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Sesamin and 4-hydroxy-sesamin from *Cinnamomum camphora*: extraction, purification and anti-inflammatory effects on the BV2 microglia by LPS-induced

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Abstract: This study optimized the extraction of sesamin and 4-hydroxy-sesamin from *Cinnamomum camphora* residue, yielding 0.3804 % under conditions of 86 ℃, 43 min, and a 1:8.39 solid-liquid ratio. The purified compounds showed 67.86 % and 78.87 % recovery rates via recrystallization. In LPS-induced BV-2 microglia, 4-hydroxy-sesamol (10-40 μmol/L) activated autophagy, reduced iNOS, COX-2, IL-6, IL-1β, TNF-α mRNA expression, and demonstrated neuroprotection without cytotoxicity, suggesting its potential in mitigating neuroinflammation.

Keywords: *Cinnamomum camphor*; response surface methodology; silica gel column chromatography; anti-inflammatory activity.

1. Introduction

Cinnamomum camphora belongs to the camphor family and is widely planted in provinces such as Fujian, Jiangxi, Guangdong, and Guangxi in China [1]. Its branches and leaves are rich in essential oils and are important raw materials for medicine, daily chemical industry, and other industries[2]. In the production process, steam distillation is commonly used to obtain the essential oil, with an extraction rate generally not exceeding 5 %, while leaving a large amount of branch and leaf residue.

According to the data, the annual production of *Cinnamomum camphora* essential oil in our country is approximately 16,000 metric tons, with residue from the essential oil extraction reaching up to 810,000 metric tons. However, less than 5 % of this residue is incinerated as fuel, while the remainder is largely left idle. This not only leads to the waste of resources but also occupies a significant amount of storage space, posing potential environmental impacts.

Our research has long focused on the utilization of *Cinnamomum camphora* branches and leaves, uncovering their rich chemical composition and diverse biological activities. Early studies revealed that extracts from *Cinnamomum camphora* contain abundant lignans, particularly sesamin and 4-hydroxy-sesamin, which exhibit significant application potential [3]. Sesamin, widely recognized for its anti-

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inflammatory [4], anti-tumor [5], neuroprotective[6], and anti-aging [10] properties, has been extensively studied globally. In contrast, 4-hydroxy-sesamin, with superior water solubility, has drawn attention for its potential anti-inflammatory activity. To explore this, we optimized extraction techniques, including ultrasound-assisted methods, to enhance the yield of bioactive compounds like sesamin and total flavonoids, which demonstrate strong antioxidant properties [14]. Additionally, novel compounds, such as a unique 1,2-seco-flavan-3-ol, have been isolated from *Cinnamomum camphora* var. *linaloolifera*, further enriching its chemical diversity [16]. Sesamin's neuroprotective and anti-inflammatory effects are mediated through the p38MAPK/NLRP3 signaling pathway[18] [19],highlighting its therapeutic potential.

Building on these findings, we conducted systematic studies on the preparation of high-purity sesamin and 4-hydroxy-sesamin from *Cinnamomum camphora* residues and investigated the anti-inflammatory activity of 4-hydroxy-sesamin. This research aims to provide a foundation for the high-value utilization of *Cinnamomum camphora* residues, addressing both resource efficiency and environmental sustainability.

2. Materials and Methods

2.1. Plant Material

The plant material used in this study was *Cinnamomum camphora* (No．20190715), harvested in March 2022 from the Haishan Fruit Orchard in Xiangyang Township, Nan'an City, Quanzhou, Fujian Province, China.The orchard is situated at an altitude of 700 meters with geographic coordinates of 118°31'10" East longitude and 25°17'38" North latitude. It was identified by Zou Shuangquan, a researcher from the College of Forestry of Fujian Agriculture and Forestry University, as *Lauraceae*, *Cinnamomum* (Sinamom), and *Cinnamomum camphora* (*G. Campora*) Specimens were stored at the Sample Room of the Fujian Province University Engineering Research Center for Conservation and Utilization of Natural Biological Resources at Fujian Agriculture and Forestry University.

2.2. Main Reagents

Analytical grade methanol, ethanol, ethyl acetate (Sinopharm Chemical Reagent Co., Ltd.); chromatography grade methanol, acetonitrile (Merck, Germany); pure water, ultrapure water (prepared in-house); sesamin, 4-hydroxy-sesamin standards (prepared in-house, purity > 98 %); thin-layer chromatography silica gel (GF254), column chromatography silica gel (100-200 mesh and 300-400 mesh) (Qingdao Haiyang Chemical Co., Ltd.); petroleum ether, ethyl acetate (Fuzhou TeWei Chemical Co., Ltd.); lipopolysaccharide (LPS) (Product No. L2630); MTT (Product No. M2128); DMEM medium (Product No. C11995500BT); fetal bovine serum (Product No. 26010-074); SDS-PAGE Gel Preparation Kit (Product No. P0012AC); ChamTM SYBR® qPCR Master Mix (Product No. Q311-02), Reverse Transcription Kit (Product No. K1622); antibodies for AMPK (Product No. 5831), p-AMPK (Product No. 50081), mTOR (Product No. 2983), p-mTOR (Product No. 5536), ULK1 (Product No. 8054), p-ULK1 (Product No. 14202), Beclin1 (Product No. 3738), p62 (Product No. 5114), LC3A/B (Product No. 12741), β-Actin (Product No. HC201).

2.3. Main Instruments

TQ-400Y high-speed multifunction grinder (Yongkang Tianqi Shengshi Industry and Trade Co., Ltd.); PR224ZH/E electronic analytical balance (OHAUS, USA); RV3V rotary evaporator (IKA, Germany); HH-W600 thermostatic water bath (Jinan Oulai Bo Biological Co., Ltd.); KQ500DE type ultrasonic cleaner (Kunshan Ultrasonic Instruments Co., Ltd.); RC 2 Control cooling circulator (IKA, Germany); Waters W2695-QDA high-performance liquid chromatograph (Waters, USA), ZF-5 portable ultraviolet analyzer (Shanghai Jia Peng Technology Co., Ltd.); cell culture incubator (Thermo Fisher, USA); optical microscope (Thermo Fisher, USA); low-temperature centrifuge (Eppendorf, Germany); microplate reader (TECAN, Switzerland); BIO-RAD electrophoresis system (BIO-RAD, USA);

chemiluminescence imaging system (BIO-RAD, USA); 7900H Real-Time PCR system (Applied Biosystems, USA).

2.4. Determination of Sesamin and 4-hydroxy-sesamin Content and Their Extraction and Purification

2.4.1. HPLC Analysis Conditions

Using the HPLC detection method defined by Guo[19], the chromatographic conditions were established, and a standard curve was generated. A precise amount of 10.8 mg of sesamin and 12.3 mg of 4-hydroxy-sesamin reference materials were dissolved in ethyl acetate and diluted to a volume of 5 mL to prepare a stock solution of the reference substances. A volume of 1.0 mL of this stock solution was mixed to yield a mixed reference solution with a sesamin concentration of 1.08 mg/mL and a 4-hydroxy-sesamin concentration of 1.23 mg/mL, labeled as solution 1.An accurate volume of 0.4 mL of the reference stock solution was mixed and diluted to 10 mL to obtain a mixed reference solution with a sesamin concentration of 0.08 mg/mL and a 4-hydroxy-sesamin concentration of 0.08 mg/mL, designated as solution 2.

2.4.2. HPLC Standard Curve Generation

The prepared mixed reference solutions were analyzed using a Diamonsil C18 reversed-phase column (250 mm × 4.6 mm, 5 μm)with acetonitrile (A) and water (B) as the mobile phase. A gradient elution program was applied as follows: 0–30 min, 30%–42% A; 30–45 min, 42%–65% A. The flow rate was maintained at 1.0 mL/min, and detection was performed at 235 nm. The column temperature was set at 30 °C, and the injection volume was 10 μL.

For solution 1, injections of 1, 6, 10, and 20 μL were analyzed, while for solution 2, injections of 2, 4, 6, 8, and 10 μL were analyzed. The peak areas were recorded, and standard curves were constructed by plotting peak area (y-axis) against the concentration of the reference substances (x-axis). The linear range and correlation coefficient (R^2) were determined to validate the method.

2.4.3. Optimization of Extraction Using Single Factor Analysis

The collected *Cinnamomum camphora* leaves underwent air-drying, followed by pulverization with a high-speed multifunctional pulverizer. The powder was subjected to extraction with organic solvents. An analytical balance was used to measure 5.000 g of *Cinnamomum camphora* leaf powder, which was then placed into a 500 ml flat-bottomed flask. The study investigated the impact of extraction solvents (methanol, anhydrous ethanol, ethyl acetate), solid-liquid ratio (1:4, 1:6, 1:8, 1:10, 1:12), extraction temperatures (80, 85, 90, 95, and 100 °C), and extraction times (20, 30, 40, 50, and 60 min) on the extraction rate of sesamin and 4-hydroxy-sesamin from *Cinnamomum camphora* leaves [21]. The extraction rate was calculated as the ratio of the content of sesamin or 4-hydroxy-sesamin measured by HPLC to the amount of *Cinnamomum camphora* leaves used for extraction. Based on single-factor experimental results, factor levels were determined, and a Plackett-Burman (PB) design (n = 12) was implemented, with the total extraction rate as the response value (**Table 1**).

Table 1.PB experimental factor levels.

Factorse	**Level**	
	Low	**High**
A-Extraction temperature (℃)	80	90
B-Extraction time (min)	30	50
C-solid-liquid ratio (g/mL)	1:6	1:10

2.4.4. Optimiz ation of Extraction Through Response Surface Methodology

A Box-Behnken Design within the response surface methodology framework was employed to evaluate the influence of multiple factors and their interactions on the extraction rate of sesamin and 4-hydroxy-sesamin. The three factors selected were extraction temperature (A), solid-liquid ratio(B) , and extraction time (C). To display the Box-Behnken Design (BBD) matrix, the relevant data were organized and presented. The BBD matrix is shown (Table **2**).

Table 2. Factor level of response surface.

Factorse		**Level**		
		-1	**0**	**1**
Extraction temperature（℃）	A	80	85	90
solid-liquid ratio（g/mL）	B	1:6	1:8	1:10
Extraction time（min）	C	30	40	50

2.5. Sesamin and 4-hydroxy-sesamin Content and Their Extraction and Purification

2.5.1. Separation of sesamin and 4-hydroxy-sesamin by Silica-gel Thin-layer Chromatography (TLC)

A small quantity of crude extract was applied to the silica gel plate using a capillary tube. The plate was then placed in a chromatography chamber with varing volume ratios of petroleum ether-ethyl acetate as the mobile phase. The spots were visualized using iodine vapor. The R_f values were calculated by measuring the distance from the center of each spot to the origin and from the solvent front to the origin. The optimal elution solvent ratio was determined based on the R_f values using the following formula:

$$R_f = \frac{Distance\ from\ the\ spot\ center\ to\ the\ origin}{Distance\ from\ the\ solvent\ front\ to\ the\ origin}$$

2.5.2. Purification Process of Sesamin and 4-hydroxy-sesamin by Silica-gel Column Chromatography

Twenty-five grams of silica gel (300-400 mesh, 0.37-0.48 mm) was activated in an oven at 120 °C for 2 hours and subsequently cooled to room temperature in a desiccator. The activated silica gel was then packed into a chromatography column (Φ15mm×300mm, column volume 60 mL) connected to an automatic fraction collector. The sample, applied in a dry state, was eluted with the selected solvent at a controlled flow rate of 3 mL/min using a peristaltic pump. The eluent was collected as 15 mL fractions. The collected eluate was subjected to HPLC content determination and recrystallization, and the recovery rate was calculated. The recovery rate represents the ratio between the content of the sample obtained by recrystallization and the content of the sample determined by HPLC.

2.5.3. Optimization Using Orthogonal Experimental Design

Based on single-factor experiments, an $L_9(3^3)$ orthogonal design was employed to optimize the purification process of sesamin and 4-hydroxy-sesamin from *Cinnamomum camphora*. The factors investigated included mixing ratio, sample loading mass, and elution flow rate, with their levels (**Table 3**).

Table 3. Orthogonal experimental design factor level table.

Level	**Mixing ratio/ g:g**	**Loading amount/ g**	**Elution flow rate/ (mL/min)**
1	1:2	1	2
2	1:2.5	1.5	3
3	1:3	2	4

2.5.4. Method Validation

Validation experiments were conducted under the optimal silica gel column chromatography conditions determined by orthogonal design. The process was repeated three times, and the effective eluates were collected, analyzed, and used to calculate the purification rates of sesamin and 4-hydroxy-sesamin.

2.6. Anti-inflammatory Activity study of 4-hydroxy-sesamin

2.6.1. Cell Culture

BV2 microglial cells (from the China Center for Type Culture Collection) were revived and cultured in DMEM complete medium containing 10% FBS and 1% penicillin-streptomycin at 37 ℃ with 5 % CO_2 and saturated humidity. Media was changed daily and cells were passaged every other day for subsequent experiments.

2.6.2. Experimental Grouping and Treatment

BV2 microglial cells in the logarithmic growth phase and in good condition were seeded onto plates. The groups were divided into the cell control group, LPS 100 ng/mL (model group), and LPS + 4-hydroxy-sesamin at 10, 20, and 40 μmol/L. When cells reached 80% confluency, LPS and 4-hydroxy-sesamin were added according to the groupings and co-incubated for 12 hours.

2.6.3. MTT Assay for BV2 Microglial Cell Viability

BV2 cells were seeded onto 96-well plates at a density of 5×10^4 cells/mL, 100 μL per well, with 6 replicate wells. Cells were cultured at 37 ℃ with 5% CO_2 and saturated humidity. Following the grouping and treatment as described in step 2.6.2 , the medium was replaced after 12 hours of incubation, MTT (5 mg/mL) was added, and incubation continued for another 4 hours before measuring the absorbance at 490 nm (OD value).

$$Cell\ viability(\%)=\frac{OD\ value\ of\ the\ experimental\ group}{OD\ value\ of\ the\ normal\ control\ group}\times 100\%$$

2.6.4. RT-qPCR Detection of IL-6, IL-1β, TNF-α, COX-2, and iNOS mRNA levels in BV2 Microglial cells

BV2 cells were seeded onto 6-well plates at a density of 1.2×10^5 cells/mL, with 2 mL per well and three replicate wells. After 12 hours of incubation, the medium was replaced and cells were treated with LPS and 4-hydroxy-sesamin as described in step 2.6.2 for an additional 12 hours. The supernatant was then discarded, and cells were washed three times with pre-cooled PBS. Total RNA was extracted with TRIzol, followed by cDNA synthesis using a reverse transcription kit. Subsequently, amplification was performed using the cDNA as a template with an amplification kit. Primers for IL-6, IL-1β, TNF-α, COX-2, iNOS, and the internal control GAPDH were designed using Primer 5 software. PCR amplification conditions were as follows: initial denaturation at 95 ℃ for 30 seconds, followed by 40 cycles of denaturation at 95 ℃ for 10 seconds and annealing at 60 ℃ for 30 seconds. The results were analyzed relative to the internal reference gene using the $2^{-\Delta\Delta CT}$ method to quantify the relative mRNA expression levels. To provide the primer sequences relevant to the study, the corresponding information was collated. The primer sequences are presented (Table 4).

Table 4. Primer sequences of IL-1β, IL-6, TNF-α, COX-2, iNOS and GAPDH genes.

Gene	Upstream Primer（5'-3'）	Downstream Primer（5'-3'）	Length（bp）
IL-6	CTGCAAGAGACTTCCATC CAG	AGTGGTATAGACAGGTCTGTTGG	131
IL-1β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG	116
TNF-α	GCCGATGGGTTGTACCTTGT	TCTTGACGGCAGAGAGGAGG	139
COX-2	CAGGAGATGGTCCGCAAGAG	GCAAATGTAGAGGTGGCCCT	130
iNOS	AGTCTTTGGTCTGGTGCCTG	TGGTAACCGCTCAGGTGTTG	198
GAPDH	GAGAAACCTGCCAAGTATGATGAC	AGAGTGGGAGTTGCTGTTGAAG	129

2.6.5. Detection of AMPK/mTOR/ULK1 Signaling Pathway and Autophagy-Related Protein Expression Levels by Western Blot

BV2 microglial cells were seeded onto 6-well plates at a concentration of 1.2×10^5 cells/mL, with 2 mL per well and three replicates. Subsequently, cells were exposed to experimental grouping and reagent interventions following the procedures described in step 2.6.2 . After a 12-hour incubation in a CO_2 incubator the supernatant was dis-carded, and the cells were washed three times with pre-cooled PBS. Total protein was then extracted by lysing the cells, and the protein concentration was determined using the BCA assay. The proteins were denatured by adding 5× loading buffer and heating at 100 ℃ for 15 minutes. Each sample, containing 30 μg of protein, was separated using SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with skim milk powder (or BSA) for 1.5 hours. Primary antibodies against AMPK, p-AMPK, mTOR, p-mTOR, ULK1, p-ULK1, Beclin1, p62, LC3, along with their respective secondary antibodies were applied. The membranes were then developed in a gel imaging system, and image analysis was performed using Image Lab software. The relative expression levels of target proteins were represented by the ratio of the integrated optical density (IOD) of the target protein to that of the internal reference protein.

3. Results and Discussion

3.1. HPLC Detection Results and Standard Curves

To establish the HPLC chromatographic conditions for sesamin and 4-hydroxy-sesamin, relevant experiments were carried out. The results indicated that within the concentration range of 0.08 mg/mL to 1.23 mg/mL for both sesamin and 4-hydroxy-sesamin, effective separation was achieved at the detection wavelength of 235 nm, with a separation degree greater than 1.5 (Figure 1).Their retention times were 30.769 min and 43.740 min, respectively. Through HPLC detection method, the linear regression equation for the standard curve of sesamin is expressed as $y = 2\times10^9x+8316.5$, $R^2=0.9997$; For the standard curve of 4-hydroxy-sesamin, the linear regression equation is $y=2\times10^9x+144585.0$, $R^2=0.9996$.

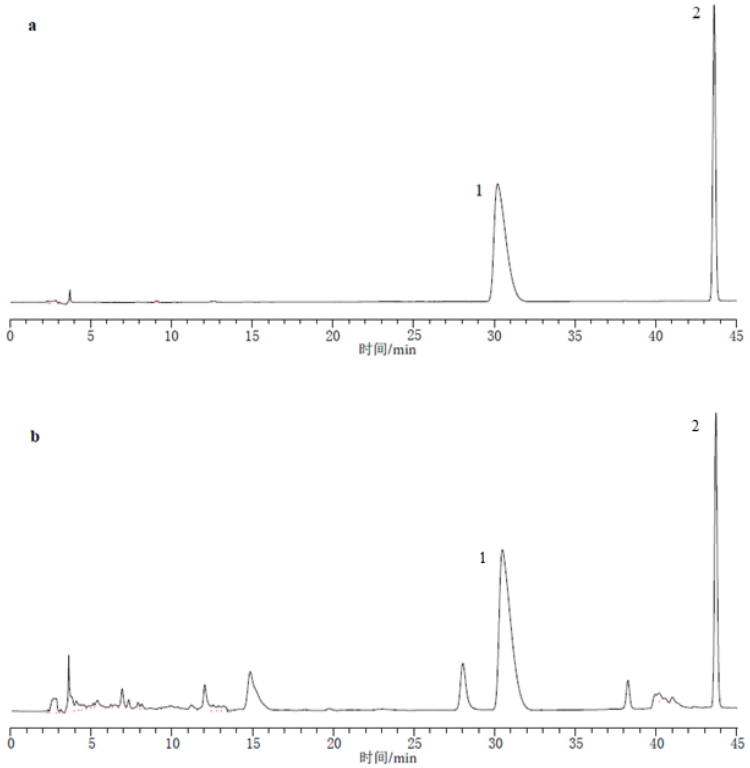

Figure 1. HPLC chromatograms of reference substance (**a**) and sample (**b**).Note: **1**.4-hydroxy-sesamin; **2**. Sesamin.

3.2. Analysis of Single-factor Experiments

In order to determine the impact of different variables on the overall extraction rate of sesamin and 4-hydroxy-sesamin, we designed a four-factor, five-level one-way experiment. The optimal conditions were determined based on the one-way experiment, specifying anhydrous ethanol as the extraction solvent, an extraction temperature of 85 ℃, a solid-liquid ratio of 1:8 g/mL, and an extraction time of 40 min.

To comprehensively analyze the factors influencing the total extraction rate and the extraction of sesamin and 4 - hydroxy - sesamin, a series of experiments were conducted. The results show that when the extraction solvent is anhydrous ethanol, the total extraction rate can reach a maximum of 0.36 %, which is significantly higher than that of ethyl acetate ($P < 0.01$) and methanol ($P < 0.05$) (Figure 2A). Under consistent conditions, the highest total extraction rate is achieved at an extraction temperature of 85 °C. The extraction rate at 85 °C is significantly higher than those at 90 °C, 95 °C, and 100 °C ($P < 0.01$), and notably higher than that at 80 °C ($P < 0.05$) (Figure 2B). The total extraction rate shows an increasing trend with the increase of the solid - liquid ratio, reaching its maximum at a ratio of 1:8 and then decreasing. At a ratio of 1:8, the total extraction rate is significantly higher than that at a ratio of 1:10 ($P < 0.01$) and significantly higher than that at a ratio of 1:4 ($P < 0.05$), while there is no significant difference in the extraction of sesamin and 4 - hydroxy - sesamin between a ratio of 1:8 and 1:6 ($P > 0.05$) (Figure 2C). The highest total extraction rate is obtained with a reflux extraction time of 40 min (Figure 2D).With an increase in reflux extraction time, the total extraction rates of sesamin and 4-hydroxy-sesamin also increased. However, when the time exceeds 40 min, structural changes induced by temperature may lead to a decrease in the total extraction rate of sesamin and 4-hydroxy-sesamin.

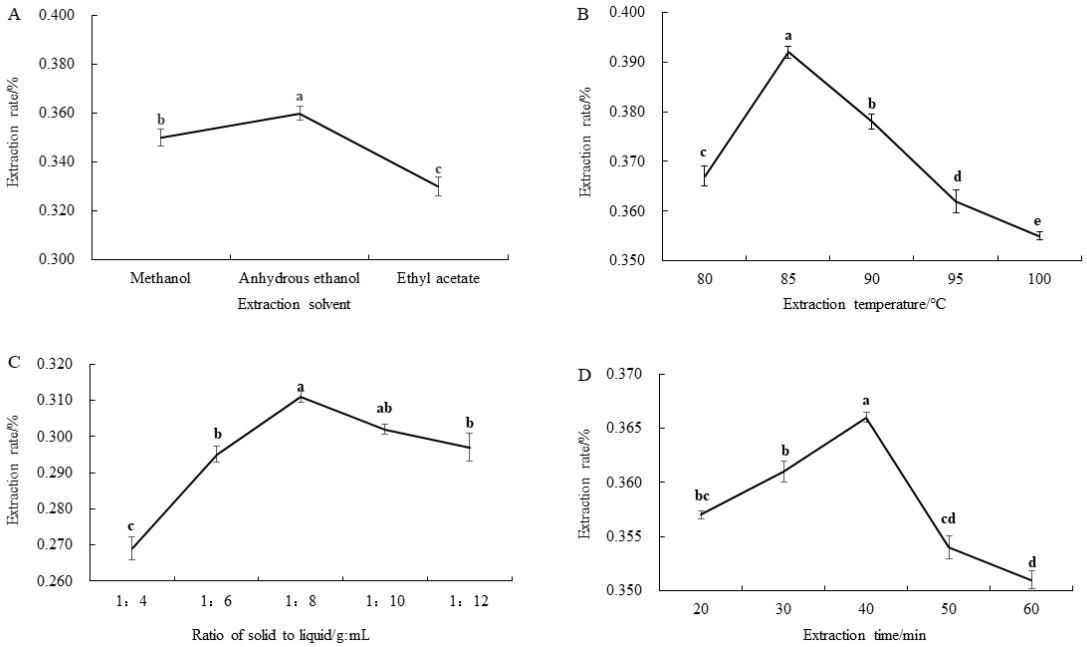

Figure 2. The effect of extraction solvent (A), extraction temperature (B), solid-liquid ratio (C), extraction time (D) on the total extraction.

Note: Multiple comparisons between date using Waller-Duncan methodology. Different letters indicated a significant difference ($P < 0.05$) based on one-way ANOVA followed by Tukey's test.

The Plackett-Burman experimental results (Table 5) revealed an F-value of 66.30 through regression and significance analyses, indicating model significance. Analysis of F-values and P-values for extraction time, temperature, and solid-liquid ratio demonstrated their significance order: extraction time > extraction temperature > solid-liquid ratio. Extraction time and temperature exhibited an extremely significant impact ($P < 0.01$), while the solid-liquid ratio showed significance ($P < 0.05$).

Table 5. Plackett-Burman experimental results.

No.	Extraction temperature (℃)	Extraction time (min)	Solid-liquid ratio (g/mL)	Total extraction rate (%)
1	90	50	1:6	0.362 1
2	80	50	1:10	0.362 7
3	90	30	1:10	0.359 5
4	80	50	1:6	0.359 6
5	80	30	1:10	0.348 5
6	80	30	1:6	0.352 9
7	90	30	1:6	0.355 6
8	90	50	1:6	0.360 9
9	90	50	1:10	0.365 4
10	80	50	1:10	0.361 6
11	90	30	1:10	0.360 5
12	80	30	1:6	0.351 8

3.3. Camphor Using Response Surface Methodology

Based on the results of the single-factor experiments, three factors were selected as independent variables. The extraction process for sesamin and 4-hydroxy-sesamin underwent optimization using the Design-Expert 8.0.6.1 software and the Box-Behnken design method. This approach also included an assessment of the interaction effects among the three key factors (extraction time, solid-liquid ratio, and extraction temperature). To present the outcomes of this optimization process, relevant data were collated and analyzed. The results and analysis of this optimization process are presented (Table 6). A detailed discussion highlights the impact of each factor and their interactions on the total extraction rate, ultimately identifying the optimal extraction conditions.

Table 6. Experimental results of response surface test.

No.	Extraction temperature （℃）	solid-liquid ratio （g/mL）	Extraction time （min）	Total extraction rate (%)
1	80	1:6	40	0.3585%
2	90	1:6	40	0.3620%
3	80	1:10	40	0.3570%
4	90	1:10	40	0.3709%
5	80	1:8	30	0.3566%
6	90	1:8	30	0.3658%
7	80	1:8	50	0.3647%
8	90	1:8	50	0.3699%
9	85	1:6	30	0.3579%
10	85	1:10	30	0.3670%
11	85	1:6	50	0.3688%
12	85	1:10	50	0.3734%
13	85	1:8	40	0.3811%
14	85	1:8	40	0.3796%
15	85	1:8	40	0.3775%
16	85	1:8	40	0.3783%
17	85	1:8	40	0.3789%

Based on the outcomes of the response surface methodology experiments, a three-factor quadratic polynomial regression equation was derived to analyze the total extraction yield (Y) of sesamin and 4-hydroxy-sesamin in relation to three independent variables: extraction temperature (A), solid-liquid ratio (B), and extraction time (C): $Y=3.791\times10^{-3}+3.985\times10^{-5}A+2.633\times10^{-5}B+3.692\times10^{-5}C+2.608\times10^{-5}AB-9.972\times10^{-6}AC-1.126\times10^{-5}BC-9.758\times10^{-5}A^2-7.222\times10^{-5}B^2-5.103\times10^{-5}C^2$.The regression coefficients of the first-order terms are arranged in the order A > C > B, indicating that the factor with the most significant impact on the total extraction efficiency is the extraction temperature, followed by the extraction time and the solid-liquid ratio. Variance analysis of this regression model confirmed that it achieves an extremely significant level ($P < 0.0001$), with a coefficient of determination (R^2) of 0.9855. The F values for extraction temperature (A), solid-liquid ratio (B), and extraction time (C) are 53.06, 23.15, and 45.53 respectively. The F values for AB, AC, and BC are 11.36, 1.66, and 2.12 respectively. The F values for A^2, B^2, and C^2 are 167.42, 91.72, and 45.79 respectively. The non-significant lack of fit ($P = 0.3092 > 0.05$) validates the model's high correlation with the empirical data (Table 7). Consequently, this model stands as an effective tool for predicting the total extraction yield of sesamin and 4-hydroxy-sesamin under different extraction conditions within the experimental domain.

Table 7. ANOVA for egression model with lignanoids contents as response value.

Source of variance	Sum of squares	df	Mean square	*F* value	*P* value	Saliency
Model	1.136×10^{-3}	9	1.262×10^{-4}	52.72	<0.0001	**
Extraction temperature（A）	1.271×10^{-4}	1	1.271×10^{-4}	53.06	0.0002	**
Ratio of solid to liquid（B）	5.544×10^{-5}	1	5.544×10^{-5}	23.15	0.0019	*
Extraction time（C）	1.09×10^{-4}	1	1.09×10^{-4}	45.53	0.0003	**
AB	2.721×10^{-5}	1	2.721×10^{-5}	11.36	0.0119	*
AC	3.978×10^{-6}	1	$3.978\times10^{-}$	1.66	0.2384	
BC	5.067×10^{-6}	1	5.067×10^{-6}	2.12	0.1891	
A^2	4.009×10^{-4}	1	4.009×10^{-4}	167.42	<0.0001	**
B^2	2.196×10^{-4}	1	2.196×10^{-4}	91.72	<0.0001	**
C^2	1.096×10^{-4}	1	1.096×10^{-4}	45.79	0.0003	**
Residual error	1.676×10^{-5}	7	2.395×10^{-6}			
Lack of fit	9.321×10^{-6}	3	3.107×10^{-6}	1.67	0.3092	
Pure error	7.44×10^{-6}	4	1.860×10^{-6}			
Summation	1.153×10^{-3}	16				

Utilizing the Design-Expert 8.0.6.1 software, three-dimensional response surface plots were constructed to illustrate the interactions of the three experimental parameters-extraction temperature, solid-liquid ratio, and extraction time-and their combined impact on the total extraction yield. The inclination of the response surface and the dispersion of the contour plots serve as indicators of the degree of interaction among the variables under consideration. To explore the interaction between extraction temperature and solid - liquid ratio on the extraction yield, relevant data were analyzed. The analysis results showed a prominently steep response surface corresponding to the extraction temperature and the solid - liquid ratio, indicating a significant interaction ($P < 0.05$) on the extraction yield (Figure 3). This steepness underscores the critical in-fluence of both extraction temperature and solid-liquid ratio on the yield. Additionally, the contour plots manifested elliptical shapes, suggesting that the relationship among extraction temperature, extraction time, and solid-liquid ratio concerning the yield of sesamin and 4-hydroxy-sesamin is intricate and inherently nonlinear within the context of the thermal reflux extraction process. These elliptical contours signify a substantial interaction between the parameters, indicating a complex interplay that is pivotal for optimizing the extraction process. Therefore, the response surface methodology plays a crucial role in elucidating these intricate relationships and guiding the optimization of extraction processes to enhance yield efficiencies.

The response surface analysis identified the optimal conditions for the reflux ex-traction of sesamin and 4-hydroxy-sesamin from *Cinnamomum camphora*: an extraction temperature of 86 ℃, a solid-liquid ratio of 1:8.39 g/mL, and an extraction duration of 43 min. Under these specified parameters, the model predicts a total extraction yield of 0.3804 %. In order to validate the reliability of the response surface methodology results, three experiments were carried out under the specified conditions. The experimental results are presented (Table 8.Verification experiment. Table 8). The experimentally obtained total extraction rate of 0.3805 % is in remarkable agreement with the predicted value of 0.3804 %, showcasing the precision of the optimization process, with an acceptably low relative error of 0.0156 %.The absolute difference between the experimental and predicted extraction rates is a negligible 0.0001 %, highlighting the minute discrepancy, indicating that the regression model established through the response surface methodology possesses a high degree of congruence with the actual experimental outcomes and can effectively predict the extraction efficiency of sesamin and 4-hydroxy-sesamin.

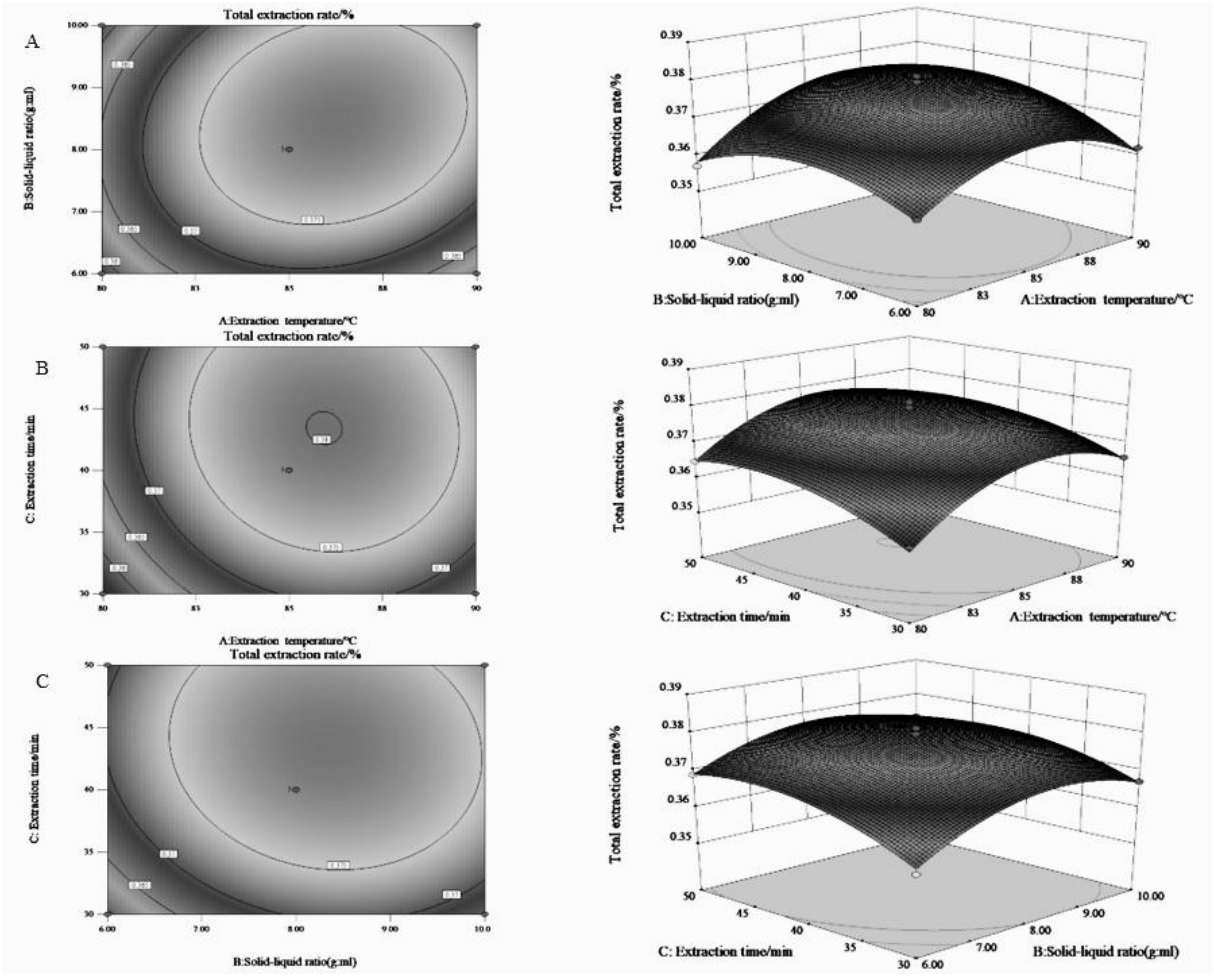

Figure 3. Contour diagram (left) and response surface diagram (right) of the influence of different factors on the total extraction rate. (A) AB; (B) AC; (C) BC.

Table 8. Verification experiment.

No.	Extraction rate/%	Average extraction rate/%	Relative error/%
1	0.3817%		
2	0.3802%	0.3805%	0.0156%
3	0.3795%		

3.4. Optimization and Validation of Silica Gel Column Chromatographic Purification for Sesamin and 4-Hydroxy-Sesamin from Camphor Using Orthogonal Experimental Design

3.4.1. Silica-gel Column Chromatographic Purification of Sesamin and 4-hydroxy-sesamin from Camphor

The R_f values of sesamin and 4-hydroxy-sesamin were determined using thin-layer chromatography (TLC) at various ratios of petroleum ether to ethyl acetate to identify the optimal elution system for silica gel column chromatography. To analyze the relationship between the ratio of petroleum ether to ethyl acetate and the Rf values of compounds, data were collected and tabulated. The results show that when the ratio of petroleum ether to ethyl acetate was 15:1, the Rf values for both compounds were below 0.2 (Table 9).Consequently, the selection of a 15:1 ratio for petroleum ether to ethyl acetate is deemed suitable as an eluent for the initial purification step to remove impurities.

When the ratio of petroleum ether to ethyl acetate is 9:1, the R_f value of sesamin was observed to be between 0.2 and 0.3, while that of 4-hydroxy-sesamin was significantly is 0.041. This allows for the separation of sesamin and the retention of 4-hydroxy-sesamin within the silica gel, thereby achieving purification of sesamin. Consequently, the choice of a 9:1 ratio of petroleum ether to ethyl acetate is suitable for the elution and further purification of sesamin. When the ratio is 3:1, the R_f value for 4-hydroxy-sesamin is elevated to 0.238, making this ratio optimal for the selective separation and subsequent purification of 4-hydroxy-sesamin.

The elution curve visually represent the elution efficiency of the eluent and determine the sample collection volume. Sesamin and 4-hydroxy-sesamin were separated and purified using 5BV petroleum ether: ethyl acetate 15:1, 5BV petroleum ether: ethyl acetate 9:1 and 5BV petroleum ether: ethyl acetate 3:1. The ethanol crude extract was subjected to silica gel column chromatography under the conditions of room temperature with 25 g of silica gel mass, an elution flow rate of 3 mL/min, a sample volume of 1.5 g, a mixing ratio of 1:1.5 and an iso-gradient elution.

Table 9. R_f values of sesamin and 4-hydroxysamsamin in different proportions.

Elution solvent	Elution solvent ratio(v/v)	R_f value of sesamin	R_f value of 4-hydroxy-sesamin
Petroleum ether：Ethyl acetate	15:1	0.111	0.008
Petroleum ether：Ethyl acetate	12:1	0.150	0.019
Petroleum ether：Ethyl acetate	9:1	0.217	0.041
Petroleum ether：Ethyl acetate	6:1	0.379	0.079
Petroleum ether：Ethyl acetate	3:1	0.555	0.238
Petroleum ether：Ethyl acetate	1:1	0.872	0.712

3.4.2. Orthogonal Experimental Analysis

The purification process was optimized using orthogonal experiments, with the total purification rate of sesamin and 4-hydroxy-sesamin as the evaluation metric (**Table** 10).

Table 10. Results of orthogonal experiment.

No.	Mixing ratio/ g:g	Loading amount/ g	Elution flow rate/ (mL/min)	Total purification rate%
1	1	1	1	21.06
2	1	2	3	20.95
3	1	3	2	23.1
4	2	1	3	25.41
5	2	2	2	23.22
6	2	3	1	22.97
7	3	1	2	22.83
8	3	2	1	20.53
9	3	3	3	20.32

Based on range (Table 11)and variance analyses (Table 12), the factors influencing the total purification rate of sesamin and 4-hydroxy-sesamin were ranked as follows: mixing ratio > sample loading mass > elution flow rate. The optimal purification conditions were determined as $A_2B_1C_2$, corresponding to a mixing ratio of 1:2.5 (g:g), a sample loading mass of 1.0 g, and an elution flow rate of 3 mL/min.

Table 11. Range analysis of orthogonal experiment.

Item	Level	Mixing ratio/ g:g	Loading amount/ g	Elution flow rate/ (mL/min)
Sum of levels	1	65.11	69.3	64.56
	2	71.6	64.7	69.15
	3	63.68	66.39	66.68
Average of levels	1	21.7	23.1	21.52
	2	23.87	21.57	23.05
	3	21.23	22.13	22.23
Optimal level		2	1	2
Range		2.64	1.53	1.53
Number of levels		3	3	3
Replicates per level		3	3	3

Table 12. Orthogonal experimental analysis of variance.

Source	squared deviations	df	Mean square	F Value	*P* Value
Mixing ratio/ g:g	11.877	2	5.938	3.421	0.102
Loading amount/ g	3.609	2	1.805	0.58	0.589
Elution flow rate/ (mL/min)	3.518	2	1.759	0.562	0.597

3.4.3. Validation Experiments

Purification was conducted under the optimal conditions determined by orthogonal experiments: a mixing ratio of 1:2.5, a sample loading mass of 1.0 g, and an elution flow rate of 3 mL/min. Five parallel tests were performed, revealing an average total purification rate of sesamin and 4-hydroxy-sesamin of 23.58%, with an RSD of 0.97% (

Table 13). These results demonstrate the rationality and feasibility of the process

No.	Total purification rate/%	Average total purification rate/%	RSD /%
1	23.52		
2	23.85		
3	23.32	23.58	0.97
4	23.42		
5	23.78		

Table 13. Verification experiments

No.	Total purification rate/%	Average total purification rate/%	RSD /%
1	23.52		
2	23.85		
3	23.32	23.58	0.97
4	23.42		
5	23.78		

The contents of sesamin and 4-hydroxy-sesamin were determined, and elution curves were generated, depicting the volume of eluent on the x-axis and the contents of sesamin-like components on the y-axis. To determine the optimal elution conditions for impurities, experiments were conducted. The results indicated that the impurities could be eluted when using a petroleum ether - ethyl acetate ratio of

15:1 .A ratio of 9:1 for petroleum ether-ethyl acetate can elute a large amount of sesamin and achieve good separation between sesamin and 4-hydroxy-sesamin, showing good resolution without significant overlap between the two compounds. A ratio of 3:1 for petroleum ether-ethyl acetate could elute a large amount of 4-hydroxy-sesamin. Through HPLC, sesamin was measured as 112.78 mg and 4-hydroxy-sesamin as 193.15 mg. After one recrystallization, sesamin was obtained at 76.53 mg, and 4-hydroxy-sesamin at 152.33 mg, with single recrystallization recovery rates of 67.86 % and 78.87 %, respectively (Figure 4). Therefore, under a flow rate of 3 mL/min, a large amount of sesamin and 4-hydroxy-sesamin could be isolated and purified. This is achieved by first eluting the impurities with 3BV volume ratio of 15:1 petroleum ether-ethyl acetate, followed by elution of sesamin with 5BV volume ratio of 9:1 petroleum ether-ethyl acetate, and ultimately elution of 4-hydroxy-sesamin with 5BV volume ratio of 3:1 petroleum ether-ethyl acetate.

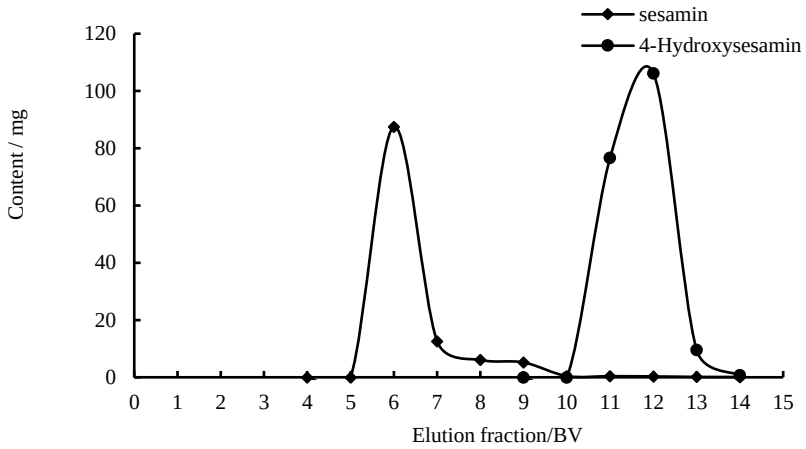

Figure 4. Elution curves of sesamin and 4-hydroxy-sesamin.

3.5. Influence of 4-hydroxy-sesamin on the Survival Rate of BV2 Microglial Cells Induced by LPS

The cytotoxic potential of 4-hydroxy-sesamin within BV2 microglial cells was meticulously evaluated using an in vitro MTT assay. To directly compare the impact of 100 ng/mL LPS exposure on BV2 micro - glial cell survival, data were collected and analyzed. The results showed that exposure to 100 ng/mL of LPS did not significantly alter the survival rate of BV2 micro - glial cells compared to the untreated control group ($P > 0.05$) (Figure 5). Additionally, treatment with 4-hydroxy-sesamin at concentrations of 10, 20, and 40 μmol/L did not demonstrate a notable effect on the vitality of LPS-stimulated BV2 microglial cells relative to the LPS-only model group ($P > 0.05$). These findings suggest that, within the tested con-centration range, 4-hydroxy-sesamin does not induce cytotoxic effects on BV2 microglial cells, thereby providing a solid basis for subsequent examination of its anti-inflammatory properties.

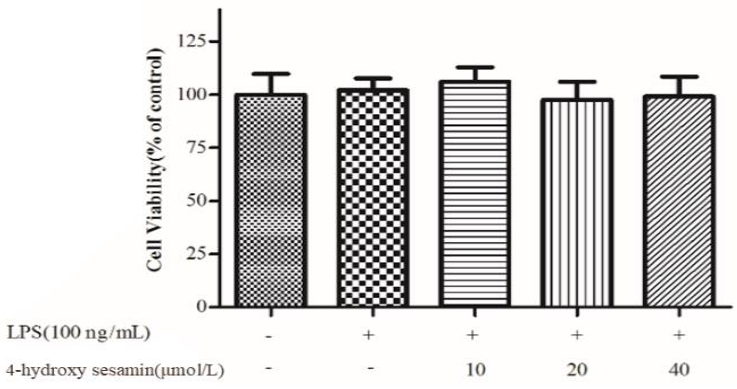

Figure 5. Effect of 4-hydroxy-sesamin on the viability of LPS-induced BV2 microglial cells($\bar{x} \pm s$,n=6).

3.6. Modulation of Inflammatory Transcript Levels by 4-hydroxy-sesamin in LPS-challenged BV2 Microglial Cells

RT-qPCR was performed to determine the effects of 4-hydroxy-sesamin on transcrip-tional levels of inflammatory markers in BV2 microglial cells. To assess the inflammatory response of BV2 microglial cells upon LPS exposure, we measured the mRNA levels of IL-6, IL-1β, TNF-α, COX-2, and iNOS. The results demonstrated that BV2 microglial cells exposed to LPS had a significant upregulation in the mRNA levels of these inflammatory factors compared to the untreated control group ($P < 0.01$) (Figure 6). In contrast, treatment with varying concentrations of 4-hydroxy-sesamin markedly reduced the mRNA expression of these pro-inflammatory mediators ($P < 0.05$, $P < 0.01$) when compared to the LPS-stimulated group. These findings underscore the potentiall of 4-hydroxy-sesamin to attenuate the LPS-induced neuroinflammatory responses in BV2 microglial cells.

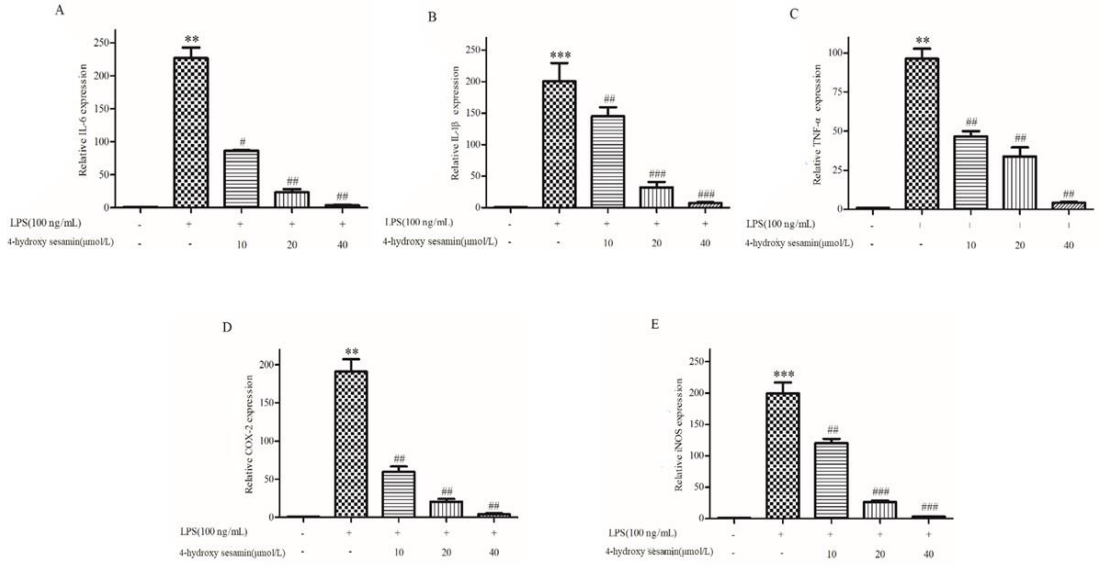

Figure 6. Effect of 4-hydroxy-sesamin on the mRNA levels of IL-6 (A), IL-1β (B), TNF-α (C), COX-2 (D), iNOS (E) in LPS-induced BV2 microglial cells ($\bar{x} \pm s$, n=3).

Different Asterisk indicated a significant difference ($P < 0.05$) based on one-way ANOVA followed by Tukey's test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared with Normal control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, compared with LPS group, the same below.

3.7. Influence of 4-hydroxy-sesamin on the Expression of Autophagic Markers Beclin1, P62, and LC3-II/LC3-I in LPS-Induced BV2 Microglial Cells

The impact of 4-hydroxy-sesamin on the expression of autophagic protein in microglial cells was assessed via Western Blot technique. For a clear illustration of the differences, the expression levels of Beclin1 protein, the LC3-II/LC3-I ratio, and p62 protein in the BV2 microglial cells of the model group after LPS stimulation, compared with those in the untreated control group (Figure 7).

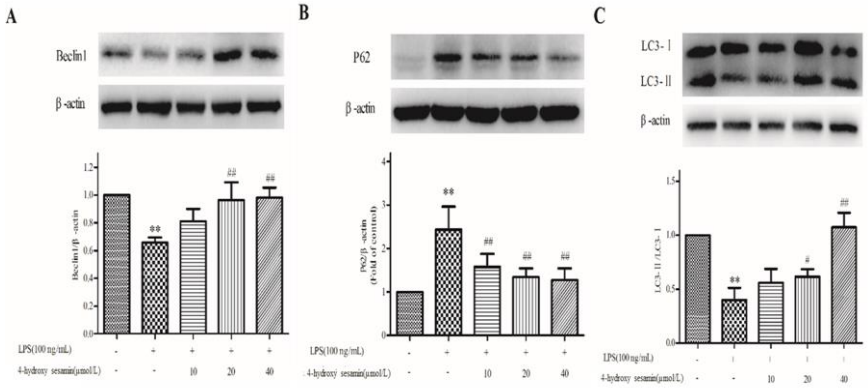

Figure 7. Effect of 4-hydroxy-sesamin on the protein expressions of Beclin1 (A), p62 (B), and LC3 (C) in LPS-induced BV2 microglial cells ($\bar{x} \pm s$, n=3).

Different Asterisk indicated a significant difference ($P < 0.05$) based on one-way ANOVA followed by Tukey's test.

Specifically, there was a significant decrease in the expression level of Beclin1 protein and the LC3-II/LC3-I ratio in the model group ($P < 0.01$), while the p62 protein levels showed a significant elevation ($P < 0.01$).These alterations denote an inhibition of autophagic processes within the cells. In contrast, treatment with various concentrations of 4-hydroxy-sesamin resulted in a significant upregulation in the expression of Beclin1 protein and the LC3-II/LC3-I ratio ($P < 0.05$, $P < 0.01$), coupled with a reduction in p62 protein levels ($P < 0.01$) compared to the model group. This suggests that 4-hydroxy-sesamin promotes autophagy in BV2 microglial cells.

3.8. Impact of 4-hydroxy-sesamin on the Phosphorylation of AMPK, mTOR, and ULK1 Proteins in LPS-Induced BV2 Microglial Cells

To investigate the impact of 4-hydroxy-sesamin on the phosphorylation status of proteins in the AMPK, mTOR, and ULK1 signaling pathways in BV2 microglial cells treated with LPS, relevant data were collected and analyzed. The findings, which demonstrate the effect of 4-hydroxy-sesamin on these signaling pathways, are presented (Figure 8). Compared to the untreated control group, LPS stimulation resulted in a significant decrease in the ratios of phosphorylated AMPK to total AMPK (p-AMPK/AMPK) and phosphorylated ULK1 to total ULK1 (p-ULK1/ULK1) ($P < 0.05$, $P < 0.01$), while the ratio of phosphorylated mTOR to total mTOR (p-mTOR/mTOR) in-creased ($P <$ 0.01), indicating inhibition of the AMPK/mTOR/ULK1 pathway by LPS. Conversely, following intervention with varying concentrations of 4-hydroxy-sesamin, there was a notable increase in the p-AMPK/AMPK and p-ULK1/ULK1 ratios ($P < 0.05$, $P < 0.01$) and a decrease in the p-mTOR/mTOR ratio ($P < 0.05$, $P <$ 0.01) compared to the LPS model group, suggesting that 4-hydroxy-sesamin is capable of activating the AMPK/mTOR/ULK1 pathway.

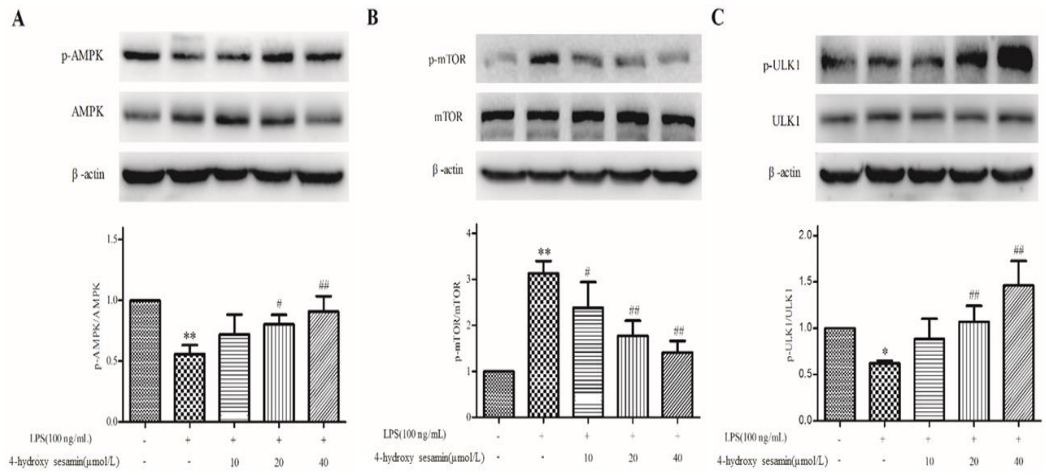

Figure 8. Effect of 4-hydroxy-sesamin on the protein expressions of AMPK (A), mTOR (B) and ULK1 (C) in LPS-induced BV2 microglial cells ($\bar{x} \pm s$, n=3).

Different Asterisk indicated a significant difference ($P < 0.05$) based on one-way ANOVA followed by Tukey's test.

4. Conclusions

This study was dedicated to sesamin and 4 - hydroxysesamin derived from *Cinnamomum camphora* High - Performance Liquid Chromatography (HPLC) was utilized to precisely establish their chromatographic conditions, ensuring effective separation. Initially, a four - factor, five - level single - factor experiment was carried out. Subsequently, the extraction process was optimized via Design - Expert 8.0.6.1 software in combination with the Box - Behnken design approach. The optimal extraction conditions were determined to be an extraction temperature of 86 °C, a solid - liquid ratio of 1:8.39 g/mL, and an extraction time of 43 min. The model - predicted total extraction yield was 0.3804 %, and the experimentally verified value was 0.3805 %, validating the reliability of the method.

The separation and purification system was established by employing Thin - Layer Chromatography (TLC) to measure Rf values, thereby identifying the optimal elution system for silica gel column chromatography. Specifically, distinct ratios of petroleum ether to ethyl acetate, namely 15:1, 9:1, and 3:1, were applied to accomplish the separation and purification of the target compounds, resulting in high recrystallization recovery rates.

Biological evaluations demonstrated that within the investigated concentration spectrum, 4 - hydroxysesamin exerted no cytotoxic effects on BV2 microglial cells. Mechanistically, it attenuated neuroinflammation by suppressing the mRNA expression of pro - inflammatory mediators, facilitated autophagic processes by modulating the expression of autophagy - associated proteins, and triggered the activation of the AMPK/mTOR/ULK1 signaling cascade.

Collectively, this study devises an effective protocol for the extraction, isolation, and purification of sesamin and 4 - hydroxysesamin. Moreover, it uncovers the anti - inflammatory paradigm of 4 - hydroxysesamin, thereby highlighting its potential utility in relevant research domains and therapeutic applications.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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