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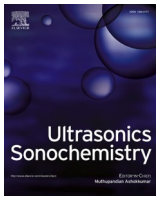

Enzymatic and ultrasound assisted β-cyclodextrin extraction of active ingredients from *Forsythia suspensa* and their antioxidant and anti-inflammatory activities

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ABSTRACT

With the proposal of the 2030 Agenda for Sustainable Development, the Chinese medicine extraction technology has been innovatively improved to prioritize low energy consumption, sustainability, and minimized organic solvent utilization. *Forsythia suspensa* (FS) possesses favorable pharmacological properties and is extensively utilized in traditional Chinese medicine. However, due to the limitations of the composition and extraction methods, its potential has not been fully developed. Thus, a combination of ultrasound-assisted extraction (UAE), enzyme-assisted extraction (EAE), and β-cyclodextrin extraction (β-CDE) was employed to isolate and purify rutin, phillyrin, and forsythoside A from FS. The results demonstrated that the efficiency of extracting enzymatic and ultrasound assisted β-cyclodextrin extraction (EUA-β-CDE) was highly influenced by the temperature and duration of hydrolysis, as well as the duration of the extraction process. According to the results of the single-factor experiment, Box-Behnken design (BBD) in Response surface method (RSM) was used to optimize the experimental parameters to achieve the maximum comprehensive evaluation value (CEV) value. The EUA-β-CDE compared with other extraction methods, has good extraction effect and low energy consumption by high performance liquid chromatography (HPLC), scanning electron microscopy (SEM), calculation of power consumption and CO_2 emission The EUA-β-CDE compared with other extraction methods, has good extraction effect and low energy consumption by HPLC, SEM, calculation of power consumption and CO_2 emission. Then, the structural characteristics of EUA-β-CDE of FS extract had significant interaction with β-CD by Fourier infrared spectroscopy (FT-IR) and differential scanning calorimetry (DSC). In addition, EUA-β-CDE extract has good antioxidant and anti-inflammatory activities. The establishment of EUA-β-CDE of FS provides a new idea for the development and application of other sustainable extraction methods of traditional Chinese medicine.

1. Introduction

Presently, the increase in global surface temperature due to the greenhouse effect triggered by carbon dioxide (CO_2) emissions is leading to global warming, along with more frequent occurrences of extreme weather events and environmental pollution issues. These conditions present significant challenges to the long-term sustainable development of humanity. The imperative to regulate CO_2 emissions is crucial for all countries worldwide. China, being the largest developing nation globally, committed during the 75th session of the United Nations General Assembly to reach peak CO_2 emissions by 2030 and attain carbon neutrality by 2060. These goals represent China's central national

Abbreviations: FS, *Forsythia suspensa*; EUA-β-CDE, enzymatic and ultrasound assisted β-cyclodextrin extraction; UAE, ultrasound-assisted extraction; EAE, enzyme-assisted extraction; β-CDE, β-cyclodextrin extraction; EUAE, enzymatic and ultrasound assisted extraction; BBD, Box-Behnken design; RSM, Response surface method; CEV, comprehensive evaluation value; HPLC, high performance liquid chromatography; SEM, scanning electron microscopy; FT-IR, Fourier infrared spectroscopy; DSC, differential scanning calorimetry; ATPE, two-phase aqueous extraction; DESE, deep eutectic solvent-assisted extraction; α-CD, α-cyclodextrin; β-CD, β-cyclodextrin; γ-CD, γ-cyclodextrin; LPS, Lipopolysaccharide; Cel, cellulase; Pap, papain; Pec, pectinase; Hem, hemicellulase.

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priority in addressing climate change [1]. The United Nations launched the 2030 Agenda for Sustainable Development, which comprises a comprehensive set of 17 sustainable development goals intricately linked by a network of 169 interconnected objectives [2]. Conventional plant extraction techniques in Traditional Chinese Medicine require significant amounts of organic solvents due to the characteristics of the active ingredients, resulting in environmental contamination pollution. For instance, methanol, a frequently employed organic solvent in the extraction of Chinese herbal medicine, can have severe repercussions such as loss of consciousness and permanent blindness if mishandled during experiments [3]. Furthermore, the efficacy of pharmaceutical ingredients may diminish the extraction rate when the extraction process fails to adequately optimize its parameters, consequently leading to increased energy consumption. Hence, this study introduces a novel FS extraction process that seeks to explore innovative environmentally sustainable extraction technologies with significant positive implications.

As a very precious Chinese medicine resource [4], *Forsythia suspense* (Thunb.)Vahl has the effects of detoxification, reduce swelling, disperse knot, and evacuate wind and heat [5]. Because FS has a remarkable effect in the treatment of a variety of diseases, it is often used in the formulation of compound drugs such as Yinqiao antipoison pill and Lianhua Qingwen capsule. In recent years, it has been determined that FS contains a variety of bioactive components including phenylethanoid glycosides, flavonoids and lignans through instrument determination. As one of the phytochemical markers for evaluating the quality of FS in the 2020 edition of Chinese Pharmacopoeia, phillyrin belongs to the lignins of FS active ingredients. Studies have shown that phillyrin has anti-inflammatory and antiviral pharmacological effects [6]. Rutin, as a flavonoids compound, has many pharmacological activities such as anti-inflammatory, antioxidant, anti-allergic and antiviral, and is widely found in nature [7]. As another phytochemical marker to evaluate FS quality in the 2020 edition of Chinese Pharmacopoeia, forsythoside A has been proved to have anti-inflammatory, antiviral and other pharmacological effects in many studies [8]. However, rutin and phillyrin have the disadvantages of low solubility and are not easy to extract, thus limiting their clinical efficacy [9]. Forsythoside A has low stability in high temperature environment and acidic or alkaline environment. Moreover, traditional extraction techniques require long heating times and large amounts of organic solvents, which is inconsistent with the Sustainable Development Goals [10]. Moreover, traditional extraction techniques require long heating times and large amounts of organic solvents, which is inconsistent with the Sustainable Development Goals. Therefore, an ecologically sustainable extraction process that can simultaneously extract rutin, phillyrin, and forsythoside A is very important.

Conventional extraction methods, such as hot reflux extraction (HRE) and organic solvent extraction, have long been utilized in the extraction of active ingredients. While organic solvent extraction boasts a high extraction rate by targeting the active components, the use of organic solvents poses environmental pollution risks due to their high toxicity [11]. On the other hand, HRE, though straightforward and efficient in extraction, necessitates prolonged heating periods during experimentation, potentially leading to the degradation of heat-sensitive components [12]. Among modern extraction techniques, several sustainable methods have emerged, including two-phase aqueous extraction (ATPE) [13], enzyme-assisted extraction (EAE) [14], ultrasonic-assisted extraction (UAE) [15], and low eutectic solvent-assisted extraction (DESE) [16]. UAE, recognized as an eco-friendly extraction technology, enhances extraction efficiency, reduces extraction time, and exhibits broad applicability by utilizing ultrasonic cavitation, mechanical shock, and thermal effects to expedite the release of bioactive compounds from cells [17]. The EAE method stands out for its energy efficiency and time-saving benefits, positioning it as a sustainable extraction approach [18,19], often combined with other methods for active ingredient extraction.

Cyclodextrins, cyclic oligosaccharides produced by converting amylose with cyclodextrin glucosyl transferase from Bacillus, play a crucial role in enhancing the stability and solubility of guest molecules [20]. Comprising 6 to 12 units of D-glucuronic acid, cyclodextrins, including α-cyclodextrin (α-CD), β-cyclodextrin (β-CD), and γ-cyclodextrin (γ-CD), form inclusion complexes with guest molecules, particularly β-CD, a widely used pharmaceutical excipient with absorbent surfaces and hydrophobic central cavities [21]. Upon entering the body, β-CD undergoes ring opening, forming linear oligosaccharides that participate in metabolic processes without causing accumulation or adverse effects, thereby improving drug solubility and stability [22]. Oligosaccharides can participate in metabolism in the body, so they do not cause accumulation or other harmful effects. And β-CD can also improve the solubility and stability of the drug. For example, in the work of Jurga Andreja Kazlauskaite et al. [23], red clover extract was prepared by a combination of ultrasonic-assisted pyrolysis and thermal reflux. The results showed that cyclodextrin greatly boosted the output of isoflavone glycosides.

To enhance the extraction efficiency of active ingredients in FS, reduce energy consumption, and mitigate environmental impact, a novel sustainable extraction method, EUA-β-CDE, was proposed in this study. The Box-Behnken design (BBD) was used to optimize the process parameters, and comparative analyses were conducted with other extraction methods. Antioxidant and anti-inflammatory assays were performed to assess the biological activity of the extract. Additionally, Fourier transform infrared spectroscopy (FT-IR) and differential scanning calorimetry (DSC) wereutilized to investigate the interaction between β-CD and the three active ingredients.

2. Materials and methods

2.1. Materials and reagents

In this experiment, FS purchased from a market near Harbin, Heilongjiang Province, China, was dried naturally under specified temperature and humidity conditions and was identified by Associate Professor Liu Yanyan, College of Veterinary Medicine, Northeast Agricultural University, Harbin, China. The FS powder, which was crushed by a pulverizer (Taisite, FW100, Tianjin, China) and filtered with a 40-mesh screen, was placed in a sealed bag and stored in a desiccator at room temperature. The origin of papain (Pap) was obtained from Maclean Biochemical Co., Ltd. (Shanghai, China). Pectinase (Pec) and hemicellulose (Hem) were acquired from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Cellulase (Cel) was acquired from Beijing Biotopped Science & Technology Co., Ltd. (Beijing, China). Lipopolysaccharide (LPS) was obtained from Sigma Aldrich Trading Co., LTD. (Shanghai, China). The chemicals rutin, phillyrin, and forsythoside A were acquired from Dalian Meilun Biotechnology Co Ltd (Dalian, China). The methanol utilized in the chromatography process was acquired from Dikma Technology, located in Beijing, China. Hydrochloric acid, citric acid, and Na_2HPO_4 were acquired from Kermel Chemical Reagents Ltd (Tianjin, China). All remaining reagents utilized were of analytical grade or superior quality.

2.2. Extraction with EUA-β-CDE

2.2.1. EUA-β-CDE procedure

Combined with the characteristics of active ingredients in FS fruit, this extraction process was established in this study, as shown in Fig. 1 (Created with BioRender.com). A total of 2.0 g FS powder, 1.5 % Pap, 6.5 % Cel, and 20 mL disodium hydrogen phosphate-citric acid buffer solutions were accurately weighed and added to a 50 mL EP tube. After mixing with a vortex mixer for 5 s, the solution was sonicated in an ultrasonic bath (Frequency: 40 kHz, Model: SB-800DTD (840 W), Ningbo Scientz Biotechnology CO., Ltd, Ningbo, China) at 32 °C for 56 min. The reaction mixture was then incubated at 85 °C for 2 min to

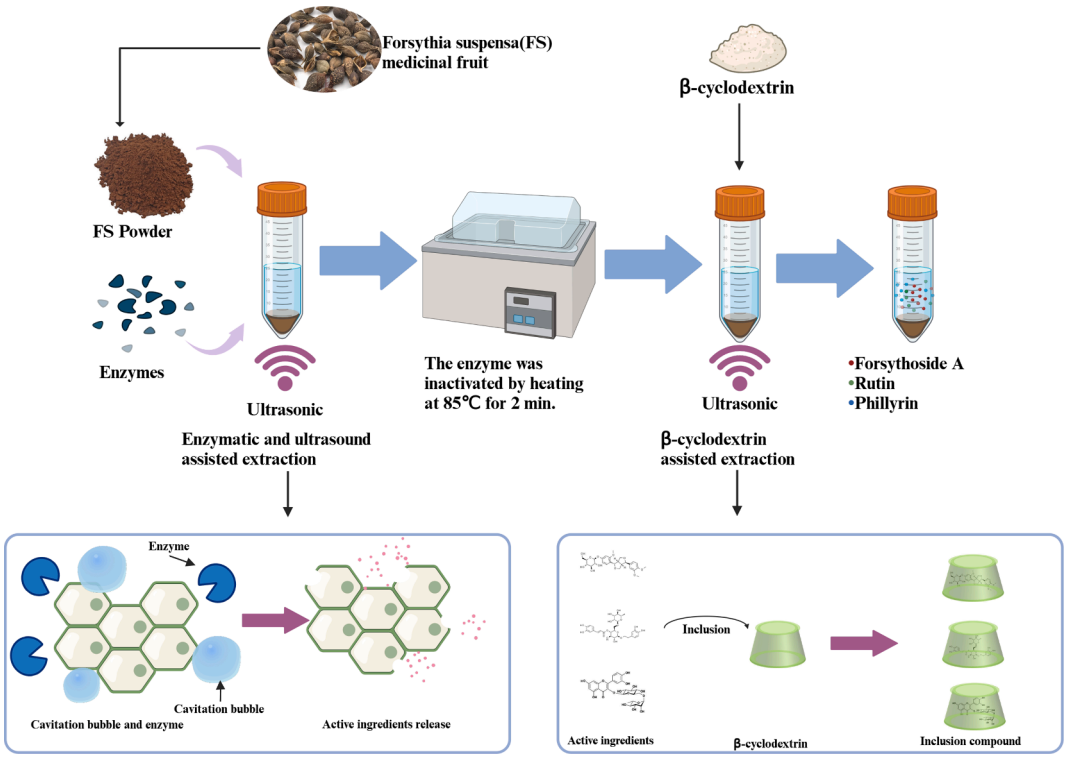

Fig. 1. The schematic of the apparatus of EUA-β-CDE.

inactivate the enzyme. 0.3 g of β-CD (98 %, Aldrich) was added to a 20 mL reaction mixture and vortexed for 5 s, the samples were sonicated at 60 °C for 82 min. Finally, the mixture was centrifuged in a centrifuge (5000 rpm, 5 min) to collect the supernatant.

2.2.2. *Single-factor experiment*

Best-fit parameters for extractive conditions were obtained by optimization of the experimental data. The sample buffer solution was prepared by adding FS powder and different enzymes into disodium hydrogen phosphate and citrate buffer solution according to certain liquid–solid ratio. Incubations were conducted with Pap, Cel, Hem, and Pec, as well as combinations of these enzymes. In addition, NaOH or HCl was used to adjust the pH of the enzymatic hydrolysate. The optimal range of ultrasonic power was 440 ~ 840 W, the amount of β-CD added was 5 ~ 45 mg/mL, the enzymolysis time and extraction time were estimated to be 30 ~ 110 min, the enzymolysis temperature was between 25 °C~45 °C, and the extraction temperature was between 40 °C~80 °C. The determination of rutin, phillyrin and forsythoside A was conducted following the procedures outlined in section 2.4. The CEV value in the resulting extraction solution was used as a criterion to ascertain the most favorable extraction conditions.

2.2.3. *Optimized EUA-β-CDE with BBD*

According to the results of the single-factor experiment, Box-Behnken design (BBD) in Response surface method (RSM) was used to optimize the experimental parameters to achieve the maximum CEV value. The optimal condition was estimated to be the value of BBD obtained with the three variables as factors, as indicated in Table 2. The matrix presented in Table 2 comprises 17 experiments that encompass three variables, each of which is manipulated at three distinct levels, in order to compute the CEV as an indication. The experimental data were examined utilizing a second-order polynomial model to assess the interplay between the independent and response variables. The experimental results were modeled using a quadratic polynomial equation, which was described by a quadratic equation:

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X_i^2 + \sum_{j<i}^{3}\beta_{ij} X_i X_j \quad (1)$$

The variables X_i and X_j are input variables that have an impact on the response function Y. The intercept is denoted as β_0. The coefficients β_i, β_{ii}, and β_{ij} represent the linear, quadratic, and interaction components, respectively.

2.2.4. *Computation of comprehensive evaluation value (CEV) via CRITIC algorithm*

As a relatively objective weighting method, the CRITIC weighting method mainly considers the strength and conflict index between data. If the standard deviation of the data is higher, the greater the variability; conversely, if the correlation coefficient between the indicators is higher, the less conflict there is, thus the weight of the data will be lower [24,25]. Initially, the indicator components were standardized by utilizing data from each individual component in the experiment. The formula used for standardization was: indicator component = (measured value − minimum value)/(maximum value − minimum value) [26]. The CRITIC algorithm was employed to compute the weight coefficients for each component in order to derive the CEV. The following are the main steps for the CRITIC algorithm [27].

i. According to the initial data matrix is established:

$$x = (x_{ij})_{m\times n} \quad (2)$$

where x_{ij} (i = 1, 2, …, m;j = 1, 2, …, n) is the original data corresponding to the j-th indicator of the i^{th} sample.

ii. The original data matrix above was standardised using the Z-score method:

$$x_{ij}^{*} = \frac{x_{ij} - \overline{x}_j}{s_j} \quad (3)$$

$$\overline{x_j} = \frac{1}{m}\sum_{i=1}^{m} x_{ij} \tag{4}$$

$$S_j = \sqrt{\frac{1}{m-1}\sum_{i=1}^{m}\left(x_{ij} - \overline{x_j}\right)^2} \tag{5}$$

where xj is the average of the j-th indicator, s_j is the standard deviation of the j-th indicator, and $X^* = (x_{ij}^*)_{m\times n}$ is the standardized matrix.

iii. The coefficient of variation of each indicator was calculated:

$$v_j = \frac{s_j}{\overline{x_j}} \tag{6}$$

where v_j is the variation coefficient of the j-th indicator.

iv. The correlation coefficient matrix $R = (r_{kl})_{n\times n}$ of matrix X* is calculated:

$$r_{kl} = \frac{\Sigma_{i=1}^{m}\left(x_{ik}^* - \overline{x}_k^*\right)\left(x_{il}^* - \overline{x}_l^*\right)}{\sqrt{\Sigma_{i=1}^{m}\left(x_{ik}^* - \overline{x}_k^*\right)^2}\sqrt{\Sigma_{i=1}^{m}\left(x_{ik}^* - \overline{x}_k^*\right)^2}} \tag{7}$$

where r_{kl} denotes the correlation coefficient between the k-th indicator and l-th indicator.

v. The independent coefficient ηj of each index was determined to evaluate the degree of correlation between different indexes:

$$\eta_j = \sum_{k=1}^{n}\left(1 - |r_{kj}|\right), j = 1, 2, \cdots, n \tag{8}$$

vi. The total amount of information for each indicator was calculated:

$$D_j = v_j\eta_j, j = 1, 2, 3, \cdots, n \tag{9}$$

vii. The weight of each indicator was determined:

$$\omega_j = \frac{D_j}{\Sigma_{j=1}^{n} D_j}, j = 1, 2, 3, \cdots, n \tag{10}$$

Finally, CEV was calculated according to the following formula:

$$CEV = \sum_{i=1}^{m} Y_{ij}w_j \tag{11}$$

2.3. HPLC was used to determine the active components in FS extract

The Waters high performance liquid chromatography system (Waters e2695) was used, and a reversed-phase C18 column (5 μm, 4.6 × 250 mm) was used in the experiment. The sample size was 10 μL, the column temperature was 25℃, and the detection wavelength was 254 nm. The mobile phases used were gradient elution solvents, namely solvent A (0.3 % aqueous acetic acid) and solvent B (methanol). 0–8 min, 30 %B ~ 33 %B; 8–24 min, 33 %B ~ 40 %B; 24–39 min, 40 %B ~ 52 %B; 39–55 min, 52 %B ~ 36 %B. The peak area of each peak corresponds to the relative concentration of the related chemical. The determination of rutin, phillyrin, and forsythoside A by HPLC was evaluated by measuring linearity of the calibration curve, precision between and within days, stability, repeatability, and recovery rate.

2.4. Comparison of different extraction methods

2.4.1. Comparison of CEV of different extraction methods

To assess the extraction effects of EUA-β-CDE, EAE, UAE, β-CDE, 70 % methanol (70 % ME) was used for the organic solvent extraction and HRE, a study was conducted. The extraction settings for EAE, UAE, and β-CDE were aligned with the optimal extraction conditions for EUA-β-CDE. The settings under which HRE was conducted were as follows: A 2 g of FS powder and 20 ml of distilled water are added to a round-bottomed flask. The flask is then immersed in a water bath at 85 °C for 2 h. The extraction method was done according to the procedure outlined in Chinese Pharmacopoeia [28]. Finally, following the steps outlined in 2.4, the rutin, phillyrin and forsythoside A contents were determined. The CEV was used as the primary indicator for comparing various extraction procedures.

2.4.2. Energy conservation

The global rapid economic growth leads to a rising need for energy. Nevertheless, conventional extraction methods employing significant organic solvents, driven by the active ingredient properties, pose a threat to environmental pollution. Thus, it is crucial to consider energy consumption and organic solvent utilization in the extraction process. Consequently, the extraction methodology was evaluated utilizing an Equation [29].

$$E_{Consumed} = P \times t \tag{12}$$

Where: Econsumed refers to the amount of energy consumed, measured in kWh; P represents the power supply, measured in kW; t denotes the duration of the extraction process, measured in hours (h).

Consumed per unit of electricity mainly comes from coal or the burning of fossil fuels. Utilizing coal or fossil fuels to generate 1 kWh of electricity results in the emission of 800 grams of CO_2 into the atmosphere through the process of combustion. Hence, the estimation of CO_2 emissions is conducted using the given equation.

$$E_{CO2} = E_{Consumed} \times 800 \tag{13}$$

where E_{CO2} denotes the amount of carbon dioxide emissions measured in kg.

2.4.3. Surface structure characterization of FS powder by different extraction methods

The morphology of FS powder before and after extraction, enzyme extraction, ultrasonic extraction, β-CD extraction and methanol extraction were observed by SEM. SEM analysis of the powder was performed using a 5000-power scanning electron microscope after it was dried.

2.5. Characterizations

In order to demonstrate the changes produced by combining the active ingredient in FS extract with β-CD, the following experiments were designed in this study.

2.5.1. FTIR analysis

The FS, β-CD, EUA-β-CDE, FS, and β-CD 20:3 physical mixtures (referred to as 20:3 FS/β-CD PM) were carefully combined with the necessary quantity of KBr. Subsequently, the resulting mixture was compressed into KBr disks. Then use the Nicolet FT – IR spectrometer (Thermo Scientific Nicolet iS20) to get the FT – IR spectrum. The scanning process involved conducting 32 scans for each sample within the spectral range of 400 cm to 4000 cm^{-1}, with an optical resolution of 4 cm^{-1}.

2.5.2. DSC analysis

Our samples were analyzed using a TA analyzer (DSC 250, America). Each specimen weighed approximately 7 mg and was carefully placed within a hermetically sealed aluminum crucible. Subsequently, the crucible was subjected to controlled heating in an environment saturated with nitrogen gas, with the temperature being gradually increased from 20 to 180 °C Cat a rate of 10 °C per minute.

2.6. Thermal stability study

The FS compound was extracted using a combination of EUA-β-CDE method. This extraction processes resulted in the production of the desired extraction solution. The solution that was obtained was subsequently transferred into eppendorf tubes and subjected to a 60 °C water bath for varying durations of 0, 1, 2, 3, 4, and 5 h, respectively. The determination of the contents of rutin, phillyrin and forsythoside A was conducted.

2.7. Antioxidant capacity of extracts of FS by different extraction methods

2.7.1. Activity of DPPH in scavenging free radicals

The DPPH solution preparation with ethanol concentration is 0.2 mM. Afterward, the reagents were categorized into three distinct groups: sample group, blank group, and control group. A 1:1 sample solution was mixed with a DPPH solution to create the sample group. The blank group comprised a combination of 1:1 sample solution and anhydrous ethanol. Lastly, the control group was composed of a 1:1 mixture of DPPH solution and water. Once thoroughly mixed, leave it in the dark for 30 min. The control group in this experiment was vitamin C (VC). The absorbance was measured at 414 nm by a microplate reader. The free radical scavenging activity of DPPH was calculated by Eq. (14).

$$DPPH\ radical\ scavenging\ acitivity\ (\%) = \left(1 - \frac{A - A_1}{A_0}\right) \times 100\% \quad (14)$$

where A_0 represents the absorbance of the control, A represents the absorbance of the sample, and A_1 represents the absorbance of the blank.

2.7.2. Activity of ABTS in scavenging free radicals

The ABTS free radical scavenging activity technique utilized is based on the T-AOC kit (S0121, Shanghai Beyotime Biotechnology Co., LTD., China) procedure. Firstly, a volume of 20 μL of peroxidase working liquid was introduced into the detection hole. As a second step, join the blank hole 10 uL of water and join in the sample hole 10 uL sample solution. Thirdly, a volume of 170 μl of ABTS working solution was introduced into each well, followed by gentle agitation, and then incubated at ambient temperature for 6 min. The control group in this experiment was vitamin C (VC). The absorbance was measured at 414 nm by a microplate reader. The free radical scavenging activity of ABTS was calculated by Eq. (15).

$$ABTS\ radical\ scaveng\ acitivity\ (\%) = \frac{A_0 - A}{A_0} \times 100\% \quad (15)$$

where A_0 represents the absorbance of the control, while A represents the absorbance of the sample.

2.8. Anti-inflammatory activity in vitro

2.8.1. Culture and treatment strategies for cells

The mouse macrophage RAW264.7 cells used in this experiment were purchased from the American Type Culture Collection (ATCC, Manassas, VA). In the laboratory, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with the addition of 12.5 % fetal bovine serum. The cells were cultured in an incubator with wet air at 37 °C and a CO_2 concentration of 5 %. Cells were used for 8–15 passages in this study.

In this experiment, RAW264.7 cells were treated with drugs, which were mainly divided into 13 treatment groups. They included EUA-β-CDE treatment group, HRE treatment group, rutin treatment group, phillyrin treatment group, forsythoside A treatment group, monomer mixture treatment group, dexamethasone treatment group, model group and control group. EUA-β-CDE treatment group included three high, middle and low dose groups with concentrations of 0.50, 0.25 and 0.125 mg/mL. The cells were treated with high, medium and low doses of HRE extracts with concentrations of 0.50 mg/mL, 0.25 mg/mL and 0.125 mg/mL, respectively. The concentration of rutin (0.0018 mg/mL), phillyrin (0.0015 mg/mL), forsythoside A (0.035 mg/mL) and monomer mixture (0.0383 mg/mL) treated cells was based on the concentration in the 0.5 mg/mL EUA-β-CDE extract. The control group was treated with dexamethasone (DEX) at 1 μM. The model group was treated with LPS (1 μg/mL).

2.8.2. Cell viability assay

RAW264.7 cells were placed in 96-well plates at a density of 1×10^4 cells/well and cultured in an incubator at 37 °C for 8–10 h. The cells were treated under different experimental conditions and cultured in an incubator at 37 °C for 24 h. Finally, the original medium was discarded, and 10 % CCK-8 solution was added and cultured in an incubator at 37 °C for 0.5–1 h. At 450 nm wavelength measurement of optical density (OD) value through a microplate reader.

2.8.3. Intracellular reactive oxygen species (iROS) determination

RAW264.7 cells were placed in 96-well plates at a density of 1×10^4 cells/well, divided into 13 groups, and incubated in the incubator for 12 h. Subsequently, 12 groups of cells were immersed in 1 μg/mL LPS solution and incubated for another 24 h. At the same time, the other group of cells did not receive any treatment and served as the control group. Subsequently, 12 groups of cells were treated with different drugs and cultured in the same experimental environment for 24 h. After discarding the original medium, a medium containing 10 mM of 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was added and the cells were placed in an incubator at 37 °C without light for the following 40 min. As a final step, the cells were rinsed with DMEM so that any residual DCFH-DA could be removed. Using a microplate reader to measure the fluorescence intensity in cells, the light of the emission wavelength of 485 nm, detection wavelength of 530 nm.

2.8.4. Determinations of inflammatory cytokines

Commercial ELISA kits for mouse immunoassay were utilized to measure the levels of TNF-α, IL-1β, IL-6, and IL-18, which are inflammatory cytokines, in the cell culture medium, following the guidelines provided by the manufacturer. RAW264.7 cells (1×10^5/well) were cultured in 1 μg/mL LPS solution for 24 h in 24-well plates, using various experimental conditions. Following a 24-hour incubation period, the supernatants of the sample was obtained using centrifugation at a force of 2500 g for 20 min at a temperature of 4 °C. Then according to the manufacturer to provide illustrations of the determination of the level of inflammatory factors, and according to the standard curve to determine the content of inflammatory cytokines in sample. The experiment was conducted three times.

2.8.5. Morphological observations

Following three PBS washes, the cells were fixed for one hour using a 10 % formaldehyde solution in PBS. After washing the cell surface with distilled water for 3 times, the cells were soaked in hematoxylin solution for 5 min at room temperature to stain them [13]. Ultimately, the cells that were marked were rinsed using distilled water and examined using an inverted fluorescent microscope.

2.9. Statistical analysis

All analyses were carried out using SPSS version 26 (IBM, USA). The GraphPad Prism 8.0.2 software was used to analyze the images. Images with a p-value < 0.05 were considered significant. All the experiments were carried out in triplicates.

3. Result and discussion

3.1. Single factor experiments

3.1.1. CRITIC algorithm and comprehensive evaluation value (CEV)

The optimization of various components in the extraction or purification process can be influenced by multiple indicators. As a result, it is common practice to combine the content of these components into a single Comprehensive Evaluation Value (CEV). The techniques currently employed to assess overall performance include the entropy method [30], principal component analysis [31], grey correlation method [32], AHP hierarchy analysis [33], and CRITIC weight. In the CRITIC procedure, objective weights are assigned to each indicator based on the contrast of intensities and the conflict evaluation associated with each indicator [34]. The intensity of contrast is measured by the standard deviation, with a positive correlation between the standard deviation magnitude of the data and the weight. Conflict representation is determined by the use of the correlation coefficient, where an increase in phase relation leads to a decrease in weight, signifying a greater role in the overall assessment and higher level of importance. The weights for rutin, phillyrin, and forsythoside A were determined using the CRITIC method. Experimental results indicated that the quantities of rutin, phillyrin, and forsythoside A were calculated as 0.2659, 0.3901, and 0.3439, respectively.

3.1.2. The increasing effect of β-CD on the content of FS extract

Cyclodextrins possess a central ring structure characterized by cavities, wherein the oxygen atoms of the –CH- groups that bond with glucoside moieties exhibit hydrophobic properties [35]. Conversely, the water-loving nature of the –OH groups situated at positions 2, 3, and 6 of the glucose unit enables it to create inclusion complexes with other molecules via weak van der Waals forces [36]. The present study was designed to evaluate the effect of β-CD on the inclusion of rutin, phillyrin and forsythoside A. The evaluation focused on comparing the content of these three components before and after the addition of β-CD to FS. As shown in the Fig. S1(d), the mixture demonstrated an increase in the levels of rutin, phillyrin and forsythoside A when β-CD extraction was used, as opposed to when it was not utilized. Hence, it can be demonstrated that the compounds rutin, phillyrin, and forsythia A present in FS may be effectively encapsulated by β-CD.

3.1.3. The influence of the type and composition of the enzyme

Chinese medicinal materials are mainly divided into plant drugs, animal drugs and mineral drugs, among which plant drugs are the most common Chinese medicinal materials. Plant cells are mainly composed of two main parts: cell wall and protoplast. The cell wall is a dense structure composed of cellulose, hemicellulose, pectin and other substances [37]. The identification of suitable enzymes that operate on medicinal plant materials, such as cellulase and hemicellulase, has the potential to disrupt the compact structure of the cell wall [38]. This disruption can facilitate the dissolving of active components. Hence, our investigation focused on the examination of enzyme varieties and their respective quantities. The data presented in Table S2 revealed that the extraction rates of rutin, phillyrin and forsythoside A were comparatively reduced in certain enzyme treated groups as compared to control samples that did not undergo any enzyme treatment. This phenomena can be elucidated by the enzymatic conversion of naturally occurring active constituents [39]. Ultimately, the plant cell wall of FS undergoes a reduction in thickness, leading to a disorganized microstructure. This structural alteration facilitates the release of the desired product. Consequently, the compounds Cel and Pap were chosen for the subsequent studies. The investigation focused on analyzing the concentration and proportion of the combined enzymes. The findings, presented in Table S3, indicated that the mixed enzyme treatment consisting of 6.5 % Cel and 1.5 % Pap yielded the greatest CEV value of 26.17. Furthermore, it is worth noting that Cel is abundantly present in several living organisms. Moreover, it is important to note that Cel is not only abundant in several organisms, but can also be synthesized by a variety of organisms, such as bacteria, fungi, mammals, and other biological entities [40]. Pap is predominantly present in the root, stem, leaf, and fruit tissues of the papaya plant [41]. The origin of both enzymes is in line with the targets outlined in the Sustainable Development Goals.

Fig. S1(a-c,e-i) illustrates the impact of varying enzyme concentration on the extraction rates of rutin, phillyrin, and forsythia A, as well as the influence of enzymatic hydrolysis pH, solid–liquid ratio (mL/g), extraction temperature (℃), ultrasonic power (W) and β-CD addition (mg/mL) on the extraction rates and CEV of active ingredients.

3.1.4. The influence of enzymolysis temperature

Similar to the majority of chemical reactions, the activity of enzymes is notably influenced by temperature. The enzymatic activity often exhibits an upward trend within a specific temperature range, since the increase in temperature leads to a greater proportion of molecules transitioning into an active state [42]. In contrast, the enzymatic activity exhibits a decline. Conversely, enzymes are classified as proteins, and elevated temperatures can induce protein denaturation, leading to a progressive decline in enzymatic activity. Hence, it can be observed that every enzyme possesses an ideal temperature, denoting the temperature at which the rate of enzymatic reaction attains its utmost level [43]. When the reaction temperature surpasses the ideal temperature, there is a steady drop in the reaction rate as the temperature increases, eventually leading to a total cessation of the reaction.

Fig. 2c illustrates the impact of enzymatic temperature on the pace at which natural chemicals are extracted from FS. The study's findings demonstrated a positive correlation between the temperature of the enzymatic process, within the range of 25–45 °C, and the pace at which natural compounds were extracted. The highest extraction efficiency was observed at enzymolysis temperatures of 35 °C and 45 °C. This observation may be attributed to variations in enzymolysis temperatures among different enzymes for FS, leading to differences in the composition of various components at different temperatures. The ideal temperature for enzymatic digestion was established to be 35 °C, taking into account energy expenditure.

3.1.5. The influence of enzymolysis time

The enzymolysis reaction is affected by many factors, and the final result of the enzymolysis reaction is also different. In fact, in different reaction processes involving the same enzyme, the content of active ingredients detected at different reaction times is different [44]. As shown in Fig. 2d, CEV gradually increased with increase in enzymolysis time, but gradually decreased after 50 min. The findings indicated that the hydrolysis impact did not exhibit a linear increase with time, and there was a certain degree of reduction in the concentration of active components. According to the efficiency of enzymolysis and actual production, an enzymolysis time of about 50 min is appropriate.

3.1.6. The influence of extraction time

Fig. 2e depicts the impact of varying extraction durations on CEV. Within the specified time interval of 30 to 70 min, the CEV exhibited a progressive increase in response to the duration of ultrasonic exposure. However, a notable drop in CEV was observed between the time range of 70 to 110 min. The potential cause for the degradation of rutin, phillyrin and forsythoside A could be attributed to the heat effect resulting from intensified ultrasonic cavitation and prolonged ultrasonic exposure. The ultrasonic time of 70 min is chosen based on considerations of energy conservation and optimal yield.

3.2. EUA-β-CDE conditions optimization using BBD

The investigation was conducted using a Box-Behnken design comprising of 17 tests. The research aimed to examine the impact of three independent factors, specifically enzymatic temperature, enzy-

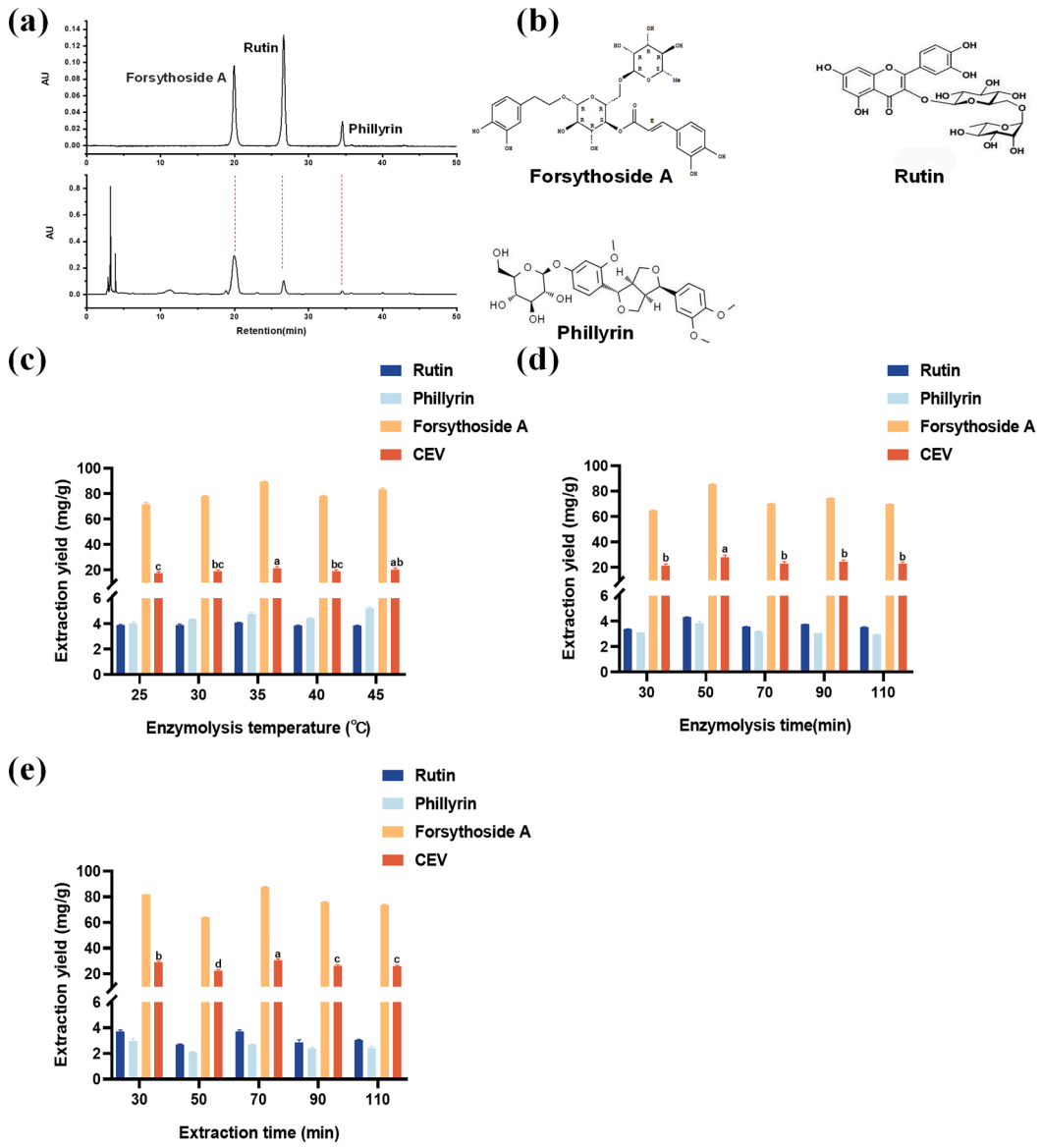

Fig. 2. (a) HPLC chromatogram of rutin, phillyrin and forsythoside A standard and EUA-β-CDE extract. (b) The structures of rutin, phillyrin and forsythoside A. The influence of enzymolysis temperature (c), enzymolysis time (d) and extraction time (e) on CEV value. Different letters indicate significant data differences between different groups ($P < 0.05$).

Table 1
Independent variables, their levels for the Box-Behnken design, and the responses obtained.

Run	X1:Enzymolysis temperature (°C)	X2: Enzymolysis time (min)	X3: Extraction time (min)	rutin	phillyrin	forsythoside A	CEV
1	30	30	70	3.49	3.46	67.04	25.3305
2	30	50	50	3.47	3.49	67.56	25.5189
3	30	50	90	3.52	4.60	68.00	26.1173
4	30	70	70	3.39	4.39	67.68	25.8871
5	35	30	50	3.34	3.41	67.48	25.4254
6	35	30	90	3.88	3.55	67.90	25.7643
7	35	50	70	3.46	3.02	68.35	25.6028
8	35	50	70	3.53	2.95	69.79	26.0909
9	35	50	70	3.59	2.69	69.67	25.9632
10	35	50	70	3.53	2.89	69.27	25.8856
11	35	50	70	3.49	3.03	68.38	25.6269
12	35	70	50	3.82	2.97	67.44	25.3694
13	35	70	90	3.48	2.80	67.93	25.3801
14	40	30	70	3.28	2.39	64.19	23.8806
15	40	50	50	3.30	2.02	65.57	24.2126
16	40	50	90	3.20	2.46	64.26	23.9089
17	40	70	70	2.93	2.78	64.16	23.9275

matic time, and extraction time, on the rate of extraction for rutin, phillyrin and forsythoside A. Table 1 displays the independent variables and response variables utilized in response surface analysis. The rutin yield ranged from 2.93 to 3.88 mg/g, the phillyrin yield ranged from 2.02 to 4.60 mg/g, the forsythoside A yield ranges from 64.16 to 69.79 mg/g, and The CEV yield ranges from 3.81 to 4.80. Furthermore, the statistical method of analysis of variance (ANOVA) was employed. Table 2 displays the outcomes of the regression model. The statistical significance of the observed results is shown by a p value of 0.0002, suggesting that the likelihood of obtaining these results by chance alone is very low. Additionally, the F value of 23.37 provides further evidence of the significance of the observed effects. The prediction made by the model holds substantial importance. Furthermore, the fit did not exhibit statistical significance ($p > 0.05$) and the pure error value was minimal, suggesting that the study's findings were highly replicable. The model's coefficient of variation was 0.8451 %, which met the acceptable range of 5 %. This suggests that the model exhibits a high level of reliability [45]. The changes of CEV between the three variables are described by response surface diagram. The model is derived by multiple regression analysis of the experimental data using second-order polynomial equations. The relationship between the response variable and the test variable can be described by a second-order polynomial equation:

$$\begin{aligned} Y =& 25.83 - 0.8655X_1 - 0.0204X_2 \\ &+ 0.0805X_3 - 0.1274X_1X_2 - 0.2255X_1X_3 - 0.0820X_2X_3 \\ &- 0.8114X_1^2 - 0.2661X_2^2 - 0.0831X_3^2 \end{aligned}$$

The ANOVA findings demonstrated the reliability of second-order polynomial models. The regression coefficient (R^2) of the response surface model, as indicated in the aforementioned equation, is 0.9678. This value shows a high level of reliability in detecting the factors and their interactions, which cannot be accurately identified through single-factor trials in isolation. Fig. 3 depicts a response surface plot in three dimensions that demonstrates the impact of variables on CEV. The graphical representation in Fig. 3a illustrates the correlation between the temperature (X_1) and duration (X_2) of enzymatic hydrolysis, and its impact on CEV. The impact of enzymatic temperature on the rate of extraction was more pronounced in comparison to enzymatic time. The extraction rate exhibited a progressive increase as the temperature rises, followed by a minor reduction upon reaching its peak value. The relationship between enzymatic hydrolysis time (X_2) and extraction time (X_3) on CEV is depicted in Fig. 3c. Both factors exhibited minimal impact on yield, but significant CEV values were achieved under conditions of moderate hydrolysis temperature and extraction time. Hence, the investigation of BBD holds significant importance in the process of optimizing EUA-β-CDE. The laboratory's validation of the response yielded a projected CEV value (26.15), obtained by the utilization of $X_1 = 32$ °C, $X_2 = 56$ min, and $X_3 = 82$ min. This observation aligns with the anticipated values presented in Table 3.

Table 2
Analysis of variance (ANOVA) for response surface model of the CEV of extraction yield.

Source	Sum of Squares	df	Mean Square	F-value	p-value	significant
Model	9.61	9	1.07	23.37	0.0002	significant
X_1	5.99	1	5.99	131.24	< 0.0001	***
X_2	0.0033	1	0.0033	0.0730	0.7948	
X_3	0.0519	1	0.0519	1.14	0.3218	
X_1X_2	0.0650	1	0.0650	1.42	0.2719	
X_1X_3	0.2035	1	0.2035	4.46	0.0727	
X_2X_3	0.0269	1	0.0269	0.5895	0.4677	
X_1^2	2.77	1	2.77	60.70	0.0001	***
X_2^2	0.2981	1	0.2981	6.53	0.0378	*
X_3^2	0.0290	1	0.0290	0.6360	0.4513	
Residual	0.3197	7	0.0457			
Lack of Fit	0.1379	3	0.0460	1.01	0.4749	not significant
Pure Error	0.1817	4	0.0454			
Cor Total	9.92	16				

a ***, most significant ($P < 0.001$); **, more significant ($P < 0.01$); *, significant ($P < 0.05$).

3.3. EUA-β-CDE compared with different extraction methods

3.3.1. Comparison of CEV of different extraction methods

In order to assess the efficacy of EUA-β-CDE in the process of extraction, a set of comparative tests were conducted. According to the data presented in Table 3, it can be observed that EUA-β-CDE exhibited superior extraction rates for rutin, phillyrin and forsythoside A compared to UAE, EAE, and β-CDE. Experiments show that the extraction efficiency of EUA-β-CDE method is higher than that of single extraction method [46]. One reason for this is that cellulose is one of the components that make up the cell wall of plants [47], and most of the active components are found in the cell wall. Thus, the active ingredient is released through the destruction of plant cell walls by enzymes. This method can not only improve the solubility of active ingredients but also improve the extraction efficiency of active ingredients [48]. In addition, ultrasound can damage the cellular structure of the plant through the cavitation effect, resulting in a reduction in the time required for the active ingredient to be transferred from the plant material to the solvent [49]. As a traditional method to extract active ingredients, HRE has been widely used in the extraction of natural herbs [50]. However, compared with the HRE method, the EUA-β-CDE method not only has higher extraction efficiency, but also reduces energy consumption. In addition, compared with the extraction technology described in the Chinese Pharmacopoeia, the EUA-β-CDE method does not require organic solvents and can avoid environmental pollution caused by organic solvents. Finally, we compared the extraction techniques in other papers. In En-Qin Xia et al. [51], Ultrasound assisted extraction method was mainly used to extract phillyrin from FS leaves. Here, EUA-β-CDE was used to extract rutin, phillyrin and forsythoside A from FS fruits. In addition, we also systematically compared this method with other conventional extraction methods to further verify its superiority. In S F Long et al. [52], ultrasonic assisted extraction of active ingredients in FS was mainly carried out using 80 % methanol solvent. However, in this study, water-based buffer EUA-β-CDE was used to extract rutin, phillyrin and forsythoside A from FS. Therefore, the EUA-β-CDE method has more significant advantages. This technique aligns with the tenets of green chemistry and contributes to the attainment of sustainable development objectives. The potential effectiveness of EUA-β-CDE as a technique for extracting plant bioactive compounds is in the use of ecologically favorable materials and methods.

3.3.2. Energy conservation

Table 3 provides an evaluation and description of the benefits associated with using EUA-β-CDE in comparison to alternative extraction techniques. Compared with 70 % methanol extraction (70 % ME) and methanol extraction (ME), the EUA-β-CDE method does not use organic solvents. When comparing the effects of EAE and HRE with EUA-β-CDE, it was shown that the latter leads to a decrease in energy consumption and CO_2 emissions. When we compared the yields of EUA-β-CDE with UAE and β-CDE, it was observed that EUA-β-CDE exhibited a greater concentration of active components. As you can see from Fig. 4, the energy consumption required to extract 1 mg/g of active compounds from EUA-β-CDE was measured to be 1.52kWh, whereas the corresponding energy consumption for UAE was estimated to be 1.72kWh. The energy consumption of β-CDE was measured to be 1.64 kWh. Hence, the findings indicated that EUA-β-CDE demonstrates a higher efficacy in extracting active constituents, while concurrently exhibiting the lowest energy usage.

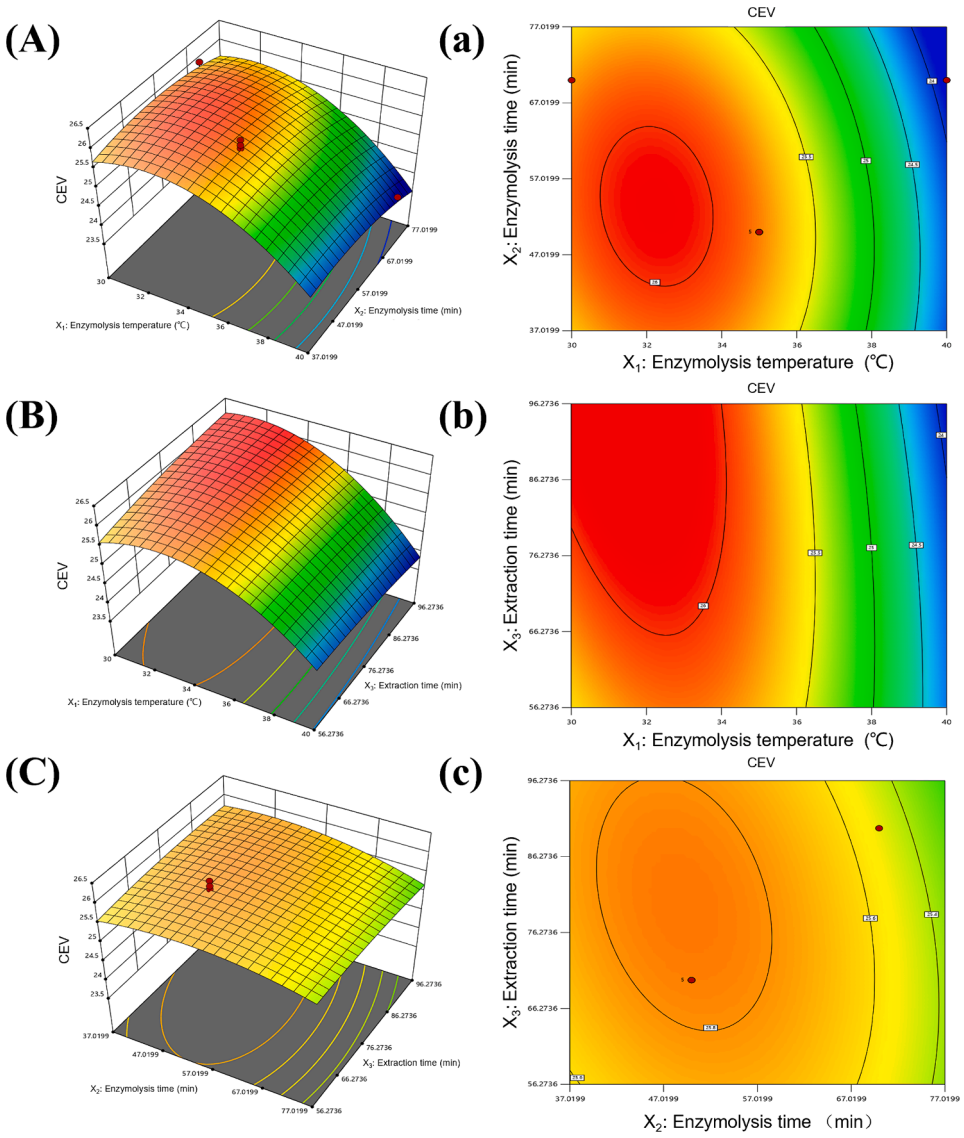

Fig. 3. The response surface plot and contour plot show the influence of the interaction between single factors on CEV. (A-a) temperature and time of enzymolysis; (B-b) enzymolysis temperature and extraction time; (C-c) enzymolysis time and extraction time.

3.3.3. SEM analysis

The mechanism of this process can be investigated by using SEM to analyze its microstructure. Fig. 5 displays the observed morphological alterations in untreated FS powder samples and samples subjected to various extraction techniques. In contrast to the untreated sample (a), the application of various procedures resulted in varying degrees of damage to the FS powder, which was evident in its altered surface characteristics. As depicted in Fig. 5b, the external layer of plant tissue subjected to EAE treatment exhibited signs of impairment and deformation. As depicted in Fig. 5c, the cell wall exhibited fracture subsequent to the application of UAE treatment, resulting in varying degrees of damage to the surface of the cell tissue. As depicted in Fig. 4d, the application of both enzyme and ultrasonic treatments resulted in noticeable cell tissue damage, accompanied by an increased presence of tissue debris. This observation suggests a synergistic impact of the enzyme and ultrasound treatments. As depicted in Fig. 5e, the external surface of plant tissues subjected to HRE treatment exhibited minor undulations. Fig. 5f demonstrates a significant presence of folds on the cell surface following the extraction process using 70 % ME. As depicted in Fig. 5g, a significant quantity of folds manifested on the cellular membrane subsequent to the ME process. As depicted in Fig. 5h, the cellular surface exhibited a rather smooth appearance subsequent to the extraction process involving β-CD, with no discernible signs of damage. The findings of this study indicate that EUA-β-CDE is a fast and efficient alternative technique for extracting rutin, phillyrin and forsythoside A.

3.4. Characterizations

3.4.1. FTIR analysis

FS, β-CD, EUA-β-CDE, and FS and β-CD physical combination of FT –IR spectrum, as shown in Fig. 6a. An absorption peak at 1245 cm^{-1} was identified, matching to the C-O bond. The bending vibrations of the –CH3 group were seen at wavelengths of 1376 cm^{-1} and 1455 cm^{-1}. The –CH2 group exhibited vibrational peaks at wavelengths of 2855 cm^{-1} and 2926 cm^{-1}. The –OH group exhibited a vibrational peak at a precise wavelength of 3296 cm^{-1}. β-CD exhibits characteristic peaks in its infrared spectrum. These include a C-H bending peak at 1418 cm^{-1}, a –CH2 vibrational peak at 2927 cm^{-1}, and a prominent –OH vibrational peak at 3354 cm^{-1}. The absorption peaks at 1245 cm^{-1}, 1376 cm^{-1}, 1455 cm^{-1}, 2855 cm^{-1}, 2926 cm^{-1}, and 3296 cm^{-1} in Fig. 6a clearly indicate the presence of distinct physical mixing between FS and β-CD. In contrast, β-CD exhibited distinct absorption peaks at 1418 cm^{-1},

Table 3
Extraction results from different extraction methods.

	rutin	phillyrin	forsythoside A	CEV	Extraction time (min)	Organic solvent consumption (mL)	Energy consumption (kWh)	CO_2 production (kg)	Energy consumed per extraction of 1 mg/g of ingredients (mg/g)
EUA-β-CDE	3.60	3.06	69.84	26.17	56 + 82	0	115.92	92.74	1.52
EAE	2.70	1.32	55.21	20.22	138	0	138	110.4	2.33
UAE	3.22	2.93	61.30	23.08	138	0	115.92	92.74	1.72
β-CDE	3.22	2.09	65.32	24.13	138	0	115.92	92.74	1.64
HRE	2.73	3.01	52.55	19.97	120	0	120	96	2.06
70 %ME	3.35	3.30	67.71	25.46	30	20	7.5	6	1.01
ME	1.08	1.08	24.02	8.97	25	20	6.25	5	0.24

2927 cm^{-1}, and 3354 cm^{-1}. The cyclodextrin exhibited a distinct peak in the FS extract, whereas the distinctive peak of the FS extract was no longer observable. The findings of the study revealed that β-CD effectively contained the active component found in FS.

The limitation of functional group vibrations in the EUA-β-CDE extract can be ascribed to the existence of β-CD, which indicates that the β-CD and the interaction between natural active ingredients. The unique spectral peaks of FS and β-CD indicate that they had weak or no interaction with one other throughout the physical mixing process. Following the interaction with β-CD, the distinctive peaks of the compounds in FS exhibited a reduction in intensity or complete disappearance [53–56]. FT-IR spectroscopy was employed to uncover possible interactions between the molecules of the host and guest. However, through the next experiment is needed to further prove that active ingredient is β-CD inclusion.

3.4.2. DSC analysis

The physical states of the guest molecules were investigated using DSC [57]. Fig. 6b displays the outcomes of DSC analysis conducted on various materials, including FS powder, a physical mixture of β-CD and FS, and EUA-β-CDE extract. The FS powder exhibited a negligible endothermic peak at a temperature of 152.08 °C, while β-CD displayed a similarly small endothermic peak at a little higher temperature of 163.28 °C. Simultaneously, the combination of FS and β-CD exhibited two distinct points of endothermic peaks at temperatures of 139.09 °C and 159.81 °C. The absorption peak of FS extract was observed at a temperature of 163.24 °C. Additionally, the endothermic peak of FS was no longer present, suggesting that the natural active ingredient was successfully included by β-CD. There was a notable interaction between FS and β-CD.

3.5. Thermal stability study

Fig. 6c illustrates the thermal stability of the active components. In a span of five hours, the concentrations of rutin and phillyrin experienced a significant decrease. This fall occurred without the introduction of the β-CDE solution. Additionally, it was observed that forsythoside A initially exhibited a rapid decline within the first hour, but thereafter reached a state of stabilization. In comparison with the initial time point (0 h), the rutin content exhibited a reduction of 10 %, while the phillyrin content experienced a decrease of 24.06 %. Additionally, the forsythoside A content demonstrated a decline of 2.92 %. The levels of rutin and phillyrin steadily reduced in the β-CDE solution, whereas the levels of forsythoside A remained stable. Compared with the initial time point (0 h), the content of rutin, phillyrin, and forsythoside A decreased by 4.9 %, 17.35 % and 0.82 %, respectively. According to the experimental phenomenon, β-CD can not only increase the content of active ingredient, but also improve the stability of active ingredient [58]. Consequently, the experimental results showed that the use of β-CD as an extractant can improve the stability of the active ingredients of natural drugs.

3.6. Antioxidant capacity

In recent years, many people have conducted studies on the antioxidant capacity of extracts with natural medicines.While antioxidants usually have the ability to scavenge free radicals. The antioxidant capacity of EUA-β-CDE and HRE extracts was evaluated by scavenging DPPH and ABTS free radicals. The scavenging rate of EUA-β-CDE and HRE extracts against both DPPH radicals and ABTS radicals exhibited an initial increase followed by stabilization,as depicted in Fig. 7. The EUA-β-CDE extract exhibited a higher antioxidant capacity compared to the HRE extract, and its antioxidant capacity gradually approached that of Vc. Different concentration rutin, phillyrin, forsythoside A and Vc on DPPH free radicals and ABTS free radical clearance as shown in Fig. 7. The monomer concentrations of rutin, phillyrin, and forsythoside A at the identical position in Fig. 7, as well as the monomer concentrations of

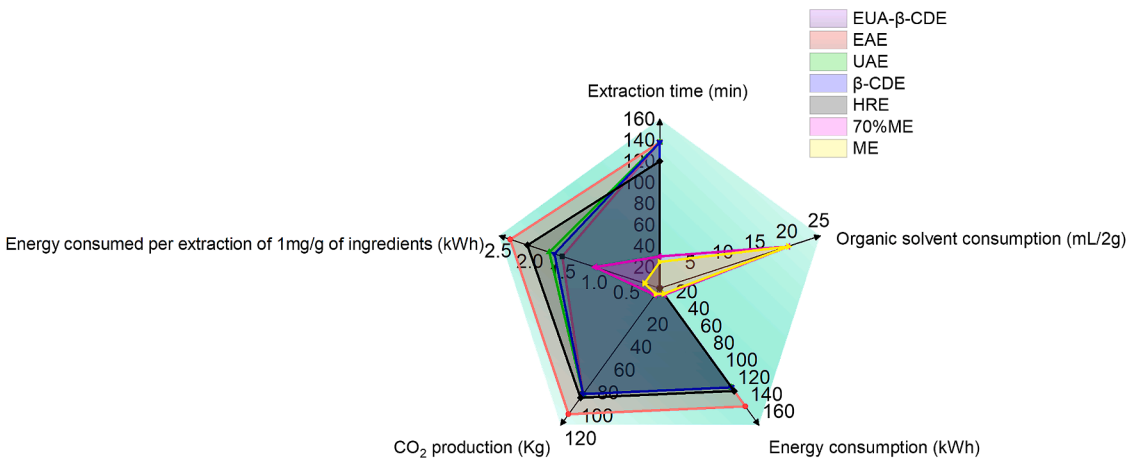

Fig. 4. Evaluation of different extraction methods based on CO_2 production, energy conservation, organic solvent use, extraction time.

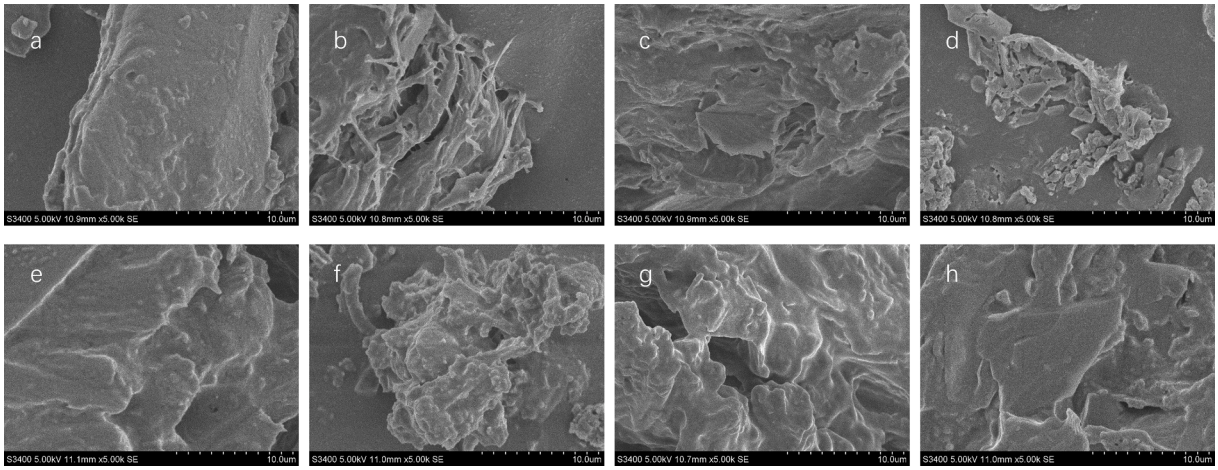

Fig. 5. 5000X SEM images of FS powder (a) in different extraction methods ; (b)EAE; (c)UAE; (d) EUAE; (e) HRE; (f) 70 % ME; (g) ME; (h) β-CDE.

the rutin and forsythoside A mixtures in Fig. 7, exhibited concentrations equivalent to the monomer concentrations of EUA-β-CDE extracts in Fig. 7 The antioxidant of the compound are primarily attributed to its hydrogen supply capacity [59] and the formation of weak interaction between β-CD and FS [60]. This interaction enhances the hydroxylation, solubility, and stability of the active compounds, facilitating its reaction with free radicals such as DPPH and ABTS [22]. Furthermore, the inclusion of β-CD in the process of inclusion enhances the concentration of FS components in the final product, resulting in an enhancement of its antioxidant effects.

The antioxidant capacity of forsythoside A and Vc exhibited an upward trend as the concentration increased, their antioxidant capacity progressively increased until it reached that of Vc. Nevertheless, the antioxidant activity of rutin and phillyrin is significantly limited (as depicted in Fig. 7), mostly due to their low concentration in the extract, which hinders their ability to exert a substantial impact. Hence, the primary factor responsible for the antioxidant capacity of FS is predominantly attributed to forsythoside A. Ultimately, the utilization of EUA-β-CDE not only enhanced the content of FS extraction, but also augmented its antioxidant properties.

3.7. Evaluation of anti-inflammatory activity

3.7.1. Cell viability

In this study, the effects of EUA-β-CDE extract and HRE extract on the activity of RAW264.7 macrophages were investigated by cell survival rate. The mass concentration of EUA-β-CDE and HRE was shown to be same, with a range of 0.5–0.125 mg/mL. See Fig. 8a. The EUA-β-CDE and HRE extracts did not have any impact on the viability of RAW264.7 cells, which remained at a level of at least 90 %. Furthermore, there was no notable disparity seen when compared to the blank group. The results showed that the administration of EUA-β-CDE and HRE extracts did not result in any cytotoxicity in RAW264.7 cells at 0.5 mg/mL.

According to the data presented in Fig. S2(a,c), the concentration of ethanol was less than 1.56 %. Additionally, the concentration of rutin was estimated at 3.13 μg/mLand that of phillyrin was 12.5 μg/mL. Cell viability remained unaltered when exposed to a concentration of 0.0625 mg/mL of forsythia A. The cell viability was found to be equal to or greater than 90 %, which had no significant difference compared with the control group. Subsequently, the ethanol content in rutin was determined to be 0.156 %. Conversely, the concentration of ethanol in forsythia A was found to be 0.625 %, suggesting that ethanol did not exhibit any interfering effects.

Fig. S2b demonstrates that cell viability remained unaffected at a rutin concentration of 3.8 μg/mL, a phillyrin concentration of 3.8 μg/mL, and a forsythoside A concentration of 0.075 mg/mL. The cell viability was found to be equal to or greater than 90 %, and there was no significant difference from the control group. Currently, the determination of ethanol in the compound monomer concentration was 0.1875 %, suggesting that ethanol did not exhibit any interfering effects.

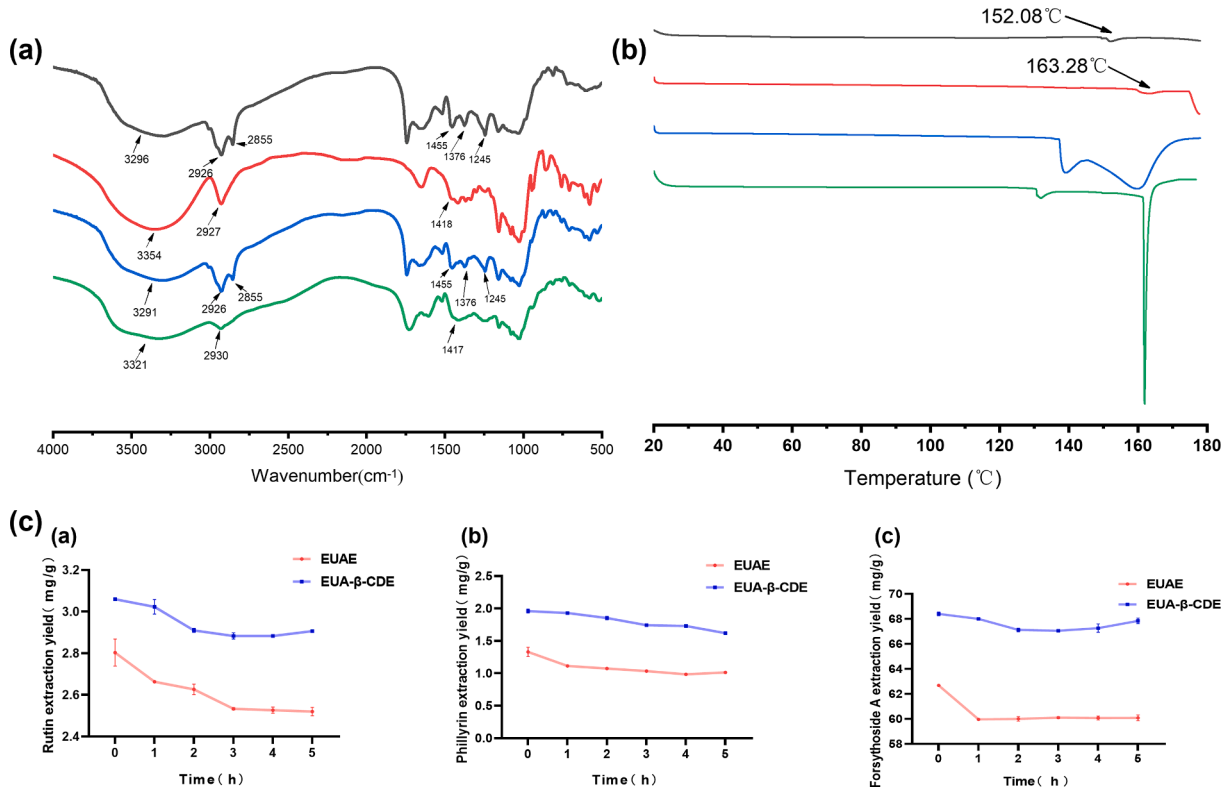

Fig. 6. (a) FTIR spectra of different substances; (b) DSC thermograms of different substances; (c) study on the thermal stability of FS extract before and after adding β-CD. FTIR spectra: (a) FS, (b) β-CD, (c) FS/β-CD PM and (d) EUA-β-CDE; DSC thermogram: (a) FS, (b) β-CD, (c) FS/β-CD PM and (d) EUA-β-CDE; Thermal stability study (a) rutin, (b) phillyrin, (c) forsythoside A.

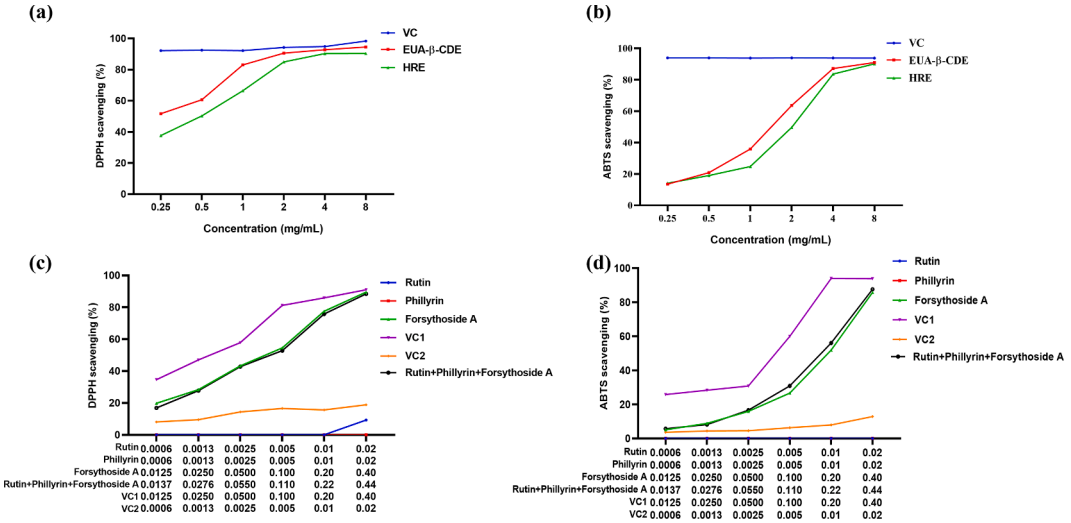

Fig. 7. The results of DPPH free radical scavenging ability (a) and (c) and ABTS free radical scavenging ability (b) and (d).

3.7.2. The production of intracellular reactive oxygen species (iROS) was determined

Endotoxins can cause overproduction of inflammatory mediators, leading to oxidative stress, inflammatory disease and even death [61,62].Multiple studies have demonstrated a robust association between oxidative stress and the advancement of inflammation [63]. Therefore, DCFH-DA was used to evaluate the number of iROS and investigate the possible role of oxidative stress injury in LPS-induced inflammation of RAW264.7 macrophages. As can be seen from the data in Fig. 8b, the production of iROS was considerably higher in the group that was stimulated with LPS compared to the control group ($P < 0.05$). The administration of EUA-β-CDE in the treatment group resulted in a dose-dependent decrease in iROS generation compared to the LPS group. The anti-inflammatory efficacy of the combination of rutin, phillyrin, and forsythoside A (0.0018 + 0.0015 + 0.035 mg/mL) was shown to be superior to that of rutin (0.0018 mg/mL), phillyrin (0.0015 mg/mL), and forsythoside A (0.035 mg/mL). The suppression of iROS levels was less pronounced in the groups treated with EUA-β-CDE extract and mixed monomers at a dosage of 0.5 mg/mL, as compared to the DEX group.

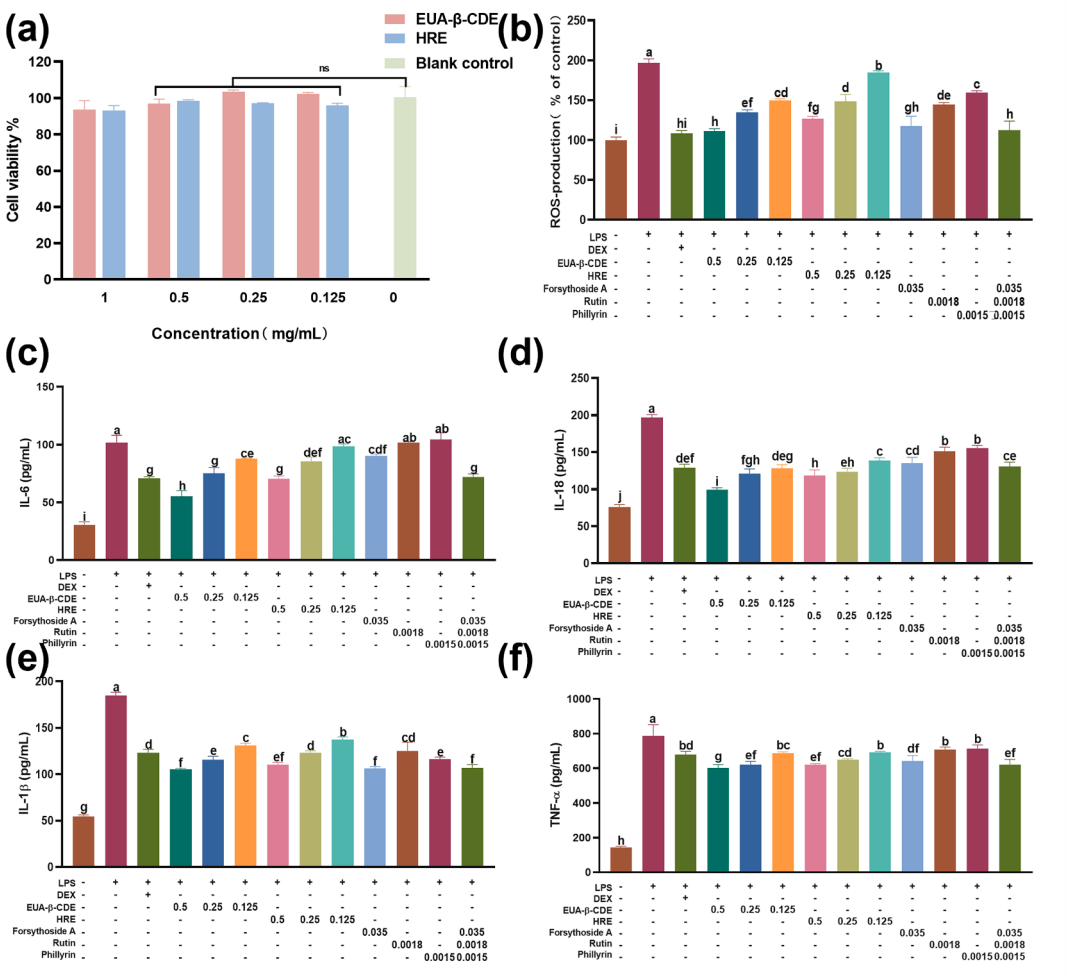

Fig. 8. (a) Cytotoxicity analysis of EUA-β-CDE and HRE extracts on RAW264.7 cells. (b) Intracellular ROS(iROS) production. The levels of LPS-induced inflammatory cytokines ((c) IL-6, (d) IL-18, (e) IL-1β, (f) TNF-α) in RAW264.7 cells were detected. Data are mean ± standard deviation (n = 3), different letters indicate significant data differences between different groups ($P < 0.05$).

3.7.3. Determination of inflammatory cytokines

During the process of cellular immunity, macrophages play a crucial role by releasing inflammatory cytokines [64]. This action is essential for the maintenance of immunological control. Therefore, ELISA was used to evaluate the effect of EUA-β-CDE extract on the production of LPS-stimulated inflammatory cytokines (IL-6, IL-18, IL-1β and TNF-α) in RAW264.7 cells. Based on the findings presented in Fig. 8c, d, e, and f, it is evident that treating cells with LPS led to a substantial rise in the secretion of inflammatory factors in comparison to untreated cells. Fig. 8 data demonstrates that dexamethasone successfully suppressed the production of inflammatory factors generated by LPS. In the concentration range of 0.5–0.125 mg/mL, EUA-β-CDE extract can reduce the release of inflammatory factors in a certain concentration dependent manner. The levels of inflammatory cytokines in the HRE extract (0.5–0.125 mg/mL) were found to be greater compared to the EUA-β-CDE extract (0.5–0.125 mg/mL) following their administration to RAW264.7 cells. In summary, the presence of rutin, phillyrin and forsythoside A in EUA-β-CDE extracts appears to possess significant anti-inflammatory properties.

3.7.4. Observation of cell morphology

The activation of macrophages is the primary and essential step in the immune response. A prominent characteristic of macrophage activation is the noticeable alteration in cell morphology, which is demonstrated by a modification in shape, increased spreading, and the development of pseudopods [65]. Nevertheless, the morphological change can be impeded by anti-inflammatory medicines [66]. The cellular morphology was examined using an optical microscope to observe the impacts of EUA-β-CDE and HRE extracts. Fig. 9a demonstrates that RAW264.7 cells often possess a spherical shape and exhibit clear cell division. As depicted in Fig. 9b, the observed cellular response to LPS induction after 24 h included the production of pseudopodia, cell expansion, and a pancake-like morphology. Conversely, Fig. 9c illustrates that only a limited number of cells in the positive medication treatment group exhibited pseudopodia formation. Fig. 9d-i illustrates the alterations in cell morphology resulting from the exposure to EUA-β-CDE and HRE at varying doses. The treatments of EUA-β-CDE and HRE exhibited variable degrees of inhibition on cell proliferation and pseudopod formation. Notably, the HRE treatment group demonstrated more pronounced morphological changes in cells compared to the same concentration (0.5–0.125 mg/mL). As depicted in Fig. 9j, k, l, and m, the mixed monomer treatment group exhibited smaller morphological alterations in cells compared to the rutin, phillyrin, and forsythoside A groups respectively. Furthermore, the degree of morphological changes in the mixed monomer treatment group was comparable to that observed in the positive drug treatment group. In line with our investigation, it was observed that FS extract exhibited anti-inflammatory properties on RAW264.7 cells stimulated with LPS, thereby mitigating the impact on cell shape characterized.

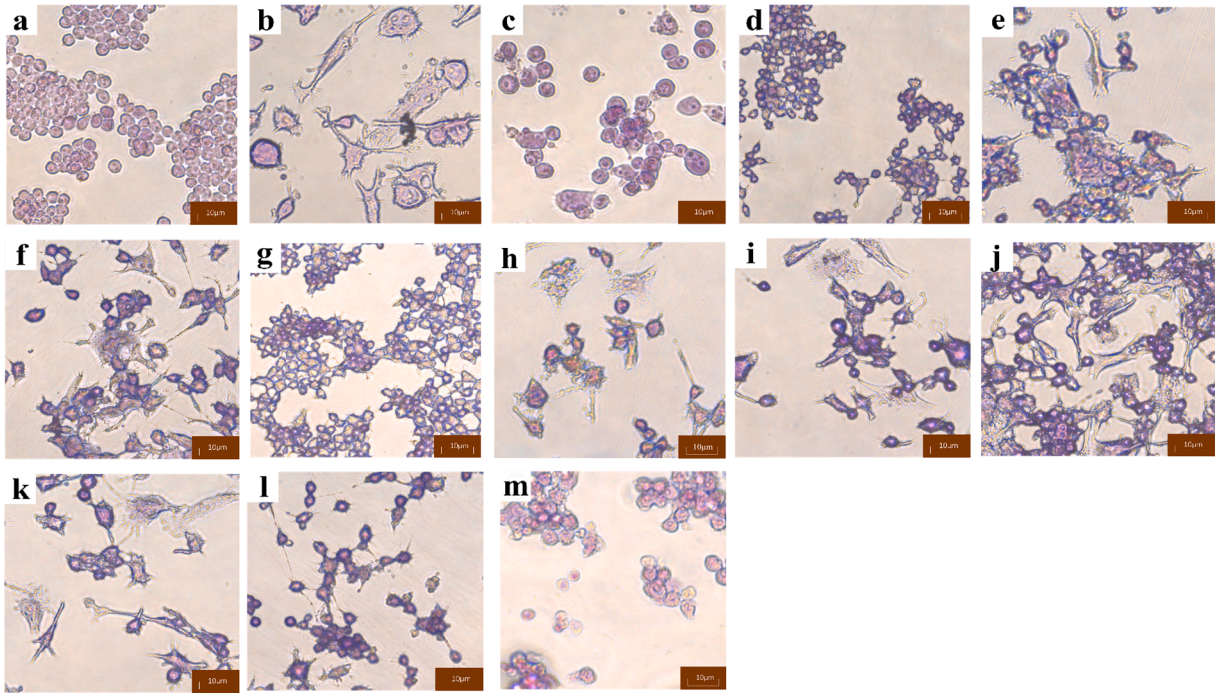

Fig. 9. Morphological changes of RAW264.7 cells were observed by optical microscope (original magnification × 400, scale; 10 μm).They included (a) control group, (b) model group, (c) DEX treatment group, (d-f) EUA-β-CDE treatment group, (g-i) HRE treatment group, (j) rutin treatment group, (k) phillyrin treatment group, (l) forsythoside A treatment group, and (m) monomer mixture treatment group. (d), (e) and (f) cells were treated with 0.5, 0.25 and 0.125 mg/mL EUA-β-CDE extracts, respectively. (g), (h) and (i) cells were treated with 0.5, 0.25 and 0.125 mg/mL HRE extracts, respectively.

4. Conclusion

The BBD experimental design was employed to improve the extraction conditions, process, parameters, and build the regression model, based on the single-factor experiments. SEM procedure revealed that the combined application of EAE, UAE, and β-CDE had a synergistic impact, leading to enhanced cell wall breakage and enzymatic hydrolysis. In order to prove the formation of clathrates between β-CD and the active ingredient, FT-IR and DSC experiments were performed. Finally, the free radical scavenging experiments and ELISA experiments proved that EUA-β-CDE extract has good antioxidant and anti-inflammatory activities. In summary, the new method of EUA-β-CDE has a good effect on the extraction of FS components. The experiments compared with different extraction methods show that EUA-β-CDE method has the potential to replace traditional methods to extract active ingredients from FS. Furthermore, the EUA-β-CDE method offers an environmentally friendly approach characterized by reduced usage of organic solvents and energy consumption. Therefore, this novel and ecologically friendly extraction approach has the potential to bring fresh thoughts into the extraction of numerous plant components.

CRediT authorship contribution statement

Xiaoyue Xiao: Writing – original draft. **Yang Zhang:** Supervision. **Kedi Sun:** Supervision. **Shuoqi Liu:** Supervision. **Qingmiao Li:** Supervision. **Yu Zhang:** Supervision. **Bello-Onaghise Godspower:** Supervision. **Tong Xu:** Supervision. **Zhiyun Zhang:** Supervision. **Yanhua Li:** Writing – review & editing. **Yanyan Liu:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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