Abstract: Deep eutectic solvents (DESs) play important roles in the extraction of active constituents in traditional Chinese medicine. Ultrasound-assisted DES has been used to extract flavonoids from *Scutellaria baicalensis*. Using the contents of scutellarin, baicalin, baicalein, wogonoside, wogonin, and oroxylin A as quantitative indices, different kinds of DESs have been optimized for extraction and betaine/acetic acid has shown the highest yield. The Box–Behnken response surface method (RSM) was utilized to select the extraction conditions with the highest yields. The optimal extraction conditions were as follows: the molar ratio of betaine/acetic acid was 1:4, the water content was 40%, the solid/liquid ratio was 1:100 g/mL, the extraction temperature was 52 °C, and the extraction time was 23 min. Compared with traditional reflux extraction using 70% ethanol as the solvent, ultrasound-assisted DES has a shorter extraction time and higher yields. Furthermore, anti-inflammatory activities of the two extracts by ultrasound-assisted DES and reflux were compared using RAW264.7 cells and the methyl thiazolyl tetrazolium (MTT) method, and they showed equal anti-inflammatory activities. The results demonstrated that the ultrasound-assisted DES method for extraction of flavonoids from scutellariae radix is simple, green, efficient, and reproducible. This research provides good method guides for the rapid and efficient extraction of flavonoids from natural sources.

1. INTRODUCTION

In recent years, the concept of green extraction solvents has attracted tremendous attention as green chemistry has become more popular. Despite various ionic liquids (ILs) have been applied for the extraction of natural compounds, there are several drawbacks such as poor biocompatibility, biodegradability, and sustainability, and IL “greenness” has been frequently challenged. Deep eutectic solvents (DESs) were introduced by Abbot et al. in 2003 and were similar to ILs but had the advantages of low cost and toxicity, and high biodegradable aspects as new green solvents. Given the above advantages, DESs have been widely applied for materials science, synthesis, biocatalysis, fast extraction, and separation from animals or plants. Generally, DES processes are performed as a liquid at room temperature and form from mixtures consisting of a hydrogen bond acceptor (HBA), such as a quaternary ammonium salt, with a hydrogen bond donor (HBD), such as alcohols, amines, and carboxylic acids. Some literature studies based on choline chloride, carboxylic acids, and HBDs have demonstrated that betaine monohydrate-based DES can be used in the extraction process with many advantages, that is, low cost, biodegradability, and nontoxicity.

*Scutellariae radix*, a traditional Chinese medicine, belongs to the Labiatae perennial herb and is beneficial to reduce fever. At present, more than 130 flavonoids have been found, and they have antipyretic, anti-inflammatory, antioxidant, immune-regulative, neuro-protective, and other pharmacological effects. As with most traditional Chinese medicines, the main extraction method of scutellariae radix is reflux with an organic solvent, which often has many disadvantages, including a long extraction time, a complex operation process, effective component reduction, and increased impurity contents. Some scholars have used DESs to extract the chemical components of *scutellariae radix*, but the quantitative indicators were tiny and the biological activities of the
chemical components were not evaluated. The results did not meet the targets of multiple indicators of the chemical components of traditional Chinese medicine.14−18

In this study, an extraction method based on ultrasound-assisted DES was used to extract flavonoids in scutellariae radix. After twelve eutectic solvents were synthesized using different mixing ratios of HBDs (choline chloride and betaine) and HBAs (urea, glucose, fructose, citric acid, formic acid, and acetic acid), a fast method for the simultaneous determination of scutellarin, baicalin, baicalein, wogonoside, wogonin, and oroxylin A was developed by HPLC. Furthermore, the response surface method (RSM) was used to optimize the extraction process according to the index of the comprehensive extraction of six chemical components. Biological activity was also evaluated in this investigation. The method will provide a reference for efficient and rapid extraction of flavonoids from Scutellaria baicalensis (S. baicalensis) using new green solvents.

2. RESULTS AND DISCUSSION

2.1. Selection of a Suitable DES. The extraction of natural products has disadvantages of relatively high toxicity efficiency with organic solvents, which leads to the loss of active ingredients and may generate considerable waste. As a result, researchers have drawn widespread attention in finding new green solvents for natural product extraction. However, the viscosity of a DES is usually very high, preventing transportation of large quantities of from the plants to solutions.19,20 Therefore, in the current study, DES containing 20% (v/v) water was screened for six flavonoids. Twelve kinds of synthetic eutectic solvents and 70% ethanol were used to extract scutellarin, baicalin, baicalein, wogonoside, wogonin, and oroxylin A based on the quantitative analysis of the external standard method and the indicator of the sum of the contents of the six chemical components. The results showed that DESs were more efficient than traditional ethanol solvents to extract flavonoids from S. baicalensis, especially the betaine/acetic acid DES (Figure 1). The total flavonoids, or the sum of the contents of scutellarin, baicalin, baicalein, wogonoside, wogonin, and oroxylin A, could reach 16.59% when the molar ratio was 1:2. The above results may be explained by the multiple hydrogen bonding interactions with flavonoids and the effect of solubility.21,22 Additionally, we did some controlled trials, including ultrasound-assisted DES extraction, ultrasound-assisted ethanol extraction, ultrasound-assisted water extraction, traditional ethanol reflux extraction, water reflux extraction, and DES extraction without ultrasound, and the sum of the contents of the six compounds was obtained as follows: 16.37 ± 0.12, 14.32 ± 0.24, 9.67 ± 0.30, 14.97 ± 0.14, 10.45 ± 0.17, and 15.21 ± 0.20%. The results showed that the extraction efficiency was as follows: ultrasound-assisted DES extraction > DES extraction without ultrasound > traditional ethanol reflux extraction > water reflux extraction > ultrasound-assisted ethanol extraction > water reflux extraction > ultrasound-assisted water extraction. Thus, the extraction efficiency of the DES is high because the flavonoids are more stable under acidic conditions, and this may be beneficial to the dissolution of the flavonoids. Therefore, the betaine/acetic acid DES was chosen to optimize the extraction process parameters.

2.2. Effect of the Ratio of Betaine/Acetic Acid. The molar ratio of HBA to HBD is a crucial factor that affects the physicochemical properties of a DES.23,24 Betaine/acetic acid was used as the eutectic solvent, and the extraction yields of scutellarin, baicalin, baicalein, wogonoside, wogonin, and oroxylin A were investigated when the betaine/acetic acid ratios were 2:1, 1:1, 1:2, 1:4, and 1:6. It can be observed from Figure 2A that the extraction rate of the total flavonoids increases with the ratio of betaine/acetic acid from 2:1 to 1:4 (mol/mol). However, the extraction rate of the total flavonoids continues to decrease with the ratio of betaine/acetic from 1:4 to 1:6 (mol/mol). Based on these results, 1:4 (mol/mol) ratio of betaine/acetic acid was used for further optimization. The experimental results show that the different ratios of solvent can change the extraction effect, which may be caused by the intermolecular interactions between the extract and the extraction liquid or between the extraction liquids.

2.3. Effect of the Water Content on Betaine/Acetic Acid. Generally, adding water to an eutectic solvent can reduce the viscosity of DESs and increase the solvability, but the optimal water content also depends on specific compounds.25,26 As shown in Figure 2B, the water ratio of the DES has a significant effect on the extraction of flavonoids. In the experiment, water contents of 0, 20, 40, 60, and 80% (v/v) were investigated based on the effects of the total content of six chemical components (scutellarin, baicalin, baicalein, wogonoside, wogonin, and oroxylin A). The results showed that as the water ratio increased, the dissolution efficiency of
the chemical components increased, and the extraction efficiency increased. However, the content of the chemical components begins to decrease when the water ratio exceeds 40%, likely due to the high water content increasing the polarity of the solvent, which reduces the interactions between the DES and flavonoids. Therefore, a eutectic solvent with a water content of 40% was used for optimization in further experiments.

2.4. Effect of Solid/Liquid Ratios on Betaine/Acetic Acid. To investigate the effect of the solid/liquid ratio on the extraction yield of flavonoids, different solid/liquid ratios (1:60, 1:80, 1:100, 1:150, and 1:200) were studied. Total flavonoids, the sum of the contents of the six chemical components, were selected as an evaluation indicator, and the results are shown in Figure 2D. With increasing liquid ratios, the total content first increased and then decreased, and when the solid/liquid ratio was 1:100, the content of the total flavonoids was the highest. The study shows that the mass transfer was influenced by the viscosity of the DES, so the flavonoid extraction decreased, and 1:100 was the optimal solid/liquid ratio.

2.5. Effect of Extraction Temperature on Betaine/Acetic Acid. Extraction temperature affects the mass transfer and thus the chemical composition. As shown in Figure 2C, the total flavonoids increased from 35 to 55 °C and then decreased from 55 to 75 °C. These results indicate that the viscosity of the extraction system dropped sharply when the temperature increased and the mass transfer strengthened, so the extraction efficiency was improved. However, the dissolution of the total flavonoids was affected when the temperature was over a certain range, and the reason might be the decomposition of the flavonoids at a high temperature. Therefore, an appropriate temperature was selected for further investigation.

2.6. Effect of Extraction Time on Betaine/Acetic Acid. Extraction time is another significant parameter for the extraction of natural products. Generally, the contents of chemical components increase with increasing extraction time. However, in this test, the flavonoids decreased when the extraction time was over 25 min as shown in Figure 2E. This result may be due to the interaction between flavonoids and DESs from polymers or because the extraction stability in betaine/acetic acid is affected by the polarity of the solvent and the temperature. Thus, extraction time plays a vital role in the extraction of flavonoids.

2.7. Optimization of Extraction Conditions for Flavonoids by RSM. RSM, a valuable statistical technique to determine the optimal values of, enables the investigation of independent variables and the effects of multiple factors and their interactions effectively to produce the highest extraction efficiency. Based on the above single-factor experimental results, the flavonoid extraction conditions (solid/liquid ratio, time, and temperature) were further optimized using the Box–Behnken design (BBD) method. To evaluate the extraction process of S. baicalensis comprehensively, the contents of scutellarin, baicalin, baicalein, wogonoside, wogonin, and oroxylin A were evaluated by weighing the comprehensive score (OD), and the OD was calculated as:

Figure 2. (A) Total flavonoids of six chemicals using DES with different betaine/acetic acid ratios; (B) total flavonoids of six chemicals using a betaine/acetic acid ratio of 1:4 (mol/mol) diluted with different percentages of water; (C) total flavonoids of six chemicals using a betaine/acetic acid ratio of 1:4 (mol/mol) with different extraction temperatures; (D) total flavonoids of six chemicals using a betaine/acetic acid ratio of 1:4 (mol/mol) with different solid/liquid ratios; and (E) total flavonoids of six chemicals using a betaine/acetic acid ratio of 1:4 (mol/mol) with different extraction times. n = 3.
where $Y$ is the content of each chemical component and $Y_{\text{max}}$ is the maximum value of the corresponding index quantity. Table 1 lists the independent variables and the levels used for BBD, indicating that the unknown factors in the model can be used to determine the best extraction process.

The model is more reliable. The coefficient of determination $R^2 = 0.9442$ shows that the model is more reliable. The coefficient of sub variation $C.V. = 1.53\%$ indicates that the model has high confidence and good accuracy. However, the “Lack of Fit” has no significance, indicating that the unknown factors affecting the extraction of flavonoids have little influence on the test results and the model can be used to determine the best extraction process conditions.

In the regression model, $P < 0.05$ of $A, B,$ and $C$ indicates that the solid/liquid ratio, temperature, and time have a significant influence on the extraction of chemical components; $P < 0.05$ of $AB, AC, BC, A^2,$ and $C^2$ shows that there are interactions between various factors. The interaction among the factors is shown in Figure 3.

### 2.8. Verification of the Models and Comparison with the Chinese Pharmacopeia Method

According to the RSM results, the optimum conditions are as follows: betaine/acetic acid molar ratio is $1:4$ (mol/mol), the solid/liquid ratio is $1:100 \text{ g/mL}$, the extraction temperature is $52 ^\circ \text{C},$ and the extraction time is 23 min. Three validation experiments were carried out according to the optimal extraction conditions, and the results show that the contents of scutellarin, baicalin, baicalein, wogonoside, wogonin, and oroxylin A were $0.73 \pm 0.04, 11.93 \pm 0.36, 2.57 \pm 0.12, 1.26 \pm 0.08, 0.41 \pm 0.2,$ and $0.17 \pm 0.04,$ respectively. Fortunately, these results show that model is more predictive and that the conditions of the extraction process are more reproducible compared with the extraction methods of the Chinese Pharmacopeia 2015, which showed contents of the six compounds of $0.57 \pm 0.09, 9.12 \pm 0.23, 2.78 \pm 0.18, 1.21 \pm 0.10, 0.12 \pm 0.02,$ and $0.10 \pm 0.02,$ respectively. Thus, this new method could be expected to be the standard extraction method of flavonoids with a green solvent from now on.

### 2.9. Anti-Inflammatory Activity in LPS-Stimulated RAW264.7 Cells

The flavonoids in scutellariae radix are regarded as the main active components and have anti-inflammatory effects; thus, the anti-inflammatory activity of the extract was assayed to evaluate the extraction results of the DES. First, the methyl thiazolyl tetrazolium (MTT) method was used to identify the effects of different concentrations of extract ($0.5 \mu \text{M} \sim 50 \mu \text{M},$ betaine/acetic and 70% ethanol extraction) on the activity of RAW264.7 cells. As shown in Table 4, the cell activity decreased after LPS stimulation, and all of these samples had a certain cytotoxic effect on RAW264.7 cells. Then, a comparative group of cell viability was applied to point out the similarities between betaine/acetic acid extraction and 70% ethanol extraction, and a $P$ value $> 0.05$ was calculated using SPSS. The results show that there is no significant difference between the two groups.

The Griess method was used to measure the NO production in the supernatant and evaluate its anti-inflammatory activity. It can be seen from Table 4 that due to LPS stimulation, the NO production of RAW264.7 cells increased approximately 18 times compared with the control group. Compared with the

### Table 1. Independent Variables and Levels Used for BBD

| level | solid/liquid ratio $(A)/ \text{g/mL}^{-1}$ | temperature $(B)/ ^\circ \text{C}$ | time $(C)/ \text{min}$ |
|-------|----------------------------------------|---------------------------------|----------------------|
| −1    | 1.80                                   | 45                              | 15                   |
| 0     | 1.100                                  | 55                              | 25                   |
| 1     | 1.120                                  | 65                              | 35                   |

### Table 2. Experimental Data and Response Values Used in BBD

| run | A  | B  | C  | comprehensive score (OD) |
|-----|----|----|----|--------------------------|
| 1   | 0  | 1  | −1 | 85.01                    |
| 2   | 1  | −1 | 0  | 87.89                    |
| 3   | 0  | −1 | 1  | 82.98                    |
| 4   | 0  | 0  | 0  | 90.87                    |
| 5   | 0  | 0  | 0  | 91.08                    |
| 6   | 0  | 0  | 0  | 93.03                    |
| 7   | 1  | 1  | 0  | 76.61                    |
| 8   | 0  | −1 | −1 | 90.11                    |
| 9   | 0  | 0  | 0  | 92.01                    |
| 10  | 1  | 0  | −1 | 83.56                    |
| 11  | 0  | 0  | 0  | 89.67                    |
| 12  | 1  | 0  | 1  | 72.89                    |
| 13  | −1 | 1  | 0  | 86.63                    |
| 14  | 0  | 1  | 1  | 85.60                    |
| 15  | −1 | 0  | −1 | 79.51                    |
| 16  | −1 | 0  | 1  | 85.28                    |
| 17  | −1 | −1 | 0  | 83.90                    |
LPS group, the extracts of different concentrations can significantly inhibit the release of NO with the increase of concentration, and the IC$_{50}$ value calculated by the software is 18.43 μM, compared with the positive control, dexamethasone (2.3 ± 0.03 μM). These results suggest that the extract of the best technology of the DES still has an anti-inflammatory activity, and the two groups of betaine/acetic acid extraction and 70% ethanol extraction have no significant differences (P value > 0.05).

All of the experimental results show that anti-inflammatory effects may be achieved by inhibiting the release of NO production, and both betaine/acetic acid extraction and 70% ethanol extraction possess equal anti-inflammatory activities.

### 3. CONCLUSIONS

In this study, a green solvent combined with ultrasonic-assisted extraction was used to extract scutellarin, baicalin, baicalein, wogonoside, wogonin, and oroxylin A from scutellariae radix simultaneously. The weight coefficient of each index was determined by RSM, and the OD was taken as an index to obtain the optimal conditions: the molar ratio of betaine/acetic acid was 1:4, the water content was 40%, the solid/liquid ratio was 1:100 g/mL, the extraction temperature was 52 °C, and the extraction time was 23 min. The results show that this method is superior to traditional 70% ethanol reflux extraction with good repeatability of the method, a shorter extraction time, and equal anti-inflammatory activities.

**Table 4. Cell Viability and NO Concentration of LPS-Stimulated Raw 264.7 Cells**

| group        | cell viability (%) (mean ± SD) | NO concentration (μM) (mean ± SD) |
|--------------|-------------------------------|----------------------------------|
|              | betaine/acetic acid extraction | 70% ethanol extraction           |
| control      | 100                           | 2.78 ± 0.31                      |
| LPS (1 μg/mL) | 89.23 ± 4.28                  | 37.21 ± 1.26                     |
| 0.5 μM       | 87.20 ± 5.35                  | 29.14 ± 1.35                     |
| 2.5 μM       | 85.81 ± 3.59                  | 86.27 ± 3.28                     |
| 5 μM         | 84.26 ± 2.73                  | 83.47 ± 3.36                     |
| 25 μM        | 82.19 ± 6.21                  | 82.17 ± 3.08                     |
| 50 μM        | 79.34 ± 3.26                  | 78.35 ± 0.28                     |
| P value      | 0.67                          | 20.79 ± 1.04                     |

LPS group, the extracts of different concentrations can significantly inhibit the release of NO with the increase of concentration, and the IC$_{50}$ value calculated by the software is 18.43 μM, compared with the positive control, dexamethasone (2.3 ± 0.03 μM). These results suggest that the extract of the best technology of the DES still has an anti-inflammatory activity, and the two groups of betaine/acetic acid extraction and 70% ethanol extraction have no significant differences (P value > 0.05).

All of the experimental results show that anti-inflammatory effects may be achieved by inhibiting the release of NO production, and both betaine/acetic acid extraction and 70% ethanol extraction possess equal anti-inflammatory activities.
DES is a green solvent used in natural product extraction and should be popularized; compared with traditional solvents, it has many advantages, including a shorter extraction time, less pollution, better reproducibility, and a better extraction effect in scale-up experiments. This study can provide a theoretical basis for the development of new green solvents and further research of scutellariae radix. Therefore, the green recovery of DES-based extractions on macroscopic resin and further pharmacodynamics and determination of the mechanism of action will be explored in further research. DES based on betaine will provide support for industrial scale-up experiments and the green extraction of traditional Chinese medicine in the future.

4. MATERIALS AND METHODS

4.1. Experimental Section. Standard scutellarin, baicalin, wogonoside, and wogonin were purchased from the National Institutes for Food and Drug Control (China, ≥98%). Baicalein and oroxylin A were purchased from Chengdu Mansite Biotechnology Co., Ltd. (Chengdu, China). Compounds for DES, betaine, glucose, and D-fructose were obtained from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Analytical-grade choline chloride, formic acid, acetic acid, citric acid, and ethanol were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The purity of the abovementioned acids was greater than 98%. RPMI-1640 medium was purchased from the Thermo company (USA).

Scutellariae radix, purchased from Zhonglu Hospital (Shandong, China), was pulverized, sieved (90 mesh), and then stored in a desiccator for further experiments.

4.2. Preparation of Deep Eutectic Solvents. DESs were prepared by heating and stirring the regents of HBA and HBD. Appropriate amounts of HBDs (such as choline chloride and betaine) and HBAs (i.e., formic acid, acetic acid, citric acid, glucose, D-fructose, and urea) were weighed. Then, 12 kinds of DESs were prepared according to a certain mole ratio and mixed in a round-bottom flask. They were heated and stirred in an oil bath at 80 °C to form a uniform and stable colorless transparent liquid. The initial compounds used to prepare DESs are listed in Table 5.

4.3. Extraction of Flavonoid Compounds from Scutellariae Radix. First, 0.1 g of scutellariae radix drug powder was weighed and placed into a 10 mL glass tube with a stopper. Then, equal amounts of different kinds of DESs were added and mixed with 4 mL of water. The solution was reacted under ultrasound at 30 °C for 10 min and centrifuged at 3500 rpm for 8 min. The flavonoids from the DES extraction solution could be obtained from the supernatant.

According to the method for the determination of scutellariae radix in Chinese Pharmacopeia 2015,0,3 0.3 g of the scutellariae radix drug powder was weighed. Then, 40 mL of ethanol (70%, vol) was added into the flask and extracted by reflux for 3 h. The solution was transferred into a 100 mL volumetric flask and mixed.

4.4. HPLC Analysis. HPLC analysis was performed on an Agilent HPLC 1260 Infinity II system equipped with a 1260 Infinity quaternary pump (G7111B), a 1260 standard autosampler (G7129A), a column compartment (OPTION 066), and a 1260 Infinity II VWD detector (G7114A). The separation of these components was completed using an Agilent 5 TC-C18 column (250 × 4.6, 5 μm). The mobile phase consisted of water containing 0.1% formic acid (eluent A) and acetonitrile (eluent B). The gradient elution program was as follows: 0–5 min, 18–24% B; 5–10 min, 24–26% B; 10–30 min, 26–40% B; 30–40 min, 40–50% B; 40–50 min, 50–100% B. The column temperature was kept at 35 °C at a constant flow rate of 0.8 mL/min, the detection wavelength was 274 nm, and the injection volume was 10 μL.

4.5. Preparation of Standard Solutions. Scutellarin, baicalin, baicalein, wogonoside, wogonin, and oroxylin A were weighed and dissolved with methanol. Then, these standard solutions were set aside in a refrigerator until their use. A specific volume of the standard solution was diluted in volumetric flasks. The mixed reference solution was composed of six chemical components with concentrations of 335 μg/mL of scutellarin, 36 μg/mL of baicalin, 167.5 μg/mL of baicalein, 85 μg/mL of wogonoside, 42.5 μg/mL of wogonin, and 18 μg/mL of oroxylin A.

4.6. RSM Experimental Design and Statistical Analyses. In this experiment, the best DES combination was selected and the best extraction process was optimized by investigating the influence of different factors on the chemical composition of scutellariae radix. Single-factor experiments were carried out to investigate the effects of different molar ratios (2:1, 1:1, 1:2, 1:4, and 1:6), water contents of DES (0, 20, 40, 60, and 80%), solid/liquid ratios (1:60, 1:80, 1:100, 1:150, and 1:200), extraction times (10, 15, 25, 45, and 55 min), and extraction temperatures (35 45, 55 65, and 75 °C). The important influencing factors were screened out. Then, the BBD method was used to test the influencing factors including solid/liquid ratio (A), extraction time (B), and temperature (C). Data processing analysis was carried out to

| entry | abbreviation | component 1 (HBA) | component 2 (HBD) | mole ratio | appearance at room temperature |
|-------|--------------|-------------------|-------------------|------------|-------------------------------|
| 1     | CCU          | choline chloride  | urea              | 1:2        | transparent liquid             |
| 2     | CCCA         | choline chloride  | citric acid       | 1:2        | transparent liquid             |
| 3     | CCG          | choline chloride  | glucose           | 1:2        | transparent liquid             |
| 4     | CCDF         | choline chloride  | D-fructose        | 1:2        | transparent liquid             |
| 5     | CCFA         | choline chloride  | formic acid       | 1:2        | transparent liquid             |
| 6     | CCAC         | choline chloride  | acetic acid       | 1:2        | transparent liquid             |
| 7     | BG           | betaine           | glucose           | 1:2        | transparent liquid             |
| 8     | BCA          | betaine           | citric acid       | 1:2        | transparent liquid             |
| 9     | BU           | betaine           | urea              | 1:2        | transparent liquid             |
| 10    | BDF          | betaine           | D-fructose        | 1:2        | transparent liquid             |
| 11    | BFA          | betaine           | formic acid       | 1:2        | transparent liquid             |
| 12    | BAC          | betaine           | acetic acid       | 1:2        | transparent liquid             |

Table 5. Initial Compounds for DES
comprehensively select the best extraction process of chemical components of scutellariae radix.

Each experiment was repeated three times. BBD was used to analyze the experimental data, and the second-order polynomial model was obtained using Design-Expert. The model was obtained by BBD after variance analysis.35–37

4.7. Anti-Inflammatory Activity Assay of NO Production. The anti-inflammatory activity assay of NO production in RAW264.7 cells was determined using the reported method.38,39 RAW264.7 cells in the logarithmic growth phase were cultivated in 96-well plates (Costar, USA) at 2 × 10⁵ cells per well in an RPMI-1640 medium with 10% FBS, 100 μg/mL of streptomycin, and 100 U/mL of penicillin. The 96-well plates were placed in an incubator maintained at 37 °C with 5% CO₂. Then, the cells were stimulated for 2 h using different concentrations of scutellariae radix extract solution (0.5, 2.5, 5, 25, and 50 μM concentrations of DES extraction and 70% ethanol extraction) and dexamethasone (Sigma, >98%), which was used as a positive control, and 0.1% DMSO as a vehicle control. In addition, lipopolysaccharide (LPS, 1.0 μg/mL) was added for 24 h, and 50 μL of Griess reagent I and Griess reagent II were mixed with 50 μL of each supernatant using an enzymatic scale to measure absorbance at 540 nm to inhibit and determine the IC₅₀ values. NO production was calculated using the standard curve generated by continuous inhibition and determine the IC₅₀ values. NO production was calculated using the standard curve generated by continuous

All experiments were repeated three times to test NO inhibition and determine the IC₅₀ values. NO production was calculated using the standard curve generated by continuous dilution of NaNO₂ in a fresh medium (R² = 0.9993), and GraphPad Prism 7 was used to calculate the IC₅₀ values. All values in this experiment are expressed as mean ± standard deviation (SD).

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