±6.96[*,***]	122.12±8.42[**]	137.50±8.12[*,**]	126.02±4.15[**]
CAT	1.71±0.16	1.36±0.05[*]	1.53±0.11[**,****]	1.64±0.02[**]	1.88±0.16[*,**]	1.59±0.21[**,****]
MDA	8.70±0.42	10.74±0.96[*]	9.69±0.45[***]	7.11±0.27[*,**]	5.30±0.33[*,**]	6.78±0.39[*,**]
Serum						
SOD	102.25±5.66	85.37±3.18[*]	95.71±2.76[**]	107.94±1.58[**]	124.22±8.76[*,**]	96.91±6.18[**]
GSH-Px	314.78±7.19	280.40±6.51[*]	307.99±8.24[**]	318.03±8.99[**]	339.34±9.11[*,**]	315.24±6.33[**]
CAT	4.24±0.16	3.82±0.13[*]	4.02±0.03[*,**]	4.23±0.13[**]	4.52±0.23[*,**]	4.26±0.52[**]
MDA	15.35±1.23	26.02±3.12[*]	21.72±1.59[*,**]	18.03±1.22[*,**]	12.31±0.56[*,**]	18.05±0.44[*,**]

[*] $p<0.01$, compared with normal control group

[**] $p<0.01$, compared with D-gal model control group

[***] $p<0.05$, compared with D-gal model control group

[****] $p<0.05$, compared with normal control group

injure the body by lipid peroxidation and speed up aging [2]. MDA is the end product of lipid peroxidation, which can cause changes in cell function, genotoxicity, and aging [30]. SOD, GSH-PX, and CAT are important anti-oxidases in the body and they play critical roles to fight against oxidation and free radical injury of the body. They can also reduce the lipid peroxidation in the body and protect the cellular structure and functions from injury by free radicals [31].

Compared with mice in the D-gal model control group, the activities of SOD, GSH-PX, and CAT in the liver homogenate, brain homogenate and serum of mice in the low-, medium-, and high-dose LIM-a and positive control (Vc) groups improved significantly, whereas the MDA content exhibited a significant decline (Table 5). All the LIM-a groups showed significant anti-aging effects with a dose–effect relationship. Compared to the model control group, the activities of SOD in the liver homogenate, brain homogenate, and serum of mice in the high-dose LIM-a group increased by 50.24, 23.77, and 45.51 %, respectively; the activities of GSH-PX in the liver homogenate, brain homogenate, and serum of mice in the high-dose LIM-a group increased by 20.20, 32.15, and 21.02 %, respectively; the activities of CAT in the liver homogenate, brain homogenate, and serum of mice in the high-dose LIM-a group increased by 30.17, 38.23, and 18.32 %, respectively; and MDA contents in the liver homogenate, brain homogenate, and serum of mice in the high-dose LIM-a group decreased by 43.11, 50.65, and 52.70 %, respectively. These results showed that LIM-a could significantly enhance the SOD, GSH-PX, and CAT activities of the D-gal model control mice, and effectively eliminate the lipid peroxidation products.

Conclusions

On the basis of single factor experiment, the optimum microwave-assisted extraction conditions of LIM using Box–Behnken design were as follows: NaOH concentration, 1.05 mol/L; ratio of raw material to liquid, 1:14.72 (g/mL); microwave time, 118.70 s; and microwave power, 320 W. Under these conditions, the extraction yield of LIM was 11.08 %, and 40.43 % higher than that of extraction by alkali extraction and acid precipitation. The results showed that microwave-assisted extraction could improve the yield of LIM. LIM-a was the main homogenous component, accounting for 75.7 % of the total amount of homogenous components. The results of anti-aging experiment indicated that LIM-a could significantly increase the weight growth rate and the antioxidase activities in the body of aged mice, and inhibit the formation of lipid peroxidation products and slow down the aging process, which suggested that LIM-a could be used as a new anti-aging drug or food product.

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